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The potential application of the endophyte *Phomopsis liquidambari* to the ecological remediation of long-term cropping soil

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ABSTRACT

A broad-spectrum endophyte, *Phomopsis liquidambari*, was used as a microbial agent to determine the effects of rapid litter decomposition on soil phenolic compounds dynamics, the soil microbial community balance and plant growth. The litter decomposition ratio was closely correlated with lignin degradation. The soil phenol concentration increased with the acceleration of litter decomposition after the first 30 days and later decreased to below the initial level. Based on denaturing gradient gel electrophoresis (DGGE) analysis, soil bacteria, especially gram-negative bacteria that have the potential to degrade aromatic compounds, were found in high abundance when the soil phenol concentration was high. When the phenolic concentration decreased, soil fungi increased in abundance. With fungal application, seed germination significantly increased to 69.87% and seedling growth was enhanced. Rapid litter decomposition by *Pho. liquidambari* initially led to higher releases of phenolic allelochemicals, which led to the enrichment of soil gram-negative bacteria. In addition, increased soil nutrients and temporarily higher concentrations of phenolics from litter decomposition strengthen seedling growth, suggesting that the endophytic fungus *Pho. liquidambari* is a suitable candidate for remediation of long-term cropping soil.

1. Introduction

Leaf litter is a fundamental natural resource containing a large carbon stock. Under natural conditions, the decomposition of leaf litter is slow and limited by various biotic and abiotic factors. Moreover, with litter decomposition, the micro-molecular phenolic allelochemicals that are present in inner leaf tissues are leached into the soil. There, they negatively affect seed germination and growth by inhibiting metabolic enzymatic activities and disturbing the synthesis of auxin (Weir et al., 2004). In addition, Li et al. (2010a) reported that the release of phenolic allelochemicals could disrupt the soil microbial balance by modifying the structures of populations and communities. In large and complex ecological systems such as tropical and temperate forests, the balance of phenolic molecules is maintained through microbe–plant interactions in soil (Inderjit, 2005). However, in long-term cropping systems, the carbon balance is disturbed by litter retention and the accumulation of phenolic allelochemicals. As the world population increases and there is less area available for farming (Li et al., 2010a), screening microbes for their ability to accelerate decomposition and eliminate allelopathic phenolics in the soil may increase the sustainability of agriculture and our ability to use lands repeatedly.

Endophytic fungi are microorganisms that live within the inner tissue of plants without causing apparent disease symptoms (Wilson, 1995). Although endophytic fungi are primarily mutualistic and commensalistic symbionts, they may not continue as endophytes throughout their life cycles (Porras-Alfaro and Bayman, 2011). As the surrounding endophytic environment is damaged (through leaf senescence, abscission, or damage), some endophytes are triggered to grow and sporulate like saprobes, and decompose recalcitrant compounds in the litter (Osono and Hirose, 2009). *Phomopsis* spp. are common endophytic fungi that exist in a wide variety of plants (Murali et al., 2006; Li et al., 2010b). As a pioneer decomposer, *Phomopsis* spp. have been demonstrated to accelerate leaf litter decomposition and soil nitrogen transformation (Dai et al., 2010; Chen et al., 2013a). Their decomposing function is directly related to lignin-degrading enzymes such as laccase, cellulose, xylanase, and β-mannanase (Promputtha et al., 2010; Chen et al., 2013b). Our previous study demonstrated that the endophyte *Phomopsis liquidambari* (strain B3) is capable of growing with the typical phenolic compound 4-HBA (4-hydroxybenzoic acid) as the sole carbon and energy source *in vitro* (Chen et al., 2011). These studies indicated that *Pho. liquidambari* is a valuable microbial resource for potential applications in agricultural soil where phenol concentration is high and leaf organic matter decomposition is slow.

