Mycorrhiza and root hairs in barley enhance acquisition of phosphorus and uranium from phosphate rock but mycorrhiza decreases root to shoot uranium transfer

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Summary

• Some phosphate rocks (PR) contain high concentrations of uranium (U), which are potentially toxic via accumulation in soils and food chains, and plant uptake of U is likely to be influenced by characteristics of roots and associated microorganisms. The relative importance of root hairs and mycorrhiza in U uptake from PR was studied using a root hairless barley (Hordeum vulgare) mutant (Brb) and its wild type (WT).

• Both plant genotypes were grown in pots with Glomus intraradices BEG 87, or in the absence of mycorrhiza, and three P treatments were included: nil P, 2% (w/w) PR and 50 mg KH2PO4-P kg−1 soil.

• Mycorrhiza markedly increased d. wts and P contents of Brb amended with nil P or PR, but generally depressed d. wts of WT plants, irrespective of P amendments. Mycorrhiza had contrasting effects on U contents in roots and shoots, in particular in Brb where mycorrhiza increased root U concentrations but decreased U translocation from roots to shoots.

• The experiment supports our understanding of arbuscular mycorrhiza as being multifunctional by not only improving the utilization of PR by the host plant but also by contributing to the phytostabilization of uranium.

Key words: Glomus intraradices, Hordeum vulgare (barley), phosphate rock, phosphorus, root hairless mutant, uranium (U) uptake.


Introduction

Direct application of phosphate rock (PR) can be an attractive alternative to commercial P fertilizers, especially in developing countries, where the poor farmers cannot afford to buy expensive, processed fertilizers (Semoka et al., 1992; Rajan et al., 1996). Depending on the origin, PRs contain various amounts of potentially toxic elements such as cadmium (Cd) and uranium (U), which accumulate in arable soil after continuous application of Cd- and U-containing P fertilizers (Williams & David, 1976; Andersson & Siman, 1991; Van Kauwenbergh, 1997). The PR deposit at Minjingu, north-east Tanzania has a proven high potential for direct application as P fertilizer with plant growth responses similar to those of superphosphates on acidic soils low in available Ca and P (Semoka et al., 1992, Msolla et al. unpublished, data). However, the relatively high U-content of Minjingu PR (Van Kauwenbergh, 1997) may pose threats to the health of mine workers and people in the mine surroundings that are exposed to the radioactive PR dust (Banzi et al., 2000). Moreover, the use of this PR as fertilizers could possibly endanger animal and human health by accumulation of U through food chains. Obviously effective measures should be taken to control the translocation of U in the cropping system and to manage the contaminated soils.
The role of arbuscular mycorrhiza (AM) in plant uptake of heavy metals has been extensively studied (Leyval et al., 1997). Root colonization by AM fungi can alleviate deficiency of micronutrients, such as copper (Li et al., 1991) and zinc (George et al., 1994; Chen et al., 2003) while in the case of metal contaminations, mycorrhiza reduced metal concentrations in shoots and increased plant growth (Leyval & Joner, 2001). The proposed underlying mechanisms include enhanced adsorption and immobilization of metals both in roots and mycorrhizosphere, which could be caused by direct binding of metals by fungal mycelium (Joner et al., 2000) and possible pH changes (Li & Christie, 2001). Mycorrhizal fungi have also been observed to enhance acquisition of 137Cs (Entry et al., 1999; Berreck & Haselwandter, 2001; Declerck et al., 2003), and 30Sr (Entry et al., 1999). Therefore, AM fungi could possibly play a role in the adaptation of their host plants to radionuclide pollutions. It was recently shown that the extraradical AM fungal mycelium took up and translocated U towards root cultures in vitro (Rufyikiri et al., 2002, 2003), but U transfer from root to shoot could not be quantified by this model system. The influence of AM fungi on U uptake under natural conditions is still unknown although such information is required to determine the importance of mycorrhizal technology for remediation of uranium-contaminated environments. In terms of nutrient transport from soil to root, AM fungal hyphae and root hairs are functionally equivalent (I. Jakobsen, unpublished data). Plant species with fine and dense root system or plant genotypes with long root hairs would depend less on mycorrhizal fungi for acquisition of P from soil (Baylis, 1975; Baon et al., 1994; Schweiger et al., 1995). However, it is still unknown whether such functional similarity is also valid for U transport from soil to root.

