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Eucalyptus urophylla root-associated fungi can counteract the negative influence of phenolic acid allelochemicals

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ABSTRACT

Root-associated fungi can potentially affect plant allelopathy by degrading or transforming allelochemicals. However, no microorganism has been identified as an allelochemical moderator in *Eucalyptus* plantations. In this study, three *Helotiales* sp. (EU03, EU04, and EU05) were isolated from the roots of *Eucalyptus urophylla*, and all three of the isolated fungi were grown in vitro with mixed phenolic acids (benzoic acid, *p*-hydroxybenzoic acid, and vanillic acid) as the sole carbon source. EU03 and EU05 can degrade 99% of the *p*-hydroxybenzoic acid in an inorganic culture medium within 9 days, while EU04 can degrade 100% of the three phenolic acids in potato dextrose broth medium within 7 days. A mixed inoculation with the three isolates can alleviate the allelopathic effects of phenolic acids and *E. urophylla* litter decomposition on *Albizia julibrissin* (Fabaceae) nodulation *ex situ*. The number of nodules on *A. julibrissin* grown with inoculated fungi plus 50 mg kg^{-1} or 100 mg kg^{-1} mixed phenolic acids were 2.17 times and 1.67 times greater than those grown without the inoculation treatments, respectively. These results highlight the role of *E. urophylla* root associated fungi, which have the potential to moderate the phenolic acid allelopathy caused by *E. urophylla*. Manipulating root associated fungi is an effective way to alleviate the effects of ecological suppression caused by *Eucalyptus* allelochemicals.

1. Introduction

Eucalyptus (Myrtaceae) is the most common genus used for afforestation or reforestation. It also forms a major commercial tree species group in southern China because of its rapid growth and adaptability (Cossalter and Pye-Smith, 2003; Gardner, 2007). However, the large *Eucalyptus* plantations in southern China have produced many problems for the local environment, such as biodiversity loss, soil erosion, and soil fertility decline (Tang et al., 2007; Yang et al., 2017). Many studies have shown that the allelopathic compounds produced by *Eucalyptus* are the main cause of decreased biodiversity (Florentine and Fox, 2003; Batish et al., 2004; Fang et al., 2009; Zhang and Fu, 2009).

A number of laboratory-based *Eucalyptus* allelopathy experiments have focused on the effects of volatilization, leaching, foliage litter decomposition, and root exudation on seed germination and the early growth of a number of different species (Sasikumar et al., 2002; Florentine and Fox, 2003; Espinosa-Garcia et al., 2008; Fang et al., 2009; He et al., 2014). However, most studies do not consider the potential role of soil microorganisms in allelopathy. A previous report showed that soil microorganisms play an important role in determining allelopathic activity (Inderjit, 2005). Many beneficial soil microorganisms can degrade allelochemicals and use them as an energy source or can detoxify the allelochemicals in the soil by transforming them to less toxic compounds (Inderjit, 2005; Ehlers, 2011). In continuous monoculture cropping systems, manipulating soil microbial populations has been shown to be an effective way of alleviating allelopathic stress (Hawari et al., 2000; Kluczek-Turpeinen et al., 2005; Jilani et al., 2008).

In recent years, an increasing number of studies have demonstrated that plant root-associated fungi have the ability to degrade allelochemicals. Ectomycorrhizal fungi, such as *Paxillus involutus* and *Laccaria laccata*, can degrade and detoxify water-soluble phenolic compounds and use the degraded products as a carbon source (Guenther et al., 1998; Munzenberger et al., 2003; Zeng and Mallik, 2006). Broad-spectrum endophytic fungi, such as *Phomopsis liquidambari*, have been shown to efficiently degrade 4-hydroxybenzoic acid and sinapic acid both in culture and in soils (Chen et al., 2011; Xie et al., 2016). Plant root-associated fungi are widespread in plant roots, and they can considerably affect soil chemical composition, microecosystems, and physical structure over their life cycle (Wang and Dai, 2011; Jumpponen et al., 2017).

