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SHORT NOTE

Conidia of *Trichoderma virens* as a phosphorus source for mycorrhizal *Pinus sylvestris* seedlings

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Abstract In this study, the mobilization and further translocation of phosphorus from conidia of saprotrophic fungus *Trichoderma virens* into *Pinus sylvestris* seedlings by nondestructive measuring of ^{32}P was assessed. The radioactive phosphorus flux from the conidia to the Scots pine seedlings forming mycorrhiza with *Laccaria laccata* and *Suillus bovinus* amounted up to 27.82% and 7.42%, respectively, on the 28th day of the experiment, while at the same time in nonmycorrhizal pine seedlings, the detected radioactivity reached only 0.56%. Our studies revealed that both ectomycorrhizal fungi: *L. laccata* and *S. bovinus*, mobilized the phosphorus from radioactive conidia of *T. virens*. On this basis, we conclude that activities of the mycosymbionts may facilitate absorption and further translocation of phosphorus from organic matter into the host plants.

Keywords *Laccaria laccata* · *Suillus bovinus* · *Trichoderma virens* · Mobilization of ^{32}P · Pine seedlings

Introduction

Elucidation of competitive interactions between ectomycorrhizal (ECM) and saprotrophic fungi when foraging for nutrients is necessary in understanding nutrient cycling in forest soils (Lindahl et al. 1999, 2001, 2002; Leake et al. 2002). Although only a limited number of studies focus on the importance of interactions between these two groups of fungi in terms of the nutrient economy, there is a developing interest among scientists in such nutrient mobilization (Cairney 2005). In view of the current knowledge, apart from absorption of easily available nutrients from the soil (Smith and Read 1997), the ECM fungi can mobilize and utilize organic forms of nutrients from forest floor debris (Read and Perez-Moreno 2003), which can strongly influence nutrient movement in the soil.

Many evidences suggest that fungal spores are considerable components of fungal biomass in soil and should receive more attention as they play essential role in ecosystem function. On average, the amount of spores produced by the fungi may vary from 7×10^{12} in case of *Calvatia gigantea* (Batsch) Lloyd up to 30×10^{9} that *Ganoderma applanatum* (Persoon) Patouillard may discharge per day from May to September and a 2.5 cm colony of *Penicillium* sp. which may contain 400×10^{7} conidia (Ingold 1971). Moreover, many reports indicate large amounts of conidia produced by *Trichoderma* species (Papavizas 1985; Polyanskaya et al. 1994). Because spores constitute a significant part of the total fungal biomass in soil, they may be an important source of phosphorus for mycorrhizal plants. According to Bååth and Söderström (1979), 20% of the total phosphorus content in the soil is contained within fungal biomass. The mechanisms facilitating ECM fungal transfer of nutrients from organic pools stored in living organisms to host plants are poorly understood. Klironomos and Hart (2001) and Lindahl (2001) suggested that ECM fungi may act as predators increasing death rates of animals and soil fungi. This is in agreement with recent studies showing a considerable degree of antagonism by a wide range of ECM fungi

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towards saprotrophic fungi (Cairney and Meharg 2002; Mucha et al. 2006, 2007). With the exception of the studies mentioned above and our previous work describing mycoparasitism of *Laccaria laccata* (Scop. ex Fr.) on saprotrophic fungi (Werner et al. 2002; Werner and Zadworny 2003; Zadworny et al. 2004), little is known about this phenomenon. Parasitism of *L. laccata* on *Trichoderma virens* (Mill. Gidd. et Foster) von Arx and *T. harzianum* (Rifai) resulting in destruction of the host cells was described by Zadworny et al. (2007). Because such activity may be essential for plant nutrition, it is necessary to assess the antagonistic potential of the other ECM fungi competing with soil saprotrophs.

Until now, most studies examining mobilization of nutrients have been done in nonsterile soil microcosms (Bending and Read 1995; Lindahl et al. 1999, 2001; Perez-Moreno and Read 2001a, b). On the contrary, our studies were performed under axenic conditions in order to provide more conclusive evidence for mobilization and translocation of phosphorus from conidia of *T. virens* by mycorrhizal fungi into *Pinus sylvestris* (L.) seedlings prior to mineralization by other soil organisms. We also examined whether the presence of the ECM fungi was positively associated with the occurrence of exudates containing phosphorus from the spores of the saprotrophic fungus.

