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Influence of Potassium Supply on Growth and Uptake of Nitrogen, Phosphorus, and Potassium by Three Ectomycorrhizal Fungal Isolates In Vitro

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ABSTRACT

Three ectomycorrhizal fungal isolates, *Cenococcum geophilum* SIV, a putative isolate of *Pisolithus microcarpus* and a *Pisolithus* sp., were cultured in vitro without potassium (K) and with three levels of K supply (as K₂SO₄). Some growth stimulation by K was observed among the fungi. Potassium in the liquid medium led to increased concentrations of nitrogen (N) and K in the fungi compared to K-free controls, and to higher phosphorus (P) concentrations in *Pisolithus* sp., but lower P in *C. geophilum* SIV. In a second experiment, the K influx rates of the fungi from external solutions followed Michaelis-Menten kinetics, indicating that K influx was an active process. Mycorrhizal fungi showed quite small $C_{\min}$ values for K influx, ranging from 2.12 to 5.39 $\mu\text{mol L}^{-1}$, in relation to K concentrations in the external solution. Such low $C_{\min}$ values might allow the external hyphae to extract K efficiently from K-deficient soils. Furthermore, the fungi had low K_m values for K uptake ($<14.85 \mu\text{mol L}^{-1}$), indicating high affinities and efficiencies of K carriers. Both *Pisolithus* sp. and *P. microcarpus* exhibited similar $C_{\min}$ and K_m values but were different from *C. geophilum* SIV. Furthermore, $I_{\max}$ values varied significantly among the fungi, ranging from 9.60 to 21.63 $\mu\text{mol g}^{-1} \text{DM h}^{-1}$, perhaps due to variation in the intracellular K concentrations in the ectomycorrhizal fungi.

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INTRODUCTION

Tropical and subtropical forest and plantation soils are often potassium (K)-deficient and improvement of K nutrition could promote tree growth in these conditions. Ectomycorrhizal fungal colonization can aid both growth and nutrient absorption by trees (Smith and Read, 1997). External hyphae on mycorrhizal roots may access soil K reserves not directly available to most plants (Wallander, 2000; Wallander and Wickman, 1999) and thus enhance K uptake by the host plants (Huang and Lapeyrie, 1996). Release of fungal protons and oxalates, which is strongly promoted by simultaneous depletion of available K and magnesium (Mg) (Paris et al., 1995, 1996), may provide mechanisms for the fungi to replace interlayer K and cause weathering of K-containing minerals in soils (Lapeyrie et al., 1987; Lapeyrie, 1988; Landeweert et al., 2002). This hypothesis has been partly supported by soil analysis showing that Douglas fir seedlings infected by the ectomycorrhizal fungus *Laccaria bicolor* S238N extracted HF-HClO₄-digestible K in a sequential extraction procedure that started with water, ammonium acetate, and HCl as extractants (Huang and Lapeyrie, 1996). Even so, there is still no direct evidence for the exploitation by ectomycorrhizas of K in soil pools, especially those unavailable to non-mycorrhizal plants. Analysis of K concentrations in plant tissues has also failed to demonstrate unequivocally the involvement of mycorrhizas in mobilization and plant uptake of soil K. There is some evidence of increased K uptake by trees from soil following mycorrhizal inoculation, but in other cases K has been found at lower concentrations in mycorrhizal plant tissues as compared with uninoculated controls (Smith and Read, 1997; Bowen, 1973).

Hyphal K acquisition by mycorrhizal spruce roots was significant and contributed a minimum of 5%–6% of total plant K uptake, indicating an important role of ectomycorrhizal mycelium in K nutrition of host plants (Jentschke et al., 2001). The favorable effects of ectomycorrhizal fungi on plant nutrition have traditionally been attributed to the capacity of extraradical mycelium to absorb dissolved nutrients from the soil solution (Smith and Read, 1997). Kinetic parameters of ion uptake (K_m , $I_{\max}$, and $C_{\min}$) may, to some extent, govern the ion uptake by mycorrhizal plants and fungi from solutions (Chalot et al., 2002). Based on work using isotopic tracers, ⁴¹K influx may be carried out by active processes and variation in the intensity of apoplastic movement of the nutrient can be observed depending on the fungal partners (Kuhn et al., 2000; Bucking and Heyser, 2000). K_m values for K absorption by *Laccaria bicolor*, *Lactarius rufus*, and *Lactarius hepaticus* were found to be 24, 18, and 96 $\mu\text{mol L}^{-1}$, respectively (Jongbloed et al., 1991), indicating variable affinities of K carriers and channels on fungal membranes. These differences between more efficient

