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Comparison of ^{233}U and ^{33}P uptake and translocation by the arbuscular mycorrhizal fungus *Glomus intraradices* in root organ culture conditions

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Abstract This study aimed to quantify and compare ^{233}U and ^{33}P uptake and translocation by hyphae of the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* in root organ culture conditions with transformed carrot (*Daucus carota* L.) roots as host. Mycorrhizal roots were grown in two-compartment Petri dishes to spatially separate a root compartment (RC) and a hyphal compartment (HC). The HC was labelled with 8.33 Bq ^{233}U ml^{-1} and 13.33 Bq ^{33}P ml^{-1}. After 2 weeks contact between hyphae and the labelled solution, ^{233}U and ^{33}P activities were measured in the RC and in the HC. ^{233}U and ^{33}P were taken up by the extraradical AM mycelium grown in the HC and this uptake represented 4.4% and 16% of the initial isotope supply, respectively. The translocation into roots developing in the RC via hyphae accounted for 5.9% and 72% of the initial isotope supply, respectively. Thus, both uptake and translocation were much higher for ^{33}P than for ^{233}U. This suggests (1) the existence in hyphal tissues of efficient mechanisms limiting the uptake and translocation of non-essential elements such as U, and (2) that the hyphae have a higher sequestration than translocation function for U, and the converse for P.

Keywords Arbuscular mycorrhizal fungus · Ri T-DNA transformed roots · Uranium · Phosphorus · Uptake

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Introduction

There is excellent evidence that the extraradical mycelium network of arbuscular mycorrhizal (AM) fungi take up essential nutrients from the soil and translocate them to plants, thus extending the rhizosphere exploration zone (for references see Jakobsen et al. 2002; Marschner 1995; Smith and Read 1997). Beside this key nutritional function, the hyphal network of AM fungi is directly involved in uptake of some non-essential heavy metals (Leyval and Joner 2001) and radionuclides (Declerck et al. 2003; Entry et al. 1999; Strandberg and Johansson 1998), which is of considerable interest for the management of polluted environments (Colpaert and Vandenkoornhuyse 2001; Leyval and Joner 2001).

Storage of waste material containing uranium (U) from mining and milling of ores poses considerable environmental problems (Shahandeh et al. 2001) through the risk of irradiation of both humans and animals due to uncontrolled radionuclide dispersion or leaching into food chains and/or water reservoirs. Remedial action is required to optimize the socio-economical value of U-contaminated sites. Long-term rehabilitation of U-contaminated sites using techniques of phytoremediation by plants and associated microbiota is now an interesting option (Huang et al. 1998; Shahandeh et al. 2001). However, a better understanding and quantification of the contribution of mycorrhizal symbionts to U bio-availability is a prerequisite for any phytoremedial option involving AM fungi.

Recently, a two-compartment root organ culture system (see review by Fortin et al. 2002) was used to study the effects of the extraradical mycelium of the AM fungus *Glomus intraradices* (MUCL 41833) on the uptake and translocation of ^{233}U (Rufyikiri et al. 2002, 2003). The authors demonstrated the capacity of extraradical mycelium to take up ^{233}U and to translocate it to the host root. The magnitude of uptake was influenced by U speciation, which is highly pH-dependent, while translocation was highly correlated with the amount of hyphae connecting the U source to the host root (Rufyikiri et al.

2002). In addition, hyphae appeared more efficient in the uptake and translocation of ^{233}U than carrot roots (Rufyikiri et al. 2003). Although uptake and translocation accounted for 5% and 11%, respectively, of the initial U supplied (Rufyikiri et al. 2003), the efficiency of this uptake and translocation has not been compared to that of an essential element such as phosphorus (P). A relatively high hyphal efficiency for P uptake and translocation was reported in numerous studies and some reviews give several references on the topic (Jakobsen et al. 2002; Marschner 1995). Such a comparison could give appreciable information on the relative extent of U uptake and translocation by fungal hyphae and on the real role of extraradical mycelium where management strategies of U-contaminated sites involve plants and AM fungi.

The present study aimed to quantify and compare ^{233}U and ^{33}P uptake and translocation by the external hyphae of the AM fungus *G. intraradices* in root organ culture conditions. Such comparisons may be useful for validating the adequacy of the experimental system and quantifying the relative contribution of the hyphae in U immobilization outside and within mycorrhizal plants.