The objective of the present work was to study effects of the AM fungus *Glomus intraradices* on uptake of U from a U-containing PR. The study was carried out with a root hairless barley mutant and its wild type and we tested the hypothesis that plant U uptake would be increased both by the presence of root hairs and by the *G. intraradices* hyphae. Our study contributes to understanding the potential role of AM fungi in reducing environmental risks and in phytostabilization of U-contaminated environments.

### Materials and Methods

#### Host plants and AM fungus

Seeds of wild type (WT) and a root hairless mutant (bald root barley, Brb) of *Hordeum vulgare* L. cv. Pallas (Gahoonia et al., 2001) were pregerminated on moist filter paper for c. 24 h and were selected for uniformity before sowing. Both plant types were grown without or with inoculum of the AM fungus *Glomus intraradices* Schenck & Smith (BEG 87). The fungus was propagated in pot culture on medic plants (*Medicago trunculata* L) grown in a soil-sand mixture for 10 wks. Inoculum from pot culture was a mixture of spores, mycelium, sandy soil and root fragments.

#### Growth medium

The growth medium was a 1 : 1 (w/w) mixture of sand and soil, which was partially sterilized (10 kGy, 10 MeV electron beam). Basal nutrients without P (Pearson & Jakobsen, 1993) were added to the mixture. The soil-sand mixture (subsequently referred to as soil) had a pH of 6.70 (1 : 2.5 soil to water) and final extractable P content of 5.9 mg kg⁻¹ (Olsen et al., 1954).

#### Experimental procedure

The experiment had three P treatments: nil (P0), 2% (w/w) PR and 50 mg KH₂PO₄·P kg⁻¹ soil (P50). The P0 and P50 treatments served as control treatments for U uptake from the U-rich phosphate rock and as treatments to evaluate the relative contribution of mycorrhizal fungi and root hairs to plant P uptake from different P sources. The phosphate rock obtained from Minjingu, Tanzania, had a particle size < 250 µm, and contained 30.3% of P₂O₅, and 310 mg kg⁻¹ of U (4367 Bq kg⁻¹). The U sources were carefully mixed into the soil together with either 70 g of the *G. intraradices* inoculum or the equivalent amount of the soil-sand mixture, and the amended growth media were filled into black ConeTainers® at 650 g per pot. Each of the six combinations of P source and inoculation were sown with three barley seeds of either the WT or the mutant. The resulting 12 treatments had three replicates and the 36 pots were set up in a randomized block design. Seedlings were thinned, 3 d after emergence, to two per pot. The experiment was conducted in a controlled environment chamber with 16 h–21°C day and 8 h–16°C night. Osram daylight lamps provided 550 µE m⁻² s⁻¹ PAR (400–700 nm). The plants grew for 4 wks from 9 March to 6 April 2003. Deionized water was added as required to maintain moisture content at 55% of water holding capacity by regular weighing. A solution of NH₄NO₃ was added to the pots 14, 21 and 24 d after sowing to provide a total of 150 mg N per pot.

#### Harvest and chemical analysis

Shoots and roots were harvested separately. Soil samples (c. 20 g f. wt) were collected for determination of hyphal length density and roots were washed. All plant samples were carefully washed with deionized water to remove adhering soil particles. Sub-samples of fresh roots were collected for the determination of AM colonization. D. wts of shoots and roots were determined after oven drying at 70°C for 48 h. Oven-dried subsamples were milled and digested in mixture of HNO₃ and HClO₄ (4 : 1 v/v) at 225°C. The dissolved samples were analysed for uranium (²³₈U) on a HR-ICP-MS (PlasmaTrace 2, Micromass) without any radiochemical treatment. The uranium isotope ²³³U was used as an internal
Phosphorus in plant samples was determined by ICP-OES (Vista axial, Varian).