Materials and methods

Organisms and growth conditions

The standard sources of *L. laccata* (Scop. ex Fr.), strain (9–1), and *Suillus bovinus* (Fr.), Kuntze, strain (15–4), were 1-month-old mycelia growing on Pp agar medium (Pachlewski 1983) at 24°C in the dark. The strains were isolated from basidiocarps under *P. sylvestris* in Poland. *T. virens* (Mill. Gidd. et Foster) von Arx, strain Z 49 originated from an agricultural soil near Poznań in Poland and was maintained on potato dextrose agar (Difco, Detroit, MI, USA) at 24°C. *P. sylvestris* L. seedlings from the province Bolewice in Poland (52°28′ N and 16°03′ E) were used in the study.

Obtaining of mycorrhizal pine seedlings

Pine seeds were soaked in water, surface-sterilized with 0.2% $HgCl_2$ (*w/v*; Polish Chemical Reagents, Gliwice, Poland) for 3 min, and washed three times (5 min each) in sterile distilled water. Seeds were germinated on 0.6% water agar (*w/v*; Difco, Detroit, MI, USA) in the dark. Subsequently, they were aseptically transferred and placed between glass walls of 300×30 mm test tubes and the rolled inside them filter paper (17×13 cm in size; paperweight 69 g/m^2, water flow 10 ml H_2O/35 s; Polish Chemical Reagents, Gliwice, Poland). The test tubes contain 100-ml growth substratum (sterile mixture of peat and perlite, 1:3 *v/v*) moistened with 50 ml of liquid Pp medium (Pachlewski 1983) with 1.25 g·L^{-1} of glucose and the concentration of other compounds reduced to 1/5. One liter of the standard medium contains 20 g glucose, 5 g maltose, 0.44 g $(NH4)_2C_4H_4O_6$, 1 g KH_2PO_4, 0.5 g $MgSO_4 \times 7H_2O$, 0.5 ml 1% solution of $C_3H_4(OH)(COOH)_3Fe \times H_2O$, 0.5 ml 0.2% solution of ZnSO4, and 50 μg thiamine-HCl (Polish Chemical Reagents, Gliwice, Poland). Three pieces (5×5 mm) of 1-month-old mycelial mats, either of *L. laccata* or *S. bovines*, were placed into the test tubes close to the roots. Control plants were nonmycorrhizal seedlings of the same age treated in the same way. All seedlings were incubated for 3 months in a growing room under lighting emitted by fluorescent tubes (Osram L36/W77 Flora; 100 μEm^{-2} s^{-1}) 16 h a day, 60% RH at 24:20°C day to night temperature ratio. Only seedlings with a large proportion of colonized roots (more than 90%) and well-developed extraradical mycelium were chosen for further experiments.

Preparation of radioactive conidia

Erlenmeyer flasks contained 50 ml of Pp agar medium, which was supplied with radioactive phosphorus in the form of $Na_2H^{32}PO_4$ (The Institute for Nuclear Studies, Świerk, Poland) at a quantity of 32.266 ng instead of KH_2PO_4, so this experiment was carrier free. Five discs (5 mm in diameter) of 2-week-old mycelial mat of *T. virens* were placed on solid media in the flasks as inoculum. As a control, colonies of the saprotrophic fungus were maintained on Pp agar medium supplied with nonradioactive phosphorus (as Na_2HPO_4 at the same quantity).

Conidial suspension was obtained by adding 75 ml of sterile distilled water to the flasks containing colonies of *T. virens*, followed by 1 h of shaking at 350 rpm. The conidial suspension of radioactive and nonradioactive fungus was flooded out on a Particle Track-etched Membrane (PTM; 10 μm thick, 0.3 μm size of the mesh, 0.24 MPa resistance to breakdown; The Institute for Nuclear Chemistry and Technology, Warsaw, Poland) and rinsed five times with 10 ml of sterile distilled water. Conidia were then suspended in sterile distilled water. To verify that ^{32}P did not increase mortality of the conidia, their germination was checked the following day under a light microscope. Suspensions of radioactive and nonradioactive conidia were placed on triplicate Petri dishes (Pol Plastic Gosselin, Gniezno, Poland) containing Pp medium and then incubated at 22°C in the dark.

Translocation of ^{32}P to *P. sylvestris* seedlings

Mycorrhizal and nonmycorrhizal seedlings were inoculated with 1 ml of radioactive conidial suspension using 20-cm-long needles (Madagay, Hungary). Radiolabeled conidia were injected between the filter paper and the glass walls of the test

tubes directly onto extraradical mycelium or on the mycorrhizal roots. In total, 13 plants—five colonized by *L. laccata*, five by *S. bovinus*, and three nonmycorrhizal plants—were inoculated with suspension of the radioactive conidia of *T. virens*. This experiment was repeated twice.