Materials and methods

Glomus intraradices Schenck and Smith (MUCL 41833) was used for the experiment. The root organ cultures were established in association with *Agrobacterium rhizogenes* (Ri T-DNA)-transformed carrot (*Daucus carota* L.) roots on modified Strullu-Romand (MSR) medium (Declerck et al. 1998, modified from Strullu and Romand 1986), as previously described (Rufyikiri et al. 2003). Briefly, carrot roots inoculated with *G. intraradices* were grown in a two-compartment system in Petri dishes separating the central root compartment (RC) from a surrounding external hyphal compartment (HC). The Petri dishes were incubated horizontally in an inverted position at 27°C in the dark for 3 weeks. Thereafter, they were set upright and the HC was filled with 25 ml liquid MSR without sucrose and vitamins and the dishes cultured for an additional week. The cultures were then ready for ^{233}U and ^{33}P labelling.

Liquid MSR medium (15 ml) without sucrose and vitamins, but labelled with 8.33 Bq ^{233}U ml^{-1} (= 0.1 μM) and 13.33 Bq ^{33}P ml^{-1}, was added to the HC after removing the old medium with a pipette. Information on the source of U is given in Rufyikiri et al. (2002). The source of ^{33}P was orthophosphate in dilute hydrochloric acid (<0.1 M) supplied by Amersham Pharmacia Biotech (UK) with a specific activity of 110 TBq $mmol^{-1}$ P and >99% ^{33}P purity (<1% ^{32}P). The stable P concentration in the liquid MSR medium was 50 μM. The pH of the labelled liquid medium was adjusted to 5.5 with 0.01 M NaOH before sterilization at 121°C for 15 min. At this pH, the saturation index log (Q/K, where Q = ion activity product and K= solubility constant) was <0, indicating undersaturation of the solution, and the calculated U speciation showed the dominant forms to be UO_2HPO_4 (aq.), $UO_2(OH)_2$ (aq.), UO_2OH^+, UO_2SO_4 (aq.), UO_2^{2+} and $UO_2PO_4^-$, representing 39.3, 24.0, 11.0, 10.5, 9.1 and 6.4% of total U, respectively, according to Rufyikiri et al. (2002).

The hyphae were maintained in contact with the solutions for 2 weeks. The limitation of capillary action, the control of metabolic activity of hyphae by formaldehyde and the control of contamination of the RC by experimental manipulations were performed as described previously (Rufyikiri et al. 2002, 2003).

At the end of the experiment, the total extraradical hyphal length, the number of spores, and the total root length were estimated in the RC using a 10-mm intersection grid method as previously described (Rufyikiri et al. 2002). The number of hyphae crossing the partition between the RC and the HC was also assessed. The pH and the ^{233}U and ^{33}P activities in the HC compartment solution were measured and the solution then removed. The compartment was rinsed three times with 15 ml distilled water before the developing hyphae were cut at the edge where they crossed the barrier and collected. For the RC, roots and the gel containing extraradical hyphae and spores were collected. The fresh weights of the different samples were measured. Roots were then divided into two sub-samples, one for assessment of ^{233}U and ^{33}P activities and the other for the measurement of root AM fungal colonization. For the first part, roots, mycelium and gel were placed separately into 20-ml glass scintillation vials, calcined at 550°C for 24 h, and the ashes dissolved in 0.1 M HCl. Aliquots (10 ml) of liquid scintillation cocktail (Wallac, OptiPhase HiSafe 3) were added to 5-ml aliquots of all solutions and ^{233}U and ^{33}P activities determined by Packard Tri-Carb 1600TR liquid scintillation counting. Values were corrected for the decay of ^{33}P. The counting efficiency was 100% for both radionuclides. Backgrounds of 0.06 ± 0.02 Bq for ^{233}U and 0.48 ± 0.16 Bq for ^{33}P were subtracted from the sample values.

The second part of the roots was prepared for microscopic examination to determine the frequency (%F) and intensity (%I) of AM fungal colonization as previously described (Rufyikiri et al. 2002, 2003).

