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Allelopathic effects of artemisinin on ectomycorrhizal fungal isolates *in vitro*

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ABSTRACT

The anti-malarial drug artemisinin is extracted from the leaves of *Artemisia annua* L. The release of artemisinin into forest soils could produce a potential risk for forest ecosystems, including effects on ectomycorrhizal fungal nutrient uptake, in areas where commercial and continual cultivation of the medicinal plant *A. annua* L. is practiced. Therefore, growth, proton and oxalate efflux, and nutrient uptake (nitrogen, phosphorus and potassium) of three isolates of *Suillus luteus* (*S. luteus* 1, *S. luteus* 13, and *S. luteus* 11) and of one isolate of *Suillus subluteus* (*S. subluteus* 12) were compared in culture solutions with different nominal artemisinin concentrations. The results showed that artemisinin inhibited significantly the growth of all studied fungi. With 25 mg artemisinin L^{-1} added, fungal biomass was decreased by 78.6% (*S. luteus* 1), 96.7% (*S. luteus* 13), 77.8% (*S. luteus* 11) and 86.8% (*S. subluteus* 12) compared with the control (without artemisinin). This could explain, at least in part, why ectomycorrhizal fungal sporocarps in forests are consistently not found near cultivated *A. annua* L. fields. The amount of proton efflux by the fungal isolates also decreased as nominal artemisinin concentrations increased, indicating the limited ability of ectomycorrhizal fungi to mobilize nutrients from soil minerals. However, nominal artemisinin significantly increased the rate of fungal oxalate efflux, suggesting membrane damage and the abnormal opening of anion channels on hyphae cell membranes. Nominal artemisinin also decreased the uptake of nitrogen, phosphorus and potassium by the fungal isolates, which may not benefit from the nutrient uptake by ectomycorrhizae. Therefore, artemisinin released from large *A. annua* L. plantations may inhibit ectomycorrhizal fungal growth, nutrition and functions in forest ecosystems in Southwest China.

Introduction

Artemisinin extracted from *Artemisia annua* L. is recommended by the World Health Organization (WHO) as a drug for the initial treatment of malaria (World Health Organization 2006). This medicinal plant is now intensively grown on a large scale for commercial use in Southwest China, because of high artemisinin concentrations in the leaves (usually greater than 10 g kg^{-1} dry leaves) (Yang et al. 2009).

Some plant species can release secondary metabolites, usually referred to allelochemicals, into soil environments to defend themselves against other plants and pathogenic microorganisms (Inderjit 1996; Duke et al. 2000). Similarly, *A. annua* L. can release artemisinin into soil mainly by rain leaching, root exudation and in certain dead plant residues. Dead leaves were shown to be the largest contributor of artemisinin entering soils accounting for more than 86% of the total soil artemisinin (Jessing et al. 2013). Artemisinin concentrations vary greatly in soils cultivated with *A. annua* L. The concentration of artemisinin reached 11.7 mg kg^{-1} soil in northern European soils for growing *A. annua* L. (Jessing et al. 2009) and 25.0 mg kg^{-1} soil in China after plant harvest (Wu, personal communication). However, in Kenyan soils this compound ranged from 10 to 2030 μg kg^{-1} (Herrmann et al. 2013; Jessing et al. 2013). This discrepancy might result from the difference in leaf artemisinin content of commercial cultivars (from 0.1% to over than 1%) and growth periods (Baraldi et al. 2008; Jiang et al. 2013; Aftab et al. 2014). Moreover, laboratory experiments indicated that artemisinin stays in the soil for a relatively long period of time. For example, after 20 mg artemisinin kg^{-1} soil was added into a sandy and loamy soil for 60 and 35 days, respectively, it was still detectable (>0.36 mg kg^{-1} soil) (Jessing et al. 2009).

Artemisinin is toxic to plants, insects, and soil microorganisms. For instance, artemisinin inhibited the germination of lettuce seeds (*Lactuca sativa* L.) and the vegetative growth of Chinese cabbage (*Brassica campestris* L.), wheat (*Triticum aestivum* L.),

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maize (*Zea mays* L.), turnip (*Raphanus sativus* L.), redroot pigweed (*Amaranthusretroflexus* L.) and rape (*B. campestris* L.) at very low concentrations (Duke et al. 1987; Bryson and Croom 1991; Lydon et al. 1997; Shen 2006; Zhao 2008; Jessing et al. 2009). This medical compound also repelled earthworms strongly at realistic field concentrations (Jessing et al. 2009) and delayed the reproduction of freshwater algae cells and *Microcystis aeruginosa* (Ni et al. 2012a,b). Both the oil and artemisinin extracted from *A. annua* L. had exhibited strong antibacterial action towards soil bacteria such as *Agrobacterium rhizogenes*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Staphylococcus aureus* and *Staphylococcus epidermidis* (Dhingra et al. 2000; Emadi and Yassa 2009). No antimicrobial effects, however, were found on other selected fungi and bacteria in laboratory and field tests, nor are there any reports on the interaction between artemisinin and ectomycorrhizal fungi (Kapoor et al. 2007; Slade et al. 2009).