Phenolic compounds are secondary substances in *Eucalyptus* and have phytotoxic effects on many other plant species (Sivagurunathan et al., 1997; Chapuis-Lard et al., 2002; Batish et al., 2004). These

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compounds have been shown to be the main substances entering the soil through the decomposition of litter and litter leachates (Chapuis-Lard et al., 2002). Lu et al. (2017) reported that soil microorganisms can alleviate the allelopathic effect of *Eucalyptus* leachates. Furthermore, the total phenolic content was lower in non-sterile soils than in sterile soils when both were treated with leachates. However, it was not clear which microorganisms played a key role. This study aimed to isolate root-associated fungi from *E. urophylla* so that they could be screened for beneficial fungi that degrade phenolic acid chemicals. In the greenhouse experiments, we also assessed if inoculation with these fungi could alleviate the negative allelopathy effects of phenolic acids and *E. urophylla* leaf litter decomposition on the nitrogen-fixing plant *Albizia julibrissin* (Fabaceae) growth and root nodulation.

2. Materials and methods

2.1. Plant sampling and soil collection

Eucalyptus urophylla roots were collected from a *E. urophylla* plantation in Shuilian Mountain Forest Park, Dongguan City, Guangdong Province, China (22° 58′ N and 113° 42′ E; 250–300 m asl). The climate is subtropical monsoon with a mean annual precipitation of 1700 mm and a mean annual temperature of 23 °C. Soil used for the greenhouse experiment was collected from an evergreen broadleaved mixed forest (most species in the area were legumes) that was near to the *E. urophylla* plantation. Plant leaf litter from *E. urophylla* was collected randomly from the E. *urophylla* plantation and air dried before the litterbag was designed.

2.2. Isolation of root-associated fungi in Eucalyptus urophylla

Eucalyptus urophylla roots were washed in deionized water, surface sterilized in 75% ethanol for 30 s and 10% sodium hypochlorite for 2 min, and then rinsed three times in deionized water. Root sections were placed in 90 mm petri dishes containing potato dextrose agar (PDA). After 3–7 days of incubation at 25 °C, the mycelia growing out of the roots were sub-cultured and individually maintained on PDA. The isolated fungi were inoculated onto inorganic screening medium (NH_4NO_3 1 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 0.5 g L^{-1}, $(NH_4)_2SO_4$ 0.5 g L^{-1}, KH_2PO_4 0.5 g L^{-1}, NaCl 0.5 g L^{-1}, and K_2HPO_4 1.5 g L^{-1} in 1 L, pH 7.0) containing 75 mg L^{-1} three phenolic acids (benzoic acid, *p*-hydroxybenzoic acid, and vanillic acid, the content of each phenolic acid was 25 mg L^{-1}) as the only carbon source. The fungi with high growth rates were selected and stored at 4 °C on PDA for further study.

2.3. Molecular identification of the selected fungi and phylogenetic analyses

The selected fungi were cultured at 25 °C in potato dextrose broth (PDB, containing 24.1 g potato and glucose extract) medium that had been shaken for 3 days at 160 rpm on an orbital shaker. For molecular identification, fungi DNA were extracted using HP Fungal DNA Kit (Omega Bio-tek). Then, the internal transcribed spacer (ITS) of each isolated fungus was amplified by primer of ITS1 (5′-TCCGTAGGTGAA CCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Yuan et al., 2010), the PCR products were sequenced by Shanghai Majorbio (Shanghai Majorbio Bio-pharm Technology Co., Ltd, Shanghai, China). The ITS sequences were used to retrieve similar sequences from GenBank using the NCBI BLAST program. The taxonomic affiliation was determined by comparing the sequences with known sequences in GenBank using BLASTN. The taxonomic, affiliation was inferred from the closest hits. The molecular analysis results identified the hosts of similar fungi and the corresponding ITS sequences were researched in GeneBank. Sequences were aligned using BioEdit and imported into the Molecular Evolutionary Genetics Analysis (MEGA) package version 5. Phylogenetic analyses were conducted using the neighbor joining tree method found in MEGA with the pairwise deletion option for handling alignment. Gaps and bootstrap tests were conducted using 1000 replicates (Zijlstra et al., 2005).

