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Negative effects of potassium supplies and calcium signal inhibitors on oxalate efflux by ectomycorrhizal fungi

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ABSTRACT

The main role of ectomycorrhizal fungi is to extract phosphorus (P) for trees. Organic acids, particularly oxalate, are also beneficial to mobilize nutrients such as potassium (K), calcium (Ca) and magnesium (Mg) from minerals and rocks in soils. In the paper, a pure liquid culture experiment was carried out to elucidate the influence of K supplies and inhibitors related to Ca signals and anion channels on the efflux of oxalate and protons by the isolates of *Pisolithus tinctorius*, *Cenococcum geophilum*, *Lactarius deliciosus* and *Boletus badius*. The fungal isolates varied greatly in the biomass and the absorption of N, P and K. Oxalate, acetate, malate, citrate and succinate were detected in the culture solutions. All the studied fungal isolates could effuse oxalate and the faster exudation was observed at low K supply than high K. The fungal K accumulation correlated negatively with the efflux rates of oxalate ($r = -0.359$, $n = 60$) and protons ($r = -0.630$, $n = 60$). The stimulation of oxalate and proton efflux by external hyphae in soil with low K could be beneficial to K mobilization from minerals. However, the inhibitors of calmodulin (trifluoperazine and ruthenium red), Ca^{2+} (verapamil) and anion channels (niflumic acid) decreased the fungal oxalate efflux at low K supply. Therefore, both Ca signals and anion channels involved in the process of the fungal oxalate exudation in low K conditions.

1. Introduction

Potassium (K), an element required slightly lower than nitrogen (N), is one of the essential major nutrients for trees in forests. K deficiency retards tree growth and limits forest productivities (Oosterhuis and Berkowitz, 1996; William and Norman, 2004; Tripler et al., 2006). Soils deficient in K are commonly found in the tropical and subtropical areas because of a deep weathering and intensive leaching. Therefore, they usually lack available K, and ectomycorrhizal trees such as pine and eucalyptus in the soil have to extract K from minerals and rocks in soils to satisfy their nutrient requirements.

Many forest trees have evolved mutualistic symbiosis with ectomycorrhizal fungi that contribute to their nutrition. In the fungus–tree associations, the fungi obtain carbohydrates from host trees and, in turn, provide the plants with mineral nutrients such as phosphorus (P) and K. Most of K in soils could not be utilized directly by plants because the available K for plants is usually less than 2% of the total soil K (Mengel and Uhlenbecker, 1993). Numerous studies have shown that mycorrhizal plants can extract nutrients, including calcium (Ca), Mg (magnesium), P and K from soil minerals (Landeweert et al., 2001; Zahoor and Zaffar, 2013). The growth of *Pinus sylvestris* seedlings was significantly stimulated by *Paxillus involutus* when microcline was used as the sole K source (Wallander and Wickman, 1999). Ectomycorrhizal inoculation might improve the growth and K nutrition of the trees in artificial plantation in nutrient-deficient soils. Yuan et al. (2004) have reported the weathering of minerals and utilization of HCl-extractable K by eucalyptus seedlings inoculated with *Pisolithus microcarpus* and the concomitant acceleration of the tree's growth rate.

Organic acids including oxalate, malate, citrate, succinate and acetate were found in the culture solutions for growing ectomycorrhizal fungal isolates. In European forest soils, the organic acids reached 1 mmol kg^{-1} in the soil and numerous 10–50 μm hollow vessels could be observed on the surface of the rocks. They were filled usually with the external hyphae of ectomycorrhizas, and high concentrations of organic acids, especially oxalate, which was detected at the hyphal ends (Landeweert et al., 2001). These organic acids could dissolve the rocks and release nutrient elements such as Ca, Mg and K. It is necessary to point out that both $[Fe(C_2O_4)_3]^{3-}$ and $[Al(C_2O_4)_3]^{3-}$ have very high chelation constants of 2.0×10^{16} and 3.9×10^{16} (Lapeyrie, 1988; Adeleke et al., 2012). Therefore, oxalate may chelate Al^{3+} and Fe^{3+} in the crystal lattice of minerals containing K, resulting in the weathering of these minerals and K release (Fox et al., 1990; Gadd, 1999). Lapeyrie et al. (1987) reported that the biological weathering of minerals was accelerated by the oxalate efflux from