Compared with other substances released from plants such as artemetin, chrysosplenol-D and chrysosplenetin, artemisinin as an allelochemical released from *A. annua* L. is quite soluble and mobile, particularly in areas with heavy rains (Baraldi et al. 2008; Jessing et al. 2009; Timoteo et al. 2010). Many soils used for the commercial cultivation of *A. annua* L. have a sandy texture and are located in forest clearings on the hillsides of mountainous areas in Southwest China. In addition, the rainfall season is from May to August, which overlaps with the growth period of *A. annua* L. It is likely that artemisinin contained in the soil and plant residues of *A. annua* L. on the soil surface might easily leach into the nearby forest soil *via* ground and surface runoff (Herrmann et al. 2013). The compound might then react with ectomycorrhizal fungi during and after the cultivation of *A. annua* L. Li et al. (2009) observed fewer ectomycorrhizal fungal sporocarps in forests near *A. annua* L. fields than those further away, indicating that the presence of artemisinin in soil could have detrimental effects on ectomycorrhizal fungi.

Many forest trees have evolved mutualistic symbioses with ectomycorrhizal fungi, in that the latter contribute to tree nutrition and growth. These fungi obtain carbohydrates from host trees and, in return, provide the plants with mineral nutrients such as phosphorus, potassium, calcium and magnesium (Jentschke et al. 2001; Landeweert et al. 2001; Adeleke et al. 2010). In the process of nutrient absorption, the extrametrical mycelia on the mantle of ectomycorrhizae enlarge soil spaces for host nutrient absorption (John et al. 1983; Landeweert et al. 2001). Proton and oxalate effused from the mycelia mobilize nutrients and increase their availability (Yuan et al. 2004). Through deep weathering and strong leaching, tropical and subtropical soils are usually deficient in available nutrients, underscoring the importance of nutrient mobilization by ectomycorrhizas for trees in these forests. To better understand the effects artemisinin might have on the fungal functions and soil nutrition in areas where *A. annua* L. is grown commercially, a pure liquid culture experiment was carried out to elucidate the influence of this allelochemical on the hyphal growth, proton and oxalate efflux, and nutrient uptake of four ectomycorrhizal fungal isolates that are widely distributed in local forests.

Materials and methods

Fungal strains

Four of the ectomycorrhizal (ECM) fungal strains used in this experiment, namely *Suillus luteus* 1 (*S. luteus* 1), *Suillus luteus* 13 (*S. luteus* 13), *Suillus luteus* 11 (*S. luteus* 11) and *Suillus subluteus* 12 (*S. subluteus* 12), were kept in the microbiology laboratory of the College of Resources and Environment, Southwest University, Chongqing, China. These ECM fungi occur on tree roots in forests that neighbour *A. annua* L. fields and were originally isolated from sporocarps in those Chongqing forest soils with a pH ranging from 4.0 to 6.4. Mycelia for inoculation were grown on Pachlewski agar medium for three weeks at 25 ± 1 °C in the dark. The medium contained the following compounds (in g L^{-1}): tartrate (0.5), KH_2PO_4 (1.0), $MgSO_4$ (0.5), glucose (20), maltose (5.0), vitamin B1 (0.1), and agar (20) as well as 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).

Experimental procedure

Twenty millilitres of the Pachlewski medium was transferred into 250 mL Erlenmeyer flasks and steam-sterilized at 121 °C for 30 min. Artemisinin (content ≥ 98%) was obtained from the Institute of Traditional Chinese Medicine, Chongqing, China, and added into culture solutions to arrive at nominal concentrations of 0, 12.5 and 25 mg L^{-1}, after sterilization. We chose these relatively high concentrations to make sure any possible effects would be discovered in this first study of the area. Each flask was inoculated with a mycelial plug (6 mm in diameter) and incubated without agitation for three weeks at 25 ± 1 °C in the dark with 12 replicates for each treatment.

Sampling and analysis

Fungal mycelia were harvested by filtration and washed with deionized water to remove the liquid culture medium from the surface. They were then oven-dried, weighed and digested with H_2SO_4–H_2O_2. Nitrogen (N) in the digests was analyzed by the 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 $pH = -\log_{10}[H^+]$. Thereafter, the culture solutions were acidified by adding 0.1 mol HCl L^{-1} to dissolve oxalate salts such as MgC_2O_4, CaC_2O_4, $Fe_2(C_2O_4)_2$ and others. Solution oxalate was subsequently analyzed by high performance liquid chromatography (HPLC; Hitachi, Japan). Sample solutions (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 the mobile phase at 0.5 mL min^{-1} and 450 psi. The standard for the oxalate solution was prepared and run before and after the sample solutions. The retention time of oxalate was 8.80 min. The rate of proton and oxalate efflux by fungal isolates was calculated by dividing the efflux amount by fungal biomass and incubation days. The amount of fungal proton and oxalate efflux in each flask was calculated using:

$$A = C_h \times V_h - C_i \times V_i$$

where A is the amount of fungal protons or oxalate efflux, C_h is the concentration of protons or oxalate in culture solution at harvest, V_h is the solution volume at harvest, C_i is the initial concentration of protons or oxalate in culture solution (here, the initial oxalate concentration is zero) and V_i is the initial solution volume.

Statistical analysis

All data were subjected to analyses of variance (ANOVA) using SPSS Statistics version 18. All parameters were checked for normality (Shapiro–Wilk) and homogeneity of variance (Levene's test), which showed all variables fit normality assumption. A two-way ANOVA was performed on the data with isolates and artemisinin level as independent factors (Duncan's multiple range test).