and less efficient mycorrhizal strains might lead to variation in the capacity of host plants to extract K from soil solutions. In addition, non-mycorrhizal seedlings of *Pinus sylvestris* and seedlings colonized by *Paxillus involutus* and *Suillus variegatus* were all able to access K in biotite, leading to stimulated growth and K uptake relative to K-free controls. However, when microcline was the K source, growth depression was induced in the seedlings (with the exception of those colonized by *P. involutus*, in which tree growth was increased), suggest that more K was extracted from microcline by seedlings colonized by *P. involutus* (Wallander and Wickman, 1999). Co-translocation of a limiting element (P) occurred with non-limiting nutrients (N, K, and Ca) by a *P. involutus* isolate (Jentschke et al., 2001). Smith et al. (1981) observed elevated concentrations of K in shoots of endomycorrhizal *Trifolium subterraneum* when plants were grown in P-deficient soils. If sufficient P was supplied to the soil to remove any mycorrhizal growth responses, then K concentrations in mycorrhizal and non-mycorrhizal plants were very similar.

Clearly, generalizations concerning involvement of ectomycorrhizal fungi in K uptake by host trees cannot be made with confidence at present. The understanding of the roles of mycorrhizal fungi in the K nutrition of trees is complicated by various properties of fungal K uptake from soil solutions and interferences in the field, especially complex interactions among nutrients in soil solutions. Moreover, interactions in nutrient uptake by higher plants and yeast have been studied in some detail (Borst-Pauwels, 1981; Marschner, 1995), but information on this aspect is quite scarce for ectomycorrhizal fungi. The objective of the present experiment was to elucidate the effects of K supply on the growth and N, P, and K uptake by ectomycorrhizal fungal isolates in liquid culture. The kinetics of K uptake by the fungi were also determined to investigate the efficiency of fungal K uptake from the culture solutions.

MATERIALS AND METHODS

Fungal Material

The three ectomycorrhizal fungi used in the experiments, namely *Cenococcum geophilum* SIV, a putative isolate of *Pisolithus microcarpus* formerly referred to as *P. tinctorius* 441 (Martin et al., 2002) and *Pisolithus* sp. (previously *P. tinctorius* 2144) were provided by Dr. Frédéric Lapeyrie of the Centre de Recherches Forestières de Nancy in France. The putative isolate of *P. microcarpus* was originally isolated in Brazil, and *Pisolithus* sp. in Australia (both from mycorrhizal *Eucalyptus* spp.); *C. geophilum* SIV was originally isolated from spruce mycorrhizas in Europe. The three fungi were maintained in culture by successive transfer on Pachlewski agar medium in the Microbiology Laboratory, Southwest Agricultural University, Chongqing, China. Mycelia for inoculation were grown on Pachlewski agar medium for 3 to 4 weeks. The medium contained

ammonium tartrate 0.5, KH_2PO_4 1.0, MgSO_4 0.5, glucose 20, maltose 5.0, vitamin B₁ 0.1, and agar 20 g L⁻¹, respectively, and 1 ml L⁻¹ microelement solution [1 L microelement solution contained H_3BO_3 8.45, MnSO_4 5, FeSO_4 6, CuSO_4 0.625, ZnCl_2 2.27 and $(\text{NH}_4)_2\text{MoO}_4$ 0.27 mg, respectively].

Experiment 1

Experiment 1 was carried out to study the effects of K on fungal growth and uptake of N, P, and K. The KH_2PO_4 in Pachlewski liquid medium was replaced by NaH_2PO_4 to give equivalent P and the experimental treatments were established by adding K_2SO_4 to the liquid medium at the following concentrations 0, 0.13, 0.65, and 3.25 g L⁻¹. The standard K concentration in Pachlewski medium was equivalent to 0.65 g K_2SO_4 L⁻¹. These concentrations are referred to as no K and low, medium, and high K, respectively, unless otherwise stated. Pachlewski liquid medium (20 mL) was transferred to a 250 ml Erlenmeyer flask and steam-sterilized at 121°C for 30 minutes. Each flask was inoculated with one 6 mm-diameter plug of mycelium and incubated without agitation for 3 weeks at $25 \pm 1^\circ\text{C}$ in the dark.