Statistical analysis of data was performed with the statistical software Statistica for Windows (StatSoft 2001). Groups of data were compared by ANOVA; significant differences were considered at $P \leq 0.05$ and mean values were ranked by Scheffé's multiple-range test.

Results

Solution pH

The pH of the solution in the HC measured at the end of the experiment was significantly higher than the initial pH of 5.5 measured in the absence of hyphae (Fig. 1). When treated with formaldehyde, the pH measured in the HC did not differ significantly from the initial pH. The pH measured in the external root-free compartment of non-mycorrhizal cultures also did not change significantly (mean of 5.6 ± 0.2).

Root and AM fungal biomass

The roots grew and ramified in the MSR medium in the RC with a root length and a root fresh weight of, 215 ± 56 cm and 853 ± 37 mg per Petri dish, respectively, at the end of the experiment (means ± SD). The roots were highly colonized by the AM fungus, with values for %F and %I of 85 ± 12 and 27 ± 3, respectively. Hyphae length and number of spores developing in the RC were 1075 ± 203 cm and 4726 ± 127, respectively. Numerous hyphae, 134 ± 38, crossed the partition between the two compartments and developed in the HC. Once in contact with the liquid MSR medium, a well-branched mycelium developed and numerous spores were produced, with a fungal fresh weight of 19 ± 4 mg per Petri dish.

Formaldehyde in the medium killed the hyphae in the HC and no further development was observed. The

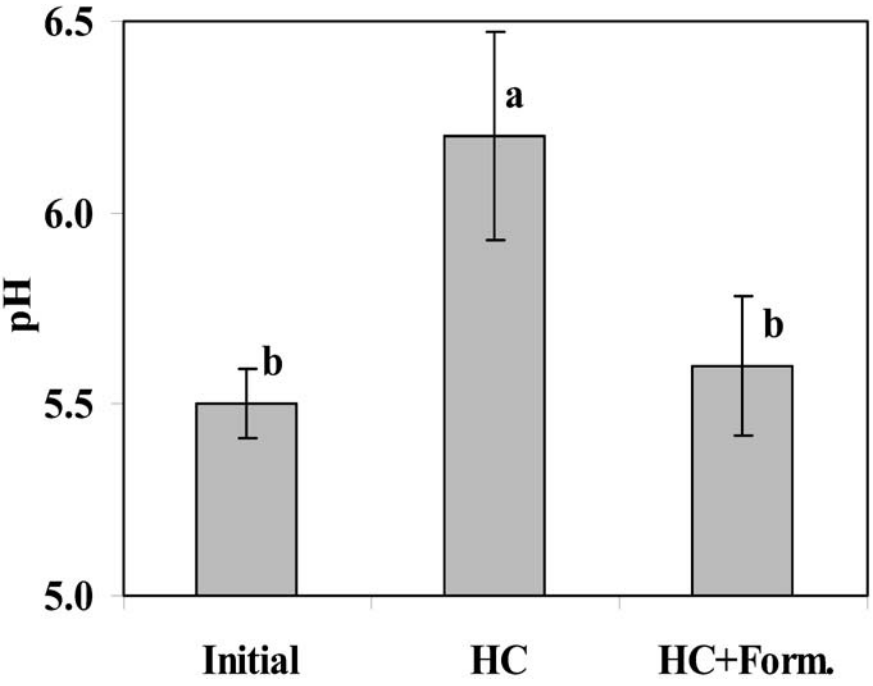

Fig. 1 pH values of the solution before (*Initial*) and after 14 days of contact between hyphae and the labelled solution in the hyphal compartment without (*HC*) and with formaldehyde (*HC+Form.*). Values are means and bars represent standard deviations ($n = 6$). Means followed by the same letters are not significantly different ($P \leq 0.05$)

Table 1 Uranium (*U*) and phosphorus (*P*) absolute activities, specific activities and activity ratios (Bq P/Bq U) for Ri T-DNA transformed carrot (*Daucus carota* L.) roots grown for 2 weeks in association with *Glomus intraradices* in a two-compartment system. Samples from the hyphal compartment (HC) consisted of solution, hyphae and spores, and from the root compartment (RC) of the gel with fungal biomass and the mycorrhizal roots. The total absorption consists of uptake by hyphae and spores in HC plus the amount translocated from the HC to the RC and located in the mycorrhizal roots and the gel with fungal biomass in RC. Values are means ± standard deviations of 6 replicates (*n.d.* not detectable)