The distribution of ^{32}P in plants was measured nondestructively, starting on the first day after inoculating the roots of seedlings with the radioactive conidial suspension. Radioactivity was measured on living plants, and each plant was used all along the experiment. Counting of the radioactivity with the SSU-3W (POLON, Bydgoszcz, Poland) scintillator NaJ(TI) (4.5×4 cm) was reduced to 1 cm by a Pb collimator (Gorączko and Koczorowska 1997). Collimator "field of waves" was 1 cm of plant length. Scintillation probe was connected to the counter of the radioactivity URS-3 (POLON, Bydgoszcz, Poland). To estimate the dynamics of ^{32}P transfer from conidia to pine seedlings, the radioactivity was counted for 100 s with a resolution of 1 cm from roots to shoots of pine seedlings and further to their buds and needles. Construction of described system is presented on Fig. 1. Radioactivity measurements were corrected for background activity and ^{32}P decay.

Quantification of inorganic phosphorus exuded from conidia

To determine whether ^{32}P bound in the conidia was actively mobilized by mycelia of *L. laccata* and *S. bovinus* or simply exuded by conidia, the radioactivity of the growth substratum in 300 ml jars (Sigma-Aldrich, St. Louis, MO, USA) supplied with radioactive conidial suspension, but containing no plants, was measured. In this experiment, glass jars were filled with 50 ml of moist growth substratum (mixture of peat and perlite, 1:3 *v/v*). Two rectangular in shape bags (6×4 cm) constructed from PTM membrane, filled with the same substratum, were added aseptically to each jar. Then, the jars were shook, sterilized, and inoculated with five mycelial plugs (5 mm in diameter) either of *L. laccata* or *S. bovinus*. Once the mycelia overgrown the substratum, each jar was supplied with 0.5 ml of the radioactive conidial suspension of *T. virens* with a pipette. In order to show whether ^{32}P was not simply exuded by the conidia, the control for this experiment not only consisted of the same growth substratum in the amount of 50 ml in the 300-ml jars without mycelia of the mycorrhizal fungi but also supplied with 0.5 ml of the radioactive conidial suspension. The additional control was performed to determine whether phosphorus was able to cross the trek membrane. Radioactive phosphorus in the form of $Na_2H^{32}PO_4$ was supplied to the growth substrate, both with and without mycorrhizal fungi. All the used systems were carrier free. The systems were incubated for 28 days and first samples were taken 6 days after adding the solution into the system and last at 28th day. At harvest, radioactivity of the growth substratum, both inside and outside the bags, was measured. In contrary to first experiment, other equipment was used to measure radioactivity. Sample radioactivity was measured by the MAZAR01 counter (POLON-IZOT, Warsaw, Poland), connected to unshielded plastic scintillation detector (TESLA SPF 34 U04 (TESLA, Czech Republic) located within a Pb layer of 5-cm-thick apparatus (Tolgryess et al. 1976). Five measurements were made for each sample. All results were corrected in relation to background radiation and half-life decay.

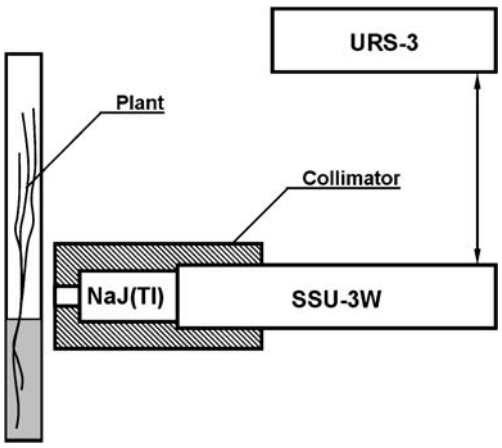

Fig. 1 Apparatus for counting of the radioactivity, constructed from the scintillation probe SSU-3W with NaJ(TI) scintillator, Pb collimator, and counter of the radioactivity URS-3

pH measurement

The pH of the growth substrate with and without mycorrhizal fungi was measured by the method previously described by Nowosielski (1974). Briefly, the pH was measured by electrode in the substratum–water suspension.