The fungal mycelium was harvested, washed free of culture medium with deionized water, and oven dried at $80 \pm 2^\circ\text{C}$ for 8 h. The dry weights of fungal mycelia were recorded and the concentrations of N, P, and K in the dry samples were determined by standard methods (indophenol blue and molybdenum blue methods followed by colorimetry and flame photometry, respectively) after digestion in H_2O_2 - H_2SO_4 solution.

Experiment 2

Experiment 2 was conducted to determine the kinetics of K uptake by the ectomycorrhizal fungi. Mycelia were cultured without agitation in Pachlewski liquid medium in which 90% of K was omitted by substituting KH_2PO_4 with NaH_2PO_4 . The cultures were grown in 200 ml beakers containing 20 mL sterilized liquid medium inoculated with a 6 mm-diameter mycelial plug. The cultures grew for 2 weeks at $25 \pm 1^\circ\text{C}$ in the dark because preliminary tests showed that the highest rates of K influx occurred after 2 weeks (data not presented). Three fungal colonies from each culture were carefully transferred to an 8 cm-diameter Petri dish. Sterilized solution containing 100 $\mu\text{mol L}^{-1}$ CaCl_2 and 0.1% glucose (w/v) buffered with 5 mmol L⁻¹ citric acid/Tris at pH 4.5 was added and then removed slowly by pipette to avoid damage to the fungal hyphae. This procedure was repeated several times to completely replace the liquid culture medium in contact with the surface of the fungal hyphae. The colonies were incubated in the Petri dish containing 40 mL of wash solution for 48 h at $25 \pm 1^\circ\text{C}$ in the dark.

The incubation solution was replaced with 40 mL solution for K absorption (KCl , $75 \mu\text{mol L}^{-1}$; CaCl_2 , $100 \mu\text{mol L}^{-1}$; pH 5.8). At regular time intervals (2, 5, 10, 20, 40, 60, 120, and 180 min), the K concentration in the solutions was measured using a K microprobe (Jiangsu Instrument Co., Taixian, China). Temperature continued to be maintained at $25 \pm 1^\circ\text{C}$ during the period of K uptake in the dark.

At the end of the K absorption period, the dry weights of the fungal colonies in each Petri dish were recorded to calculate the amount ($\text{mg g}^{-1} \text{DM}$) and rate ($\mu\text{mol g}^{-1} \text{DM min}^{-1}$) of K absorption by the ectomycorrhizal fungi according to K depletion of the solutions.

Data Analysis

Fungal growth rates in Experiment 1 were subjected to two-way analysis of variance with replication using the SAS package (Statsoft, SAS Institute Inc., Cary, NC, USA). Multiple regression equations were derived to fit the K uptake kinetics in Experiment 2 using SigmaPlot 2000 for Windows (SPSS Inc., USA).

RESULTS

Effects of Potassium Concentration in Culture Medium on Growth of Ectomycorrhizal Fungi

As shown in Figure 1, the three fungi varied greatly in growth rate in the sequence *C. geophilum* SIV > *Pisolithus* sp. > *P. microcarpus*. The mean growth rates ranged widely from 0.43 to $2.92 \text{ mg DM day}^{-1}$. The K concentration in the culture medium strongly influenced the growth of the ectomycorrhizal fungi, but the highly significant interaction term reflects the different patterns of influence. Thus, *C. geophilum* SIV showed much higher growth rates when K was applied compared with the control, with a smaller increase as K concentration increased. In contrast, growth of *Pisolithus* sp. increased only at the highest K concentration studied and the growth rate of *P. microcarpus* was almost unaffected by K supply.