Sub-samples of fresh roots were cleared in 10% KOH and stained with Trypan blue by a modification procedure of Phillips & Hayman (1970), omitting phenol from solutions and HCl from the rinse. Percentage root colonization and root length were determined by the grid line intersect method (Giovannetti & Mosse, 1980). External hyphae were extracted from soil samples using a modified membrane filter technique (Jakobsen et al., 1992). Duplicate 2 g soil samples were blended with 250 ml water and hyphae in 3 ml aliquots were collected on 25 mm membrane filters (1.2 µm) and stained with Trypan blue. Hyphal length was recorded in 25 random fields of view per filter. The length of stained hyphae on the filters was determined by the grid line intercept method at ×200 magnification (Tennant, 1975).

Statistical analysis

Data were subjected to three-way ANOVA to compare mycorrhizal status, barley genotypes and P application levels using GenStat for PC/Windows 6.1 (GenStat Committee, 2002).

Results

Colonization of roots and soil by G. intraradices

Phosphate rock reduced the proportion of root length colonized, but this effect coincided with an enhancement of root length (Table 1). By contrast, mycorrhiza development was markedly depressed by the soluble P treatment. Roots of the Brb mutant given nil P or PR had higher mycorrhiza colonization than the corresponding WT roots. Roots of non-inoculated plants remained uncolonized (Table 1).

Despite the decreased root colonization in response to P applications, there were no significant differences in the hyphal length density in soil among the different P treatments, while G. intraradices in association with Brb produced more extraradical hyphae than WT (P < 0.05) (Table 1). Some hyphae were also present in pots with non-inoculated plants, but P application or genotype had no significant influence in this case. These hyphae were probably dead or saprophytic and were assumed to be present also in the inoculated treatments.

Plant growth

In general, the WT barley produced higher d. wts than Brb (P < 0.001). The two barley genotypes responded differently to mycorrhiza and P applications (Fig. 1). In WT barley, shoot d. wt slightly increased with P applications, but was suppressed by mycorrhiza. The corresponding root d. wts were influenced neither by mycorrhiza nor P. On the other hand, the Brb mutant grew poorly without P and both shoot and root d. wts responded dramatically to the P supplies.

Furthermore, mycorrhiza increased root growth of the Brb amended with nil or PR (Fig. 1).

Root length of WT barley was unaffected by mycorrhizal colonization, while mycorrhiza increased root to shoot ratio (P < 0.001). There were significant interactions between P supply and genotypes in root length density, as root length and root to shoot ratio of Brb increased in response to both mycorrhizal colonization and the supply of P (P < 0.001; Table 1).

Plant phosphorus nutrition

Inoculation, P application and genotypes all had significant influences on P concentrations in both shoots and roots of barley plants (P < 0.001; Table 2). In general, the supply of PR or soluble P led to increased plant P concentrations, which were higher in WT barley than in the mutant. Mycorrhizal colonization decreased P concentration of WT at all P treatments and also for Brb given soluble P. Phosphorus concentrations of Brb plants markedly responded to mycorrhiza when nil P or PR was applied (Table 2).

Compared with nil P treatment, plant P content of both barley genotypes increased dramatically with application of PR or soluble P. Mycorrhizal colonization increased the P
Table 1  Mycorrhizal colonization rates, hyphal length density, root length and root to shoot ratio of wild type barley (*Hordeum vulgare*) (WT) and a root hair mutant (Brb) grown under different phosphorus applications