To clarify the effects of rapid litter decomposition by *Pho. liquidambari* on the release of soil phenolic allelochemicals and to

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determine whether such decomposition influences the balance of soil microbes or plant growth, we investigated the dynamics of phenolic allelochemical concentrations and microbial communities in *Atractylodes lancea* litter and soil containing *Pho. liquidambari*. The growth of plant seedlings and the activity of the defensive enzymes were also evaluated to investigate the effectiveness of using the endophyte *Pho. liquidambari* as a novel microbial agent for plant growth. Our study is the first to investigate the influence of rapid decomposition by an endophytic fungus *in vitro* on surrounding biological factors.

2. Materials and methods

2.1. Inoculation of the endophytic fungal strain

The endophytic strain B3 was isolated from the inner bark of *Bischofia polycarpam* (Shi et al., 2004) and identified as *Pho. liquidanbari* (Chen et al., 2011). The fungus was stored at 4 °C on potato dextrose agar (containing 200 g L^{-1} potato extract, 20 g L^{-1} glucose, and 20 g L^{-1} agar, pH 7.0). It was first activated in 100 mL potato dextrose broth (containing 200 g L^{-1} potato extract and 20 g L^{-1} glucose, pH 4.5), in Erlenmeyer flasks (250 mL) for 3 days at 160 rpm in an orbital shaker at 28 °C, to create a seed culture broth. Submerged fermentation was then carried out in a 5 L fermentation vessel (Biotech-5JPZ, Jiangsu, China), containing 3 L sterilized medium (20 g L^{-1} CMC-Na as carbon source, 200 g L^{-1} potato extract, 1 g L^{-1} NH_4Cl, 3 g L^{-1} KH_2PO_4, 1.5 g L^{-1} $MgSO_4{\cdot}7H_2O$, and 10 mg L^{-1} vitamin B_1, pH 4.5), and 10% seed culture broth was maintained for 4 days at 25 °C and 150 rpm with an aeration rate of 180 L h^{-1}. To evaluate the dry weight, the mycelia from 10 mL of culture broth was washed with sterile water twice and dried in an oven at 80 °C to a constant weight. A total of 80.24 g (equal to 7.98 g dry weight) of fungal mycelia was collected and washed twice with sterilize deionized water. The mycelia were then diluted with distilled water to a final volume of 5 L. The solution was used as the microbial agent.

2.2. Litter decomposition experimental design

The soil for the experiment was yellow-brown loam and was obtained from three randomly selected 1 m × 1 m (length × width) sites that were 5–10 cm below the surface of a long-term planting of the traditional medicinal herb *A. lancea.* The experimental station was located in Jurong, Jiangsu Province (31°37′N, 118°57′E). The soil contained 14.68 g kg^{-1} total carbon, 1.22 g kg^{-1} total nitrogen, 334.57 mg kg^{-1} of available phosphorus, and 123.33 mg kg^{-1} of available potassium and had a pH of 6.69. Soil moisture content was 28.2%. The soil was first passed through a 1 mm sieve and then prepared as flowerpot soil. Plant litter from *A. lancea* was collected randomly from the soil surface (mentioned above) by hand and stored at 4 °C for 3 days. The C/N ratio of the litter was 49.5. The litter was washed twice with sterile distilled water and cut into 2–4 cm sections before the litterbag was designed.

Plant litter decomposition and lignin degradation were studied using the buried litterbag method in flowerpots (23 cm diameter × 22 cm height) (Conn and Dighton, 2000). For the $B3^+$ treatment, 400 mL of microbial agent (containing 6.38 g dry weight of fungi) was mixed with 4.5 kg of soil and translocated to a flowerpot. A plastic litterbag (mesh size 0.8 mm) filled with 2.5 g of dry plant litter was buried 5 cm below the surface of the pot soil. Pots were placed in a room with a diurnal temperature range of 15–25 °C, and 65–80% relative humidity. A control treatment (Ck treatment) without the microbial agent and with an inactivated fungal agent (B^- treatment: the agent was sterilized at 121 °C for 20 min before being added to the soil) were processed in an identical manner. Soil moisture was maintained at 20–30% by spraying the soil surface with water. For each treatment in each time interval, pots were created in triplicate. After incubation for 30 and 60 days, three litterbags of each treatment were first dried in the laboratory (45 °C for 3 d) to measure the rate of litter decomposition, and then used for lignin level detection by the 72% sulphuric acid method (Klason, 1893). Meanwhile, soil samples obtained from 5–10 cm below the soil surface in the pots were sieved (1 mm mesh) and stored at −20 °C for soil phenolic compound detection and analysis of the microbial community.