Effects of Potassium Concentration in Culture Medium on Nitrogen, Phosphorus, and Potassium in Ectomycorrhizal Fungi

The lowest and least variable N concentrations and uptake values of all three fungi were observed in the zero-K controls. When K was added to the medium there was a marked increase in fungal N concentration and uptake that varied among the three fungi (Fig. 2). The presence of K in the medium increased

Figure 1. Mean growth rate of the three ectomycorrhizal fungi in liquid medium with different K concentrations. Bars: S.D. By analysis of variance, the F ratios for fungal isolate, K concentration and the interaction term (fungal isolate $\times$ K concentration) were all highly significant ($P < 0.001$).

fungal N concentration 3.75- to 4.93-fold, with *P. tinctorius* 2144 showing the largest response and isolate 441 showing the smallest. Fungal N uptake in the presence of K followed the sequence: *C. geophilum* SIV $>$ *P. microcarpus* $>$ *Pisolithus* sp., but the last two showed similar N uptake at the highest K addition level.

The three fungi also varied significantly in P concentration and uptake (Fig. 3), both of which were generally highest in *Pisolithus* sp. Higher P

Figure 2. Effects of K concentration in the culture medium on ectomycorrhizal fungal N concentration and uptake. ■ *Pisolithus* sp.; □ *P. microcarpus*; ● *C. geophilum* SIV; bars: standard deviation.

Figure 3. Effects of K concentration in the culture medium on ectomycorrhizal fungal P concentration and uptake. ■ *Pisolithus* sp.; □ *P. microcarpus*; ● *C. geophilum* SIV; bars: S.D.

concentrations were observed in *P. microcarpus* than *C. geophilum* SIV, while P uptake showed the opposite trend. Potassium supplied in the medium led to a pronounced decrease in P concentrations in *C. geophilum* SIV but an increase in the *Pisolithus* isolates. The P concentration in *P. microcarpus* increased markedly in the low-K medium compared with the K-free control and then decreased as K concentration in the medium increased. Added K in the medium enhanced P uptake by both *Pisolithus* sp. and *C. geophilum* SIV, but P uptake by *P. microcarpus* showed an increase in the low-K medium compared with the control and then decreased as K concentration in the medium increased.

As would be expected, fungal K concentrations and uptake values were lowest and least variable in the zero-K medium (Fig. 4), with variable responses among the three fungi to increasing K concentration in the culture medium. Fungal K concentrations changed in the sequence *Pisolithus* sp. > *P. microcarpus* > *C. geophilum* SIV in contrast to K uptake, which followed the sequence *Pisolithus* sp. > *C. geophilum* SIV > *P. microcarpus*.

Figure 4. Effects of K concentration in the culture medium on ectomycorrhizal fungal K concentration and uptake. ■ *Pisolithus* sp.; □ *P. microcarpus*; ● *C. geophilum* SIV; bars: standard deviation. Bars: S.D.

Kinetics of Potassium Uptake by Ectomycorrhizal Fungi

The time-dependent depletion of K concentration in solutions attributable to the ectomycorrhizal fungi can be expressed by:

$$C = C_0 + ae^{-bt}$$

where C is the K concentration in solution; t is the time; and a , b , and C_0 are constants (Fig. 5). According to the equation above, as time $t \rightarrow \infty$, $C = C_0$. Therefore, C_0 could be considered as the minimum K concentration ($C_{\min}$) in solution when each fungus initiated net influx and the values derived were 2.12 (*Pisolithus* sp.), 3.53 (*P. microcarpus*) and 5.39 $\mu\text{mol L}^{-1}$ (*C. geophilum* SIV) (Table 1). According to the depletion of K concentrations in the solutions, the time-dependent K uptakes ($\mu\text{mol g}^{-1}$ DM) of the fungi were obtained and are shown in Figure 6. The amount of K taken up followed the sequence *Pisolithus* sp. > *P. microcarpus* > *C. geophilum* SIV.

The rates of K influx (I), derived from the changes in external concentrations (C), were calculated by the Michaelis-Menten (1974) saturation equation as follows:

$$I = \frac{I_{\max}C}{K_m + C} \text{ (Jongbloed et al., 1991; Caassen and Barber, 1974)}$$

Figure 5. Time-dependent depletion of K^+ concentration in solutions by the three ectomycorrhizal fungi. Pt2144: *Pisolithus* sp.; CgSIV: *Cenococcum geophilum* SIV; Pt441: *Pisolithus microcarpus*.