Statistical analysis

Individual data of the repeated experiments were analyzed jointly. Analysis of variance and the Student's *t* test were conducted using the statistical analysis software Statistica PL 1998 (StatSoft Polska Inc., USA). Data presented in percent were transformed before the analysis according to the C. I. Bliss equation: $\arcsin\sqrt{\text{percentage}/100}$ (Snedecor and Cochran 1976).

Results

Concentration of ^{32}P in conidia of *T. virens*

The amount of detected radioactive phosphorus allocated to conidia was 5.14 ng. This value indicates that 0.31% of the

^{32}P supplied to the medium was incorporated into the conidia. Moreover, proliferation rates and development of the fungal mycelia growing on the radioactive medium were not reduced in comparison with the control (without ^{32}P). In addition, the number of germinated spores (86.25%) containing ^{32}P was similar to the control (88.15%) and the differences were not statistically significant (Student's *t* test).

Behaviour of *T. virens* conidia

In contrast to good development of *T. virens* mycelium on the growth substratum without mycorrhizal fungi during the first days after inoculation with its conidia, germination of the conidia was drastically reduced in the presence of *L. laccata* and *S. bovinus* colonies. Moreover, pH of the growth media with ECM fungi differed from that without them. The pH of the substratum decreased to 4.5 in the presence of *L. laccata* and to 4.0 in the presence of *S. bovinus* in comparison with 5.5 of the control.

Translocation of ^{32}P to mycorrhizal and nonmycorrhizal plants

Comparison of the amounts of ^{32}P in mycorrhizal vs. nonmycorrhizal seedlings indicated that the symbiosis significantly facilitated translocation of the phosphorus ($p<0.001$). The proportion of detected radioactivity in the shoots of pine seedlings colonized by *L. laccata* were as high as 27.82% of the radioactivity of *T. virens* conidia supplied to the substratum (Fig. 2). Translocation of ^{32}P to pine seedlings colonized by *S. bovinus* was also observed. These plants took up to 7.42% of ^{32}P contained within the conidial suspension of *T. virens* (Fig. 2). The transfer rate of ^{32}P was faster in the early phase of the experiment, and the maximum radioactivity was detected on the 28th day of the experiment. In contrast, in nonmycorrhizal seedlings growing on substratum supplied with suspension of the radioactive conidia, the proportion of detected radioactivity reached only 0.56% at 28th day (Fig. 2).

Quantification of the exudation of inorganic phosphorus from the conidia

Six days after inoculation with radioactive conidia, the proportion of detected radioactivity inside the bags made of PTM membrane was 0.03% of that detected outside bags, whereas in the presence of *L. laccata* and *S. bovinus* colonies, the proportion achieved 3.52% and 2.54%, respectively, at the same time of the experiment. The exudation of the radioactivity increased during the experiment and reached 24.86% in the presence of *L. laccata* and 29.74% in the presence of *S. bovinus* in the bags, in contrast to nonmycorrhizal substratum where the radioactivity increased in the bags only up to 2.58% on the 28th day after inoculation. The differences between mycorrhizal and nonmycorrhizal treatments were statistically significant ($p<0.001$).

Discussion

Although the ability of ECM fungi to mobilize organic forms of nutrients has been recognized previously (e.g., Perez-Moreno and Read 2000), this study offers the first demonstration that these fungi can provide plants with the nutrients stored in the spores of other soil fungi. This observation is of great importance because fungal spores are abundant in soils and can be an additional source of nutrients for ECM fungi and later for their host plants.

In this study, the proportion of detected radioactivity in the shoots of mycorrhizal seedlings reached 27.82% of the radioactivity added as the conidial suspension to the substratum for seedlings in symbiosis with *L. laccata* and 7.42% for plants in symbiosis with *S. bovinus*. In contrast, the amount of ^{32}P translocated from conidia to nonmycorrhizal pine seedlings was negligible (0.56%). In the present study, the amount of phosphorus translocated to mycorrhizal seedlings from conidia of *T. virens* was significantly smaller than those translocated from pollen and nematodes, in which levels of mobilized phosphorus reached 97% and