Table 1
Primers used for the PCR-DGGE analyses.

Target group	Name	Sequence (5′–3′)	Reference
Bacterial 16S rRNA	GC[a]-F357	TAC GGG AGG CAG CAG	Muyzer et al. (1993)
	517R	ATT ACC GCG GCT GCT GG	
Fungal 18S rRNA	GC[b]-EF4	AGG GGR TGT ATT TAT TAG	Smit et al. (1999)
	Fung5R	GTA AAA GTC CTG GTT CCC C	

R = A/G.
[a] (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GCC G).
[b] (CGC CCG CGC CGC GCG GCG GGC GGG GCG GGG GCC CCG GGG).

2.3. Phenolic acids analysis

Soil phenolic acids were quantified by high-performance liquid chromatography (HPLC) (Hartly and Buchan, 1979). Each soil sample (10 g) was extracted in 15 mL 1 M NaOH for 24 h and then shaken at 180 rpm for 30 min and filtered. The filtrate was acidified to pH 2.5 with 12 M HCl, and then centrifuged at 2000 × *g* for 10 min. The supernatant was filtered through a 0.22 μm membrane. We used an Agilent 1100 Infinity HPLC equipped with a UV detector and Agilent ChemStation Software (Santa Clara, CA, USA) for the quantitative and qualitative analysis of phenolic acids. An Agilent C18 column, 200 mm × 4.6 mm, with a particle size of 5 μm was used for the HPLC. The mobile phase consisted of acetonitrile and 1.3% acetic acid at a ratio of 17:83 (v/v). The flow rate was 0.6 mL min^{-1}, the injection volume was 20 μL, the detection wavelength was 260 nm, and the column temperature was 35 °C.

2.4. DNA extraction and PCR amplification

Soil DNA was extracted using an UltraClean Soil DNA kit (Carlsbad, CA, USA). Bacterial 16S rRNA was amplified using an universal forward primer F357-GCclamp and a reverse primer 517R; and fungal 18S rRNA was amplified using the forward primer EF4-GCclamp and the reverse primer Fung5R (Table 1). The 50 μL reaction mixture consisted of 5 μL of 10× PCR buffer (Mg^{2+} free), 3 μL of 25 mM $MgCl_2$, 4 μL of 2.5 mM dNTPs, 0.5 μL of Taq polymerase (5 U μL^{-1}, Takara Biotechnology, Japan), 1 μL of each primer at 50 mM, and 1 μL of DNA template (10–15 ng). The touchdown PCR strategy used for amplification of bacterial 16S rRNA and fungal 18S rRNA included denaturation at 94 °C for 4 min, followed by 8 touchdown cycles of denaturation at 94 °C for 40 s, annealing at 58 °C (−1 °C per cycle) for bacteria (57 °C for fungi) for 1.5 min with an extension at 72 °C for 2 min, followed by 26 cycles of 94 °C for 40 s, annealing at 52 °C for 1.5 min with an extension at 72 °C for 2 min and a final extension at 72 °C for 10 min.

2.5. Denaturing gradient gel electrophoresis analysis

To separate the amplified sequences of the bacteria and fungus, DGGE analyses were performed with the DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA) as follows: the PCR products were separated on a 7.5% (w/v) acrylamide-bisacrylamide gel, using denaturing gradients of 30–60% for bacteria and 40–70% for fungi (the 100% denaturant