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ectomycorrhizal fungus hyphae, resulting in the release of otherwise unavailable K from minerals. Wallander and Wickman (1999) found high concentrations of citrate and oxalate in culture media containing biotite that were used to grow pine seedlings colonized by *Suillus variegates*. In addition, a large amount of protons were detected in culture media for culturing ectomycorrhizal fungal isolates (Rosling, 2009). Protons can replace interlayer K in 2:1 clay minerals and even destroy their lattice structures, whereby making K available for plants (Wallander, 2000). Thus, the release of protons and organic acids, particularly oxalate, may be a mechanism for ectomycorrhizal fungi to mobilize and utilize unavailable K from minerals and rocks in soils. Moreover, the growth stimulation and concomitant increment of nutrient uptake by mycorrhizal plants is usually observed in poor soils (Huang and Lapeyrie, 1996; Pradeep and Vrinda, 2010). Simultaneous depletion of K and Mg in culture media leads to the weathering of phlogopite by ectomycorrhizal fungi in pure culture, which might be related to the oxalate efflux of ectomycorrhizal fungi (Yuan et al., 2004).

Plant roots release organic anions through their channels on plasma membranes and Ca^{2+} signals participate in this process. Many factors such as channel phosphorylation and allostery, hormones, and cytosolic cations and anions could influence Ca^{2+} signal network and organic anion efflux (Roberts, 2006). For example, aluminum and copper stimulated significantly the efflux of citrate by *Arabidopsis thaliana* roots and malate by wheat roots. The stimulation by the metal ions and concomitant reduction in Ca^{2+} flow across plasma membranes of roots were inhibited by Ca^{2+} signal and anion channel inhibitors (Yang et al., 2003). K^+, as a companying ion of organic acids, activates voltage-gated channel of anions through membrane polarization in efflux of organic acids by guard cells in stemma behaviors (Hedrich and Jeromin, 1992). K^+ may thus act as a signal factor whereby regulating the efflux of organic acids by plant cells. In our previous studies, we also found that external K supplies could influence the efflux of acetate by the isolates of *Pisolithus tinctorius* and *Lactarius deliciosus* grown in culture media, which was also related to Ca^{2+} signal network (Zhang et al., 2014). It is interesting to understand the efflux of organic acids, particularly oxalate by ectomycorrhizal fungi in response to K supply and Ca^{2+} signal network.

In general, the role of organic acids in mineral weathering on the soil scale remains controversial and the evidence on the influence of external K supply and Ca^{2+} signals on the efflux of oxalate and protons by ectomycorrhizal fungi is limited. Therefore, the objectives of this study are (i) to study efflux of oxalate and protons by four ectomycorrhizal isolates under different K level supplies in liquid medium; (ii) to explore how Ca^{2+} signals and anion channels to control oxalate and proton efflux by fungi.

2. Materials and methods

2.1. Fungal strains

Four ectomycorrhizal fungal strains used in the experiment, namely *P. tinctorius*, *Cenococcum geophilum*, *L. deliciosus* and *Boletus badius*, were kept in the microbiology laboratory of the College of Resources and Environment, Southwestern University, Chongqing, China. *L. deliciosus* and *B. badius* were originally isolated from subtropical acidic forest soils (pH 4.00–4.52) in Chongqing. *C. geophilum* from a temperate neutral forest soil (pH 6.47) in Inner Mongolia (all from mycorrhizal *Pinus* spp.) and *P. tinctorius* was isolated from a tropical eucalyptus soil (pH 5.77) in Puer, Yunnan, China. Mycelia for inoculation were grown on Pachlewski agar medium for 2 weeks at 25 ± 1 °C in the dark. The medium contained (g L^{-1}) ammonium tartrate 0.5, KH_2PO_4 1.0, $MgSO_4$ 0.5, glucose 20, maltose 5.0, vitamin B1 0.1, agar 20 and 1 ml L^{-1} microelement solution. (1 L microelement solution contained (mg) H_3BO_3 8.45, $MnSO_4$ 5.0, $FeSO_4$ 6.0, $CuSO_4$ 0.625, $ZnCl_2$ 2.27 and $(NH_4)_2MoO_4$ 0.27).