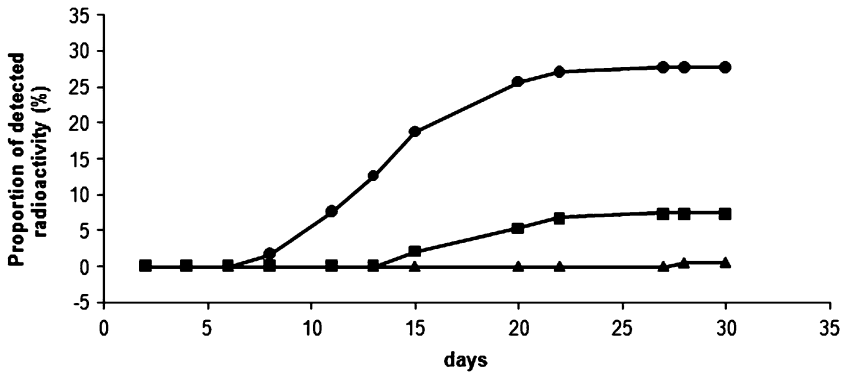

Fig. 2 Proportion of the detected radioactivity of ^{32}P translocated from the conidia of *T. virens* to shoots of Scots pine seedlings colonized by *L. laccata* (*circle*), *S. bovinus* (*square*), or uncolonized plants (*triangle*)

65%, respectively (Perez-Moreno and Read 2001a, b). The high values of phosphorus translocation in studies by Perez-Moreno and Read (2001a, b) may be due to measuring of the total amounts of phosphorus taken up from the substrata, whereas in the present study, only translocation of the radioactive phosphorus to the shoots was measured. Moreover, it is also possible that the mycelia of the ECM fungi captured large amounts of the isotope from the labeled conidia but translocated only a small part to the plants. Perez-Moreno and Read (2001a, b) have also suggested that such high amounts of phosphorus translocated from pollen and nematodes could be a result of their better exploitation activated by the presence of soil fungi or bacteria. Because the present studies were performed under axenic conditions, no other microorganisms were present that could mobilize phosphorus from the conidia and later exude it as orthophosphate for the ECM fungi to take up. Thus, it is necessary to consider what facilitates and causes acceleration of essential nutrient transport from an organic source to host plants.

Mycorrhizal fungi are known to decrease the pH of their growth media, sometimes very significantly (Hung and Trappe 1983). Assuming that such activity is regarded as a symptom of antagonistic activity (Rasanayagam and Jeffries 1992; Yamaji et al. 2005), this could also account for the death of conidia and release of phosphorus. In light of the observed decrease in pH of the growth media with mycorrhizal fungi, such a possibility should not be ruled out, especially since exudation of phosphorus from the conidia of *T. virens* introduced to the control growth medium was ten times lower than it was in the presence of mycorrhizal fungi. Labeled spores could also lose viability very quickly and accumulate phosphorus in a form of easily accessible to mycorrhizal fungi. However, the relatively small amount (2.58%) of phosphorus released by conidia of *T. virens* in the control conditions is evidence that exudation of phosphorus from the spores may have occurred primarily due to the activity of mycorrhizal fungi (24.86% and 29.74%).

The second explanation is that phosphorus translocation to mycorrhizal plants may result from an active absorption by ECM fungi during interactions with saprotrophic fungi. Lindahl et al. (1999, 2001) found that ECM fungi could compete successfully with hyphae of saprotrophic fungi. The findings of Lindahl (2001) and Klironomos and Hart (2001) also confirm that the ECM fungi accelerate nutrient cycling in the soil, giving their host plants access to essential nutrients present in living organisms, which means that mycorrhizal fungi may act as biotrophic, necrotrophic, or parasitic fungi on a wide range of other soil organisms.

One can question radioactivity counting in our study, since quenching of the radioactivity was not taken into consideration when the results of our experiments were evaluated. Certainly, the radioactivity counting through the glass of tubes may be burdened with an error. However, in the method used, the measurements of the phosphorus amount in our plants were possible because of the conversion phenomena, i.e., the beta minus particles were braked by the glass and in consequence the X radiation was excited. The unabsorbed photons were detected by scintillator. Because the yield of this conversion (i.e., beta particles on X quanta) was stable in our experimental system, the measured of the X emission was proportional to the amount of ^{32}P. Since the amount of the ^{32}P in roots and shoots was quantified identically through the glass wall of the tubes, the proportion of detected radioactivity may be overestimated due to absorption of the radioactivity by the growth substratum in the root zone.

In the present study, the uptake of phosphorus by the plants is a result of an activity of ECM fungi connected with destroying and further translocation of contents of saprotrophic fungus conidia into host plants. Difference in the P translocation between the two different ECM fungi (*L. laccata* and *S. bovinus*) is the interesting topic emerging from this study. This has implications in the area of composition of the soil fungi communities, since depending on the species present, nutrient cycling may differ as well.

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