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In vitro effect of artemisinin on microbial biomasses, enzyme activities and composition of bacterial community

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ABSTRACT

The anti-malarial drug artemisinin is extracted from the leaves of *Artemisia annua* L. The potential risk of artemisinin released from leaf debris and root exudation of this medicinal plant is unknown for microbes in soils where commercial cultivation of *A. annua* L. is practiced. Thus, a soil incubation experiment was conducted to compare microbial biomass, enzyme activities, and bacterial community compositions by different concentrations of artemisinin (without artemisinin, CK; 10 mg kg^{-1} soil, LA; and 20 mg kg^{-1} soil, HA). The results showed that this compound decreased significantly soil microbial biomass carbon and nitrogen, and activities of dehydrogenases and urease, implying the inhibition on the growth, reproduction, and metabolism of some bacteria, taking into account of the facts that the microbial biomass includes those of bacteria and dehydrogenases cannot act on their own without viable bacterial hosts. There were 13064, 11738, and 10107 16S rDNA sequences picked up from soils of CK, LA, and HA, respectively, by MiSeq Illumina sequencing, which were attributed into 541 (CK), 453 (LA), 414 bacterial phylotypes (HA). Soils received artemisinin showed lower bacterial richness and diversity indexes than CK. Less group and number of bacteria present in soils could encourage over reproduction of any single bacterial taxon and thus increase the risk of plant disease occurrence. All soils studied were dominated by three bacterial phyla (Acidobacteria, Proteobacteria, and Actinobacteria), with these three phyla accounting for 60.32-70.43% of the total bacteria. However, most of bacterial phyla and predominant phylotypes varied significantly in their abundances between CK and artemisinin treatment. Among the top 20 predominant bacteria, five to seven were unique in each of soils studied. Therefore, artemisinin released from *A. annua* L. in cultivation might exhibit selective antibacterial action towards soil bacteria and cause great changes in bacterial activities and community structure, which could influence, at least in part, soil processes and functions.

1. Introduction

About 40% of the population living in tropical and subtropical areas is threatened by malaria (Dhingra et al., 2000a). Artemisinin extracted from the leaves of *Artemisia annua* L. is recommended by the World Health Organization (WHO) as a drug for the initial treatment of this disease (World Health Organization, 2006). Semi-synthesis of this anti-malarial compound is now possible (Paddon et al., 2013). However, chemical synthesis or in vitro production of artemisinin is not economically feasible, and *A. annua* L. has to be grown artificially for extraction of artemisinin.

Secondary metabolites released by some plant species, 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 soils mainly by defoliation and root exudation. The greenhouse experiment conducted by Jessing et al. (2013) showed that artemisinin released from dead leaves during decomposition were the largest contributor of this chemical entering soils, accounting for more than 80% of the total artemisinin in soils. Artemisinin and its synthesis precursors such as artemisinic acid and artemisitene were detected in agar medium for the tissue culture of *A. annua* L when roots were formed (Weathers et al., 1994). However, this metabolite is present in subcuticular and extracellular spaces in glandular trichomes on the surfaces of leaves, stems, corolla, and floret receptacles of this medicinal plant (Wetzstein et al., 2014). The release of artemisinin by rain leaching into soils should not be neglected because of frequent rains in tropical and subtropical areas where *A. annua* L is grown, and moreover, laboratory experiments indicated that this anti-malarial compound stays in the soil for a relatively long period of time. After 20 mg kg^{-1} artemisinin was

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added into a sandy and loamy soil for 30 and 35 days, respectively, it was still detectable (> 0.36 mg kg^{-1} soil) (Jessing et al., 2009). Therefore, the continuous input of this metabolite into soils over a growing season can cause accumulation (Jessing et al., 2013). The concentration of artemisinin in field soils reached 2.30 mg kg^{-1} soil in Kenya (Jessing et al., 2011), 11.7 mg kg^{-1} soil at Aarselv in the middle of Denmark during the growth period of *A. annua* L. (Jessing et al., 2009), and even about 20.0 mg kg^{-1} soil in Chongqing, Southwest China, after the harvest (Wu, personal communication). There are adverse effects of artemisinin on soil environments because it is toxic to plants, insects, and microorganisms. For example, artemisinin inhibited the seed germination and the vegetative growth of lettuce (*Lactuca sativa* L.), Chinese cabbage (*Brassica campestris* L.), wheat (*Triticumae stivum* L.), maize (*Zea mays* L.), turnip (*Raphanus sativus* L.), redroot pigweed (*Amaranthus retroflexus* L.) and rape (*Brassica 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). Morvillo et al. (2011) found that soybean yield was affected adversely by the presence of *A. annua* L. in an intercropping system, indicating negative allelopathic interactions. This medical compound was also toxic to beetles (Duke et al., 1987) and repelled earthworms (*Eisenia fetida*) strongly at realistic field concentrations (10% and 50% effect concentrations were 5.24 ± 2.64 and 21.57 ± 4.73 mg kg^{-1}, respectively) (Jessing et al., 2009). In laboratory tests, both the oil and artemisinin extracted from *A. annua* L. showed a strong antibacterial action towards several soil bacteria such as *Agrobacterium rhizogenes, Pseudomonas aeruginosa, Bacillus subtilis, Bacillus cereus, Staphylococcus aureus* and *Staphylococcus epidermidis* (Dhingra et al., 2000b; Emadi et al., 2011). In addition, the number of cultural bacteria on agar medium and ^{3}H-leucine incorporation into bacteria from the soil for cropping *A. annua* L. were reduced compared to the control without *A. annua* L., indicating that the cultivation of this plant might impact soil bacterial compositions and activities (Herrmann et al., 2013).