Table 1
Kinetic parameters of K uptake by the three ectomycorrhizal fungi (mean $\pm$ S.D.)

Fungus	K_m ($\mu\text{mol L}^{-1}$)	$I_{\max}$ ($\mu\text{mol g}^{-1} \text{DM} \cdot \text{h}^{-1}$)	$C_{\min}$ ($\mu\text{mol L}^{-1}$)
<i>Pisolithus tinctorius</i> 2144	5.47 ± 3.26	21.63 ± 5.18	2.12 ± 0.73
<i>Pisolithus tinctorius</i> 441	5.20 ± 1.54	9.60 ± 2.04	3.53 ± 0.84
<i>Cenococcum geophilum</i> SIV	14.85 ± 5.98	13.14 ± 4.23	5.39 ± 0.92

where $I_{\max}$ is the maximum influx rate when K in solution does not limit uptake, and K_m , the Michaelis constant, is the external K concentration at which the net influx of K is equal to half of $I_{\max}$. As can be seen from Table 1, the three fungi showed a range of $I_{\max}$ values: 9.60, 13.14 and 21.63 $\mu\text{mol g}^{-1} \text{DM h}^{-1}$ for *P. microcarpus*, *C. geophilum* SIV, and *Pisolithus* sp., respectively. In contrast, the two *Pisolithus* isolates exhibited similar K_m values, which were much lower than that of *C. geophilum* SIV, and no significant variation was observed in $C_{\min}$ for K uptake by the two *Pisolithus* strains. Moreover, the K_m for K uptake by *C. geophilum* SIV was approximately three times higher than those of the two *Pisolithus* isolates, which differed little, if at all, in K_m value.

Figure 6. Time-dependent change in K uptake by the three ectomycorrhizal fungi. Pt2144: *Pisolithus* sp.; CgSIV: *Cenococcum geophilum* SIV; Pt441: *Pisolithus microcarpus*.

DISCUSSION

The external hyphae of ectomycorrhizas are responsible for the uptake and subsequent transfer of nutrients to their hosts. In general, good growth and development of hyphae in soils can extend spatially the zone of nutrient extraction from soils. The major impact of mycorrhizal fungi on the area available for nutrient absorption by the seedlings of *Pinus taeda* was provided by the extraradical hyphae, which led to an increase of approximately 40-fold in the case of *P. tinctorius* and 25-fold for *C. geophilum* in relation to the uncolonized controls (Rousseau et al., 1994). In return, essential nutrients, in particular N and P, influenced the growth of the ectomycorrhizal fungi (Smith and Read, 1997). The development of external hyphae on the root surfaces of *Afzelia africana* inoculated with four ectomycorrhizal fungi was not related to mineral nutrition (Bâ et al., 1999). However, in forests and plantations, the roots of trees are associated with guilds of large numbers of ectomycorrhizal fungi and relatively few of these may be involved in tree nutrient acquisition in any particular location. There is also very little information available on the effects of K on the growth of mycorrhizal fungi. In the present experiments, K stress in the culture solution depressed the growth of the ectomycorrhizal fungi, which showed different responses to K supply. It therefore seems reasonable to suggest that K in the soil solution would similarly affect the growth of external hyphae and their development on the surfaces of their host roots. The growth of *C. geophilum* SIV was very much poorer in K-free solution than solutions containing K, but there were no differences in the growth of *Pisolithus* sp. in controls and K-containing solutions except at the highest K concentration. It is possible that the K-free solutions were contaminated with K introduced with the mycelial plugs, but we did not determine the K status of the inocula. Nevertheless, the results do indicate that growth and development of mycorrhizal fungi can vary in response to varying K supply and imply that K in soil solutions may have less impact on the extraradical hyphae of *Pisolithus* sp. than of *C. geophilum* SIV.

Potassium accumulation in the fungi in the first experiment was apparently well correlated with accumulation of P. This may be the result of accumulation of polyphosphate in the fungal cells and is consistent with the findings of Orlovich and Ashford (1993), whose data indicate that in *Pisolithus tinctorius*, polyphosphate is uniformly distributed in the fungal vacuoles and is stabilized by K^+ .