<table>
<thead>
<tr>
<th>Barley genotype</th>
<th>P application treatment</th>
<th>Root colonization (% of root length)</th>
<th>Hyphal length density (m g⁻¹)</th>
<th>Root length (m per pot)</th>
<th>Root to shoot ratio (g g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Non-inoculated</td>
<td>Inoculated</td>
<td>Non-inoculated</td>
<td>Inoculated</td>
</tr>
<tr>
<td>WT</td>
<td>PO</td>
<td>0</td>
<td>24.3</td>
<td>1.5</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>PR</td>
<td>0</td>
<td>18.9</td>
<td>1.2</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>P50</td>
<td>0</td>
<td>1.8</td>
<td>2.0</td>
<td>6.6</td>
</tr>
<tr>
<td>Brb</td>
<td>PO</td>
<td>0</td>
<td>88.2</td>
<td>1.1</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td>PR</td>
<td>0</td>
<td>69.1</td>
<td>2.0</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>P50</td>
<td>0</td>
<td>1.5</td>
<td>1.9</td>
<td>6.2</td>
</tr>
</tbody>
</table>

Significance of Inoculation (I) *** *** ns ***
P application (P) *** ns *** ***
Genotype (G) *** * *** ***
I × P *** ns ns ***
I × G *** ns ns ***
P × G *** *** ***
I × P × G *** * ***

Table 2  Phosphorus (P) and uranium (U) concentrations in wild type barley (*Hordeum vulgare*) (WT) and a root hair mutant (Brb) grown under different phosphorus applications

<table>
<thead>
<tr>
<th>Barley genotype</th>
<th>P application treatment</th>
<th>Shoot P concentration (mg g⁻¹)</th>
<th>Root P concentration (mg g⁻¹)</th>
<th>Shoot U concentration (ng g⁻¹)</th>
<th>Root U concentration (ng g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Non-inoculated</td>
<td>Inoculated</td>
<td>Non-inoculated</td>
<td>Inoculated</td>
</tr>
<tr>
<td>WT</td>
<td>PO</td>
<td>1.28</td>
<td>1.19</td>
<td>1.11</td>
<td>1.13</td>
</tr>
<tr>
<td></td>
<td>PR</td>
<td>2.68</td>
<td>2.64</td>
<td>2.30</td>
<td>2.23</td>
</tr>
<tr>
<td></td>
<td>P50</td>
<td>4.47</td>
<td>4.10</td>
<td>2.09</td>
<td>1.87</td>
</tr>
<tr>
<td>Brb</td>
<td>PO</td>
<td>1.03</td>
<td>1.41</td>
<td>1.47</td>
<td>2.87</td>
</tr>
<tr>
<td></td>
<td>PR</td>
<td>1.04</td>
<td>2.45</td>
<td>1.41</td>
<td>3.63</td>
</tr>
<tr>
<td></td>
<td>P50</td>
<td>3.03</td>
<td>2.91</td>
<td>2.05</td>
<td>1.99</td>
</tr>
</tbody>
</table>

Significance of Inoculation (I) *** *** ns ***
P application (P) *** *** ***
Genotype (G) *** *** ns ***
I × P *** ns ns ***
I × G *** ** ns ***
P × G *** *** ***
I × P × G *** ** ns ***

Table 1: P0, PR and P50 represent application of nil P, 2% (w/w) phosphate rock and 50 mg KH₂PO₄-P kg⁻¹ soil, respectively.

Table 2: P0, PR and P50 represent application of nil P, 2% (w/w) phosphate rock and 50 mg KH₂PO₄-P kg⁻¹ soil, respectively.

By ANOVA; ** P < 0.001; * P < 0.05; ns, not significant.
content of Brb plants receiving nil P or PR, whereas in other treatments, mycorrhizal plants contained less (or similar, only for WT under nil P application) P as compared to corresponding non-inoculated plants (Fig. 2).

Uranium uptake and partitioning

Shoot U concentrations in barley plants were significantly increased by the application of PR, but hardly affected by genotypes ($P = 0.394$) or mycorrhizal colonization ($P = 0.359$). In contrast, root U concentrations varied with genotype, inoculation and P application ($P < 0.001$). Root U concentrations were higher in the WT than in the mutant, and the mutant had higher root U concentrations in the presence than in the absence of mycorrhiza when nil P or PR was supplied (Table 2).