Treatment	Absolute activity (Bq per Petri dish)				Specific activity (Bq g^{-1} fresh wt. or Bq ml^{-1})		Ratio
	^{233}U		^{33}P		^{233}U	^{33}P	
	Mean	% Input	Mean	% Input			
Without formaldehyde in HC							
Input solution	125 ± 0.1	100	200 ± 0.2	100	8.33 ± 0.01	13.33 ± 0.01	1.6
Final solution	106 ± 6	85	9 ± 2	4.5	7.07 ± 0.42	0.60 ± 0.08	0.08
Hyphae/spores	5.5 ± 1.3	4.4	32 ± 2	16	289 ± 45	1,684 ± 95	5.9
Without formaldehyde in RC							
Gel/fungal mass	4.9 ± 0.3	3.9	14 ± 2	7	0.33 ± 0.06	0.93 ± 0.3	2.9
Mycorrhizal roots	7.4 ± 1.2	5.9	143 ± 12	72	8.6 ± 0.9	168 ± 14	20
Total absorption	17.8	14.2	189	95	–	–	–
With formaldehyde in HC							
Final solution	124 ± 0.5	99	197 ± 1	98.3	8.26 ± 0.4	13.13 ± 0.08	1.6
Hyphae/spores	0.92 ± 0.1	0.7	0.32 ± 0.11	0.16	541 ± 43	188 ± 21	0.35
With formaldehyde in RC							
Gel/fungal mass	0.10 ± 0.2	0.08	0.24 ± 0.2	0.12	0.007 ± 0.004	0.016 ± 0.01	2.3
Mycorrhizal roots	n.d.	–	0.20 ± 0.1	0.1	n.d.	0.56 ± 0.3	–

numbers of hyphae that crossed the partition between the two compartments and the hyphal fresh weight in the HC were 63 ± 18 and 1.7 ± 0.8 mg per Petri dish, respectively, and no spores were formed.

^{233}U and ^{33}P activities

At the end of the experiment, 14.2% of the initial ^{233}U supply in the HC had been absorbed and 4.4% was located in the AM fungal mycelium developing in the HC. The remaining 9.8% was found in the RC, i.e. in the gel with fungal biomass (3.9%) and in the mycorrhizal roots (5.9%) (Table 1). The translocation of ^{233}U by hyphae from the HC to the RC amounted to 69% of total U

absorption (9.8% out of the 14.2%). The ^{33}P activity in the fungal mycelium developing in the HC and in mycorrhizal roots and gel in the RC amounted to 95% of the initial supply in the HC, and 83% of this total absorption was translocated to the RC. Both ^{33}P uptake by the fungal mycelium developing in the HC and its translocation from the HC to the RC were much higher than for ^{233}U. Indeed, the $^{33}P/^{233}U$ activity ratio decreased from 1.6 in the input solution to 0.08 after 2 weeks of contact with the mycelium, while it increased to 5.9, 2.9 and 20 in the fungal mycelium, gel with fungal biomass and mycorrhizal roots, respectively.

The activities in formaldehyde-killed hyphae were 1.9 times higher for ^{233}U and 9 times lower for ^{33}P than in living hyphae. ^{233}U and ^{33}P were observed at very low activities in the gel with fungal biomass, while only ^{33}P was detected at low activity in roots developing in the RC. ^{233}U and ^{33}P were not detected in the RC in control cultures without hyphae.

Discussion

A two-compartment root organ culture system recently improved to allow large hyphal biomass production in the labelled hyphal compartment (Rufyikiri et al. 2003) was used to quantify and compare the uptake and translocation of ^{233}U and ^{33}P by extraradical fungal hyphae of *G. intraradices*. Phosphorus was chosen for this comparison because numerous studies have demonstrated without ambiguity its high translocation by fungal hyphae both in vivo (Jakobsen et al. 2001; Li et al. 1991; Pearson and Jakobsen 1993) and, more interestingly, in root organ culture conditions (Joner et al. 2000; Koide and Kabir 2000.; Maldonado-Mendoza et al. 2001; Nielsen et al. 2002).