There are many large *A. annua* L. plantations, accounting for about 80% of global artemisinin production, in Chongqing, Southwest China, because of high artemisinin concentrations in the leaves (usually greater than 1.5 g kg^{-1} dry leaves) (Yang et al., 2009). When this plant is introduced in a new area, the soil ecosystems are exposed to high concentrations of artemisinin released by root exudation and defoliation. However, little information is known about the impacts of this allelochemical on soil microbial activities and community compositions. The main objectives of the present experiment were to realize the changes of soil microbial biomasses, enzyme activities, and bacterial community compositions in vitro as affected by artemisinin, and to give some information for evaluating the potential risk of *A. annua* L. cultivation to soil health and functions.

2. Materials and methods

2.1. Experimental soil and treatments

The cultivated horizon of a purplish soil (entisols, pH 6.32) without growing *A. annua* L. in history, which is widespread in *A. annua* L. cultivation areas in Chongqing, Southwest China, was used in our incubation experiment. The soil was loamy in texture and contained 13.05% of clay (< 0.002 mm) and 2.31% of organic matter.

1.00 kg fresh soil (68.5% of maximum field moisture capacity) was placed in a plastic container. 25 mL aqua solutions containing artesunate at 0, 0.54, and 1.09 g L^{-1}, respectively, were mixed homogenously with the soil in each container to reach artemisinin concentrations (mg kg^{-1} soil) at 0 (CK), 10 (low artemisinin, LA) and 20 mg (high artemisinin, HA). These nominal artemisinin concentrations are very close to realistic field concentrations found in the middle of Denmark (11.7 mg kg^{-1} soil) (Jessing et al., 2009) and Chongqing, Southwest China (about 20.0 mg kg^{-1} soil) (Wu, personal communication), which might make sure real effects would be discovered in this first study of the area. Each of container was covered with a microporous film (50 μm in diameter), and incubated at 30 °C for 60 days. There were five replications for each treatment. During the incubation period, the soil moisture was kept at 70 ± 2.5% of maximum filed water capacity by adding sterile water every week. This moisture content is optimal for the growth of *A. annua* L. in cultivation.

2.2. Soil sampling and analysis

Before sampling, soil was mixed again homogeneously. Each of soil samples taken from containers was divided into two parts. One part of them was N_2-frozened immediately for microbial biomass measurement and bacterial 16S rDNA pyrosequencing. The soil microbial biomass carbon (MBC) and nitrogen (MBN) were determined on a 15-g oven-dry equivalent fresh soil sample by the chloroform–fumigation–extraction method (Lin et al., 1999). 16S rDNA extraction (1.00 g oven-dry basis of fresh soil), amplification, pooling, MiSeq Illumina sequencing, and data processing were performed in Majorbio Biotech Co., Ltd (Shanghai, China) as described by Qiu et al. (2012). The other part of soil samples was air dried to determine organic matter (OM) by the dichromate oxidation method (Bao, 2008). Soil dehydrogenases activity was assayed by the method of Del Egido et al. (2017) based on the spectrophotometrical measure of red formazan released after incubation of soil samples for 1 h at 37 °C with 2, 3, 5- triphenyltetrazolium as the substrate. The method of estimating urease activity comprised incubation of soil with an aqueous urea solution, extraction of ammonium with mixture solution of 1 N KCl and 0.01 N HCl and colorimetric NH_4^+ determination by a modified indophenol reaction (Guan, 1986). Phosphatase activity was determined by colorimetric estimation of the *p*-nitrophenol released by phosphatase after soil was incubated with buffered sodium *p*-nitrophenyl phosphate solution (pH 6.50) and toluene at 37 °C for 1 h (Guan, 1986). β-glucosidase activity was determined according to the spectrophotometrical measure of *p*-nitrophenyl (pNP) released after 1 h of soil incubation at 37 °C with *p*-nitrophenyl-β-D-glucopyranoside as the substrate in modified universal buffer (pH 6.00) (Guan, 1986).

2.3. Statistical analysis

Statistical analyses, including ANOVA and mean differences by least significant differences (LSD), were performed to compare the treatment effects of different artemisinin concentrations using the SAS statistical software package (2002, version 9.1.3). All results were considered significantly different at $P < 0.05$ unless noted otherwise.

3. Results

3.1. Soil OM, MBC, and MBN

All tested soils showed no significant difference in OM, ranging from 29.43 to 32.05 mg kg^{-1} soil. However, artemisinin addition decreased significantly soil MBN compared with CK and the similar trend was also found with soil MBC and microbial C/N ratio (not significant between CK and LA) (Table 1).

3.2. Soil enzyme activities

Artemisinin decreased dehydrogenase activities in soil compared to CK (not significant between CK and LA (Table 2). Urease activities followed a similar trend to dehydrogenase, with CK showing the highest activity (55.47 mg NH_4-N kg^{-1} h^{-1}) and HA showing the lowest (46.07 mg NH_4-N kg^{-1} h^{-1}). However, all soils had similar phosphotase and β-glucosidase activities irrespective of artemisinin addition, ranging from 3.45 to 3.82 mg pNP kg^{-1} h^{-1} (Phosphotase) and from 32.44 to 36.17 mg Glu kg^{-1} h^{-1}(β-glucosidase).