The U contents of barley shoots were affected by P supplies only ($P < 0.001$) (Fig. 3), but genotype and inoculation interacted significantly ($P < 0.05$). This was caused by presumably contrasting mycorrhiza effects on U content in shoots of Brb and WT barley supplied with PR. Root U contents were influenced by both P application and genotype ($P < 0.001$). Wild type barley accumulated much more U in its roots than Brb. Significant interactions between genotype and inoculation ($P < 0.001$) indicated that mycorrhizal colonization increased root U content of Brb but decreased that of WT barley. The U uptake efficiency, estimated as uptake per unit root length, was clearly highest for the PR treatment and was likewise higher for WT than for Brb barley (Table 3). The presence of mycorrhiza also enhanced U uptake efficiency, but only in the Brb mutant.

Generally only a small fraction (< 15%), of U had been translocated from roots to shoots of barley plant. Non-inoculated Brb had much higher shoot to root ratios than the corresponding inoculated treatments (Fig. 4). This was also true for the WT barley except for the PR treatment.

Discussion

This work is the first to demonstrate mycorrhizal effects on U uptake from U-contaminated soil by a crop plant and subsequently on U translocation from roots to shoots. The root hairless barley used in present work also represented a powerful tool to investigate the relative contribution of root hairs and mycorrhizal fungi to U uptake.

Mycorrhiza formation is influenced by the P nutrition status of the host plant (Thomson et al., 1991) and root colonization in the present study was accordingly reduced in P fed barley plants. Root colonization by G. intraradices did not
enhance P uptake and growth of WT barley at any P treatment and this confirms that growth of barley with well-developed root hairs is generally not aided by mycorrhiza (Fay et al., 1996; Plenchette & Morel, 1996; Khaliq & Sanders, 2000) except under extreme P deficiency (Jakobsen, 1983; Baon et al., 1993). On the other hand, the mycorrhiza responsiveness in plant P uptake in the root hairless Brb mutant is in accordance with our previous characterization of the mutant with respect to mycorrhiza (I. Jakobsen, unpublished data). Although the enhanced P uptake in mycorrhizal Brb plants (nil P and PR treatments) did not result in increased shoot growth, we conclude that mycorrhiza can be essential to support growth of plants root with none or poorly developed root hairs. The mycorrhiza status is therefore essential if such plant species are selected in, e.g. revegetation and phytoremediation programmes.

For non-mycorrhizal plants uptake of U was much higher in WT barley with root hairs than in Brb, irrespective of P treatments. On the other hand, Brb had a higher U uptake in the presence than in the absence of G. intraradices. The important role of AM fungus and root hairs in U uptake become more obvious from their impact on specific U uptake efficiencies. The higher U uptake efficiency of normal barley roots compared with that of hairless roots of Brb and the improved U uptake efficiency of Brb by G. intraradices, could explain the differences in U uptake among treatments. In general, an enlarged absorbing area provided by root hairs or mycorrhiza will be of key importance in plant uptake of U.

Less than 15% of the total U uptake was transferred to the shoots suggesting the presence of a specific mechanism in barley for retaining U in roots. This is consistent with other studies showing that plant species differ in U accumulation, and that this accumulation predominately occurs in the roots (Sheppard & Thibault, 1984; Zafrir et al., 1992; Saric et al., 1995). Non-inoculated Brb plants had markedly higher shoot to root ratios of total U uptake than the corresponding inoculated treatments, and Brb had general higher ratios than WT. This means that U partitioning to shoots was inhibited by the presence of root hairs or AM fungus. Such results support previous findings that mycorrhizal fungi significantly contributed to the U immobilization by mycorrhizal roots (Rufyikiri et al., 2003). Hyphae of G. intraradices had higher U flux rates than host roots (Rufyikiri et al., 2003) and U accumulation was observed in fungal vesicles of mycorrhizal Cynodon dactylon.