There were large variations in the concentrations and uptake values of N, P, and K in leaves of ectomycorrhizal *Afzelia africana* depending on fungal inoculation (Bâ et al., 1999). The concentrations of K and P in the leaves of Douglas fir seedlings infected by *Laccaria bicolor* S238N were increased by approximately 36% and 55% over non-mycorrhizal controls (Huang and Lapeyrie, 1996). Smith et al. (1981) observed elevated concentrations of K in shoots of mycorrhizal *Trifolium subterraneum* when the plants were grown in P-deficient soils. If sufficient P was supplied to the soil to remove any mycorrhizal

growth response, then K concentrations in both groups of plant were very similar. Fungal K accumulation is strongly influenced by the form of N available (NH_4^+ vs. NO_3^-) as well as by other ions (Smith and Read, 1997). This suggests that the important role played by mycorrhizal fungi in nutrient uptake is influenced by complex nutrient interactions. Carefully designed experiments to investigate K nutrition of mycorrhizal fungal isolates and mycorrhizal plants need to take all these potentially confounding factors into account. The fungal concentrations of N, P, and K in our experiments changed differently in response to varying K supply in the culture solution. In general, K supply promoted increases in the concentrations of N and K in the different ectomycorrhizal fungi to different extents, while P concentrations increased in one fungus (*Pisolithus* sp.) and decreased in another (*C. geophilum* SIV). These results support earlier reports of complex interactions in nutrient uptake by ectomycorrhizal fungi.

Potassium transporters in plants and fungi have received much attention (Rodriguez-Navarro, 2000). The process is complicated by the fact that both the surface potential and the membrane potential influence the apparent affinity of the ion for the carrier. The kinetic parameters of K influx depend upon cellular pH and K concentrations (Boxman and Roelofs, 1988). The fungi were, therefore, pre-cultured in buffer solution to avoid interferences from cellular pH before determining the kinetic parameters of K influx in the present experiments. The K influx rates of all three fungi from external concentrations satisfied Michaelis-Menten kinetics and support the hypothesis of active influx (Kuhn et al., 2000). The results support the hypothesis that mycorrhizal fungi may show quite small $C_{\min}$ values for K influx, ranging from 2.12 to 5.39 $\mu\text{mol L}^{-1}$, in relation to K concentrations in soil solutions. Such low $C_{\min}$ values could allow the external hyphae of mycorrhizas to extract K efficiently from K-deficient soils. Furthermore, the ectomycorrhizal fungi exhibited K_m values ranging from 5.20 to 14.85 $\mu\text{mol L}^{-1}$ for K influx, indicating high affinities of K carriers. However, soil solutions consist of complex ions and an appreciable inhibition by these ions of K influx into mycorrhizal hyphae might be expected in field conditions. It may be of interest that two strains of *Pisolithus* showed similar $C_{\min}$ and K_m values (and therefore perhaps efficiencies and affinities of K carriers) that differed from those of *C. geophilum* despite the large geographical distances between the locations where the *Pisolithus* strains were originally isolated.

$I_{\max}$ for K uptake by the three fungi varied widely. Glass (1976) proposed that K influx into plant roots was regulated via allosteric inhibition of the K carrier by internal K concentrations, resulting in changes in kinetic parameters, especially $I_{\max}$ reduction at high cellular K concentrations. Similar effects of cellular K on kinetic parameters for K influx have also been observed in cereal crops. However, it is not certain whether this is also true for ectomycorrhizal fungi. In Experiment 1, the mycorrhizal fungi varied significantly in K concentrations even in the medium with a low K concentration (20% of "normal" K concentration). We can therefore suggest that the mycorrhizal fungi used

for determining kinetic parameters (cultured in medium with 90% of the K omitted) could have variable K concentrations. We must, therefore, be cautious in interpreting differences in $I_{\max}$ for K influx for the three fungi studied as differences in the properties of K carriers.

Potassium nutrition of ectomycorrhizal fungi in association with host trees still remains unclear. Fungal isolates cultured under well-controlled conditions are usually employed to avoid interferences in the field and to simplify the experimental methods. The results obtained from such studies are valuable but could differ from plant-fungus associations in the field. These potential differences must be taken into consideration in the interpretation of data from fungal cultures.

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