The uptake of ^{233}U by the extraradical mycelium developing in the HC and its translocation from the HC to the RC were of the same order as that previously reported (Rufyikiri et al. 2003), but much lower than for ^{33}P. The high P uptake and translocation indicated that the system was functional and that the low uptake and translocation of U were not due to an experimental artefact. Thus, fungal hyphae are less efficient at taking up and translocating U than P.

The high P translocation observed in this study corroborated previous observations that P translocation by extraradical mycelium was much more efficient than for other elements, both macro- and micronutrients (Jakobsen et al. 2002; Marschner 1995). Comparing the uptake and translocation of ^{32}P, ^{65}Zn and ^{35}S by hyphae of *G. mosseae*, Cooper and Tinker (1978) reported a similar, relatively high efficiency for ^{32}P, while the rate of ^{65}Zn translocation was very low. As suggested by Bago et al. (2002), differences between elements indicate that sophisticated metabolic processes occur along hyphae to regulate both uptake and translocation of elements in AM fungi.

Uranium has no known role in plant nutrition and, like other heavy metals, is tolerated in small quantities but results in toxicity when accumulated to high concentration (Ebbs et al. 2000). Some reports have shown that mycorrhizal fungi markedly alleviate metal toxicities by reducing metal uptake into mycorrhizal plants (Joner and Leyval 1997; Rufyikiri et al. 2000). Beside the sequestration of metals in intraradical fungal hyphae (Joner and Leyval 1997; Weiersbye et al. 1999), often evoked as a mechanism of AM fungal protection against metal toxicity for plants, a limited translocation of heavy metals by the extraradical hyphae into host roots might also be important for reducing heavy metal exposure of host root tissues. This was observed for ^{233}U relative to ^{33}P in this study and also for ^{65}Zn relative to ^{32}P by Cooper and Tinker (1978). However, the mechanisms by which the symbiotic partners distinguish between nutrients and non-essential trace elements or between adequate and excess amounts of a given element, leading to selective uptake and translocation by hyphae, remain unknown.

In a previous study (Rufyikiri et al. 2003), we observed that Cu-extractable U represented only a small fraction (15%) of the total U content of mycelium. It was suggested that the formation of stable complexes or precipitates was the main mechanism of U accumulation in fungal hyphae in contact with U, and this was assumed to contribute to the low translocation of U. The present study showed a corresponding higher accumulation rather than translocation of U in the extraradical hyphae relative to P, as indicated by the P/U ratio, which was more than 3 times higher for translocation than for the uptake. Various mechanisms may be involved, including high affinity of U with hyphal functional groups (hydroxyl, phosphate, carboxylic and amino), resulting in complexation and precipitation. Uranium was shown to accumulate both extracellularly on the cell wall surface and intracellularly throughout the cytoplasm of *Saccharomyces cervisiae* (for references, see Suzuki and Banfield 1999). Interaction of uranyl ions with amino-ligands and polymers such as chitin and chitosan by complexation and adsorption has been reported by other authors (Guibal et al. 1996) and chitin is known to be a component of the walls of extraradical and intraradical hyphae (Smith and Read 1997). However, in this study, the pH conditions (5.3–6.5) favoured the formation of neutral uranyl phosphate and hydroxyl species and only a small fraction of the total U in the solution was present in cationic form (UO_2^{2+} and UO_2OH^+) (Rufyikiri et al. 2002). This would impair the bio-adsorption of U as neutral and anionic complexes might interact weakly with the negatively charged cell surface (Suzuki and Banfield 1999). Therefore, formation of stable complexes and precipitation of U, for instance as uranyl phosphate, seems to be the main mechanism of U accumulation in hyphae.

In conclusion, this study has provided for the first time fundamental information on the relative contribution of the AM fungus *G. intraradices* to the uptake and translocation into plant roots of U (a non-essential and toxic element) versus the essential nutrient P. The high

efficiency at which hyphae took up and translocated P indicates the adequacy of the experimental system. Both U uptake and translocation by external hyphae were much less important than that of P, due to differences in chemical behaviour responsible for interactions with various components of the fungal mycelium. Uranium showed relatively more uptake than translocation when compared to P, suggesting that hyphae have higher sequestration than translocation for U and the converse for P.

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