### Table 3 Root uranium (U) uptake efficiency of wild type barley (Hordeum vulgare) (WT) and a root hair mutant (Brb) affected by phosphorus (P) application and inoculation treatments

<table>
<thead>
<tr>
<th>Barley genotype</th>
<th>P application treatment</th>
<th>Root U uptake efficiency (ng m⁻¹)</th>
<th>Inoculated</th>
<th>Non-inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>P0ᵃ</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>PR</td>
<td>12.6</td>
<td>11.5</td>
<td>11.5</td>
</tr>
<tr>
<td></td>
<td>P50</td>
<td>0.73</td>
<td>0.84</td>
<td>0.84</td>
</tr>
<tr>
<td>Brb</td>
<td>P0</td>
<td>0.12</td>
<td>0.28</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>PR</td>
<td>2.59</td>
<td>5.51</td>
<td>5.51</td>
</tr>
<tr>
<td></td>
<td>P50</td>
<td>0.33</td>
<td>0.29</td>
<td>0.29</td>
</tr>
</tbody>
</table>

**Significance** of inoculation (I), P application (P), Genotype (G), I × P, I × G, P × G, and I × P × G by ANOVA.

ᵃP0, PR and P50 represent application of nil, 2% (w/w) phosphate rock and 50 mg KH₂PO₄-P kg⁻¹ soil, respectively.

ᵇBy ANOVA; *** P < 0.001; * P < 0.05.
roots (Weiersbye et al., 1999). Similarly, using a compartmented pot system, Joner & Leyval (1997) demonstrated that $^{109}$Cd added to the hyphal compartment was adsorbed by extraradical hyphae and subsequently transported to plant roots, but transfer from fungus to plants was restricted by fungal immobilization. The extensive fungal colonization of Brb would have contributed to U immobilization in the fungal tissue and lowered U concentration in shoots. Moreover, the lower shoot to root ratios in U content of non-inoculated WT than non-inoculated Brb provided evidence for the similar impacts of root hairs and AM fungi in U partitioning. The negative impact of the PR and P50 treatments on partitioning of U to shoots in non-mycorrhizal Brb plants in particular corresponded to a higher root-shoot ratios in P treated plants.

Additionally, U speciation is known to be highly pH-dependent (Langmuir, 1978; Mortvedt, 1994; Ebbs et al., 1998). It was established that soluble uranyl cations or uranyl-sulfate species that are stable under acidic conditions were translocated to a higher extent to roots through fungal tissues, while phosphate and hydroxyl species dominating under acidic to near neutral conditions or carbonate species dominating under alkaline conditions were rather immobilized by hyphal structures (Rufyikiri et al., 2002). The results obtained under soil pH 6.7 in present work confirmed this immobilization processes. Under solution culture conditions Ebbs et al. (1998) demonstrated that U was presumably taken up predominantly as the free uranyl cations at pH 5.0, as at this pH the U content and concentration were the highest and lowest in shoots and roots, respectively. It deserves further in vivo studies to investigate the role of mycorrhizal fungi in U uptake and redistribution under acid conditions.

Phytoremediation received much research interests in recent years (Salt et al., 1998; McGrath & Zhao, 2003) and attention was also paid to phytoremediation of radionuclides (Zhu & Shaw, 2003; Dushenkov, 2003). Obviously, the potential importance of arbuscular mycorrhiza in phytoremediation of metal–contaminated soils should be taken into account due to its ubiquity in natural ecosystems (Smith & Read, 1997). It is generally agreed that at high concentrations of any nonessential metals AM fungi often improve plant resistance against toxic effects by enhancing metal retention in the roots, thereby reducing metal concentrations in shoots (Leyval & Joner, 2001). Consequently, arbuscular mycorrhiza could possibly help phytoremediation of metal contaminated sites (Leyval et al., 2002). Phytostabilization is proposed as an alternative management practice when it is considered impossible to clean up heavily contaminated environments or waste deposits. The present study demonstrates a potential role of arbuscular mycorrhiza in such phytostabilization of U contaminated environments, possibly by reducing U translocation in the plant-soil continuum and food chains, thereby reducing environmental risks. However, there is still a need to carry out systematic studies to screen suitable plant species/genotypes and compatible symbionts, and to understand the function of critical factors influencing U acquisition.

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**References**