2.2. Culture of fungal isolates

The KH_2PO_4 in Pachlewski liquid medium was replaced by NaH_2PO_4 to give equivalent P and the K treatments were established by adding K_2SO_4 to the liquid medium at the following concentrations (mg K L^{-1}): 8, 40 and 200. Forest soils in tropic areas are usually considered as K-deficient if available K is less than 40 mg kg^{-1} (Yuan et al., 2005). Therefore, these K concentrations in the culture media are referred to as low K (K_1), normal K (K_2) and high K (K_3), respectively. A total of 20 mL of Pachlewski liquid medium was transferred into a 100 mL Erlenmeyer flask and steam-sterilized at 121 °C for 30 min. Each flask was inoculated with a mycelial plug (6 mm in diameter) and incubated without agitation for two weeks at 25 ± 1 °C in the dark.

2.3. Experimental treatments

Thereafter, the culture solutions were poured out and the mycelia were washed with the sterilized distillation water to remove culture solution on their surfaces. Concerning the absence of Ca^{2+} in Pachlewski medium and the importance of Ca^{2+} in plasma membrane integrity, anion channel activation and cytosolic Ca^{2+} concentrations, 5 mL of $CaCl_2$ sterilized solution (0.1 mol L^{-1}) was then added into each Erlenmeyer flask to incubate fungal mycelia for 24 h. Fungal mycelia were washed with sterilized water and $CaCl_2$ solution was replaced by Pachlewski liquid medium (20 mL) with the same K concentrations as previously described. Simultaneously, the inhibitors related to calmodulin (trifluoperazine, TFP), Ca^{2+} (verapamil, VP; ruthenium red, RR) and anion channels (niflumic acid, NIF) were added into the culture solutions, respectively. Their concentrations in the mediums were 150.0 μmol L^{-1} TFP, 8.0 μmol L^{-1} RR, 150.0 μmol L^{-1} VP and 15.0 μmol L^{-1} NIF. The control was established exactly in the same way except that no inhibitors were added. The fungal mycelia were cultured statically for 48 h at 25 ± 1 °C in the dark with 6 replicate flasks in full randomized design.

2.4. Harvest and analysis

Fungal mycelia were harvested by filtration and washed with deionized water to remove the liquid culture medium from the surfaces. They were then oven-dried at (80 ± 2 °C) for 24 h, weighed and digested with H_2SO_4–H_2O_2. Nitrogen (N) in digests was analyzed by Kjeldahl procedure, phosphorus (P) by molybdenum blue colorization (Murphy and Riley, 1962) and potassium (K) by flame photometry (Ohyama et al., 1991).

Filtrate pH was detected using a PHS-3C pH meter (Shanghai Analysis Instrument Company, China). The proton concentration in solution was obtained according to $pH = -\log10\ [H^+]$. Thereafter, the culture solutions were acidified by 0.1 mol L^{-1} HCl and then analyzed for organic acids by high-performance liquid chromatography (HPLC; HITACHI, Japan). Samples (20 μL) were injected into an Ion-300 organic acid analysis column (Phenomenex, Torrance, CA, USA) with 2.5 mmol L^{-1} H_2SO_4 as mobile phase at 0.5 mL min^{-1} and 450 psi. The retention time was 9.57 min for oxalate, 11.52 min for citrate, 13.31 min for malate, 14.53 min for lactate, 15.95 min for succinate, 17.47 min for formate and 20.72 min for acetate. Standards of organic acids were prepared and analyzed before and after the sample solutions.

2.5. Statistical analysis

All data were subjected to analysis of variance using SPSS 20.0 model (ANOVA, Duncan's multiple range test and Pearson's correlation coefficient). All parameters were checked for normality (Shapiro–Wilk) and homogeneity of variance (Levene's test), which showed that all variables fit normality assumption. Differences obtained at levels of $P < 0.01$ were considered significant.