2.4. Identification of the optimal culture concentration of phenolic acids for fugal-mediated degradation

An inorganic culture medium was used as the basic control medium. Then 25 to 200 mg L^{-1} of each of three phenolic acids (benzoic acid, *p*-hydroxybenzoic acid, and vanillic acid) were mixed and used as the sole carbon source. All the treatments were incubated in the dark at 25 °C on a shaker rotating at 160 rpm. After 9 days cultivation, the fungal mycelia were collected using filter paper, washed twice with sterilized distilled water, the dry biomass of fungi was recorded after drying at 60 °C for 24 h. The used culture medium was tested to determine the concentration of the three phenolic acids left in the medium. There were three replicate cultures for all treatments.

The contents of the three phenolic acids were identified and quantified with gas chromatography (Agilent 7890, California, USA) coupled to a mass selective detector (Agilent 5975). An HP-5MS column (30 m × 0.25 mm × 0.25 μm, Agilent) was used for GC–MS. The temperature program was set as follows: initial temperature of 60 °C, increasing at a rate of 3 °C min^{-1} to 70 °C with a 2 min hold. Following the hold, the temperature was increased to 93 °C at 5 °C min^{-1} with a 2 min hold, followed by an increase to 280 °C at a rate of 10 °C min^{-1}. The 1 μL samples were injected in the split mode (1:10).

External calibration curves were used to quantify the test concentrations of the phenolic acids. In order to eliminate possible experimental and injection error, octadecanoic acid was used as the internal standard to calculate the tested concentrations, according to the ratio of tested allelochemicals to the internal standard.

2.5. Phenolic acids degradation in liquid culture by the isolated fungi

Based on the maximum fungal biomass and high phenolic acids degradation section 2.4, the optimal concentration of each phenolic acid for fungal degradation was 100 mg L^{-1}. Therefore, a culture solution containing 100 mg L^{-1} of each three mixed phenolic acids was used to evaluate the ability of the three selected fungi to degrade identified allelochemicals from *E. urophylla*. An isolated colony of each fungal species was used to inoculate 100 mL of inorganic culture solution containing 100 mg L^{-1} of the three phenolic acids cultured at 25 °C with shaking (160 rpm). At 3, 5, 7, and 9 days, 3 mL of the culture solution was taken from each culture to determine the concentrations of the three phenolic acids. A modified method based on Proestos et al., (2006) was used for the silylation procedure. Briefly, aliquots of the subsamples (0.5 mL each) were dried with nitrogen evaporators (Flyde MD 200) at room temperature. The residue was dissolved in 200 μL acetonitrile and silylating reagent (BSTFA + 1% TMCS) and placed in a water bath at 70 °C for 1 h. After cooling, the reaction mixture was filtered with a 0.45 μm organic membrane and then immediately analyzed using an Agilent-5975 GC–7890 MS. Their degradation abilities in full nutrient medium were tested by replacing the inorganic culture medium with PDB.

2.6. Greenhouse experiment

Nitrogen-fixing *Albizia julibrissin* was used to test if inoculating soil with the selected fungi could decrease the allelopathy effects of phenolic acids and *E. urophylla* leaf litter decomposition on legume-mutualism and plant growth. The stored fungi were activated in PDA at 25 °C and cultured as fungal inoculums in PDB medium as described previously. The fungal mycelia were collected, washed five times with sterilized distilled water before they were added to the soils. Surfaced-sterilized *A. julibrissin* seeds were germinated prior to sowing and then three of seeds were planted into a pot. Each pot contained 300 g of field soil. The plants were thinned to one per pot within the first week. The