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# Degradation of a model pollutant ferulic acid by the endophytic fungus *Phomopsis liquidambari*

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## HIGHLIGHTS

- *P. liquidambari* effectively degraded ferulic acid in MSM and soil.
- The metabolic mechanism of ferulic acid by an endophytic fungus was first studied.
- Transcription of the *fdcB3*, *lacB3* and *pcaB3* genes was induced in *P. liquidambari*.
- *P. liquidambari* laccase played an important role during ferulic acid degradation.
- *P. liquidambari* is a potential microbial agent to mitigate polluted environments.

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## ABSTRACT

Biodegradation of ferulic acid, by an endophytic fungus called *Phomopsis liquidambari* was investigated in this study. This strain can use ferulic acid as the sole carbon for growth. Both in mineral salt medium and in soil, more than 97% of added ferulic acid was degraded within 48 h. The metabolites were identified and quantified using GC–MS and HPLC–MS. Ferulic acid was first decarboxylated to 4-vinyl guaiacol and then oxidized to vanillin and vanillic acid, followed by demethylation to protocatechuic acid, which was further degraded through the β-ketoadipate pathway. During degradation, ferulic acid decarboxylase, laccase and protocatechuate 3,4-dioxygenase activities and their gene transcription levels were significantly affected by the variation of substrate and product concentrations. Moreover, ferulic acid degradation was determined to some extent by *P. liquidambari* laccase. This study is the first report of an endophytic fungus that has a great potential for practical application in ferulic acid-contaminated environments.



## 1. Introduction

Ferulic acid (or 4-hydroxy-3-methoxycinnamic acid) exists in a free form or covalently linked to lignin and other polymers in the plant cell wall, and its distribution is nearly ubiquitous in the plant kingdom (Mathew and Abraham, 2006). However, with the development of agro-industrial operations, ferulic acid is now present in high concentrations in many industrial effluents and residues, including those produced in wine-distilleries, olive oil processing industries, table olive industries and pulp paper processing, etc. (Beltran De Heredia et al., 2001). Ferulic acid is considered a high-priority environmental pollutant because it exhibits high toxicity on the growth of organisms at a low concentration (Fiorentino et al., 2003).

The methods of photocatalytic degradation and chemical oxidation have been studied over the long term for their abilities to promote the degradation of ferulic acid in the environment (Gernjak et al., 2003; Amat et al., 2005). The use of solar photocatalysis for degradation of ferulic acid has proven to be a potential method. However, this methodology has the major drawbacks of limited stability of photocatalysts and the difficulty in recovering the catalyst for re-use (Amat et al., 2005). Chemical oxidation, such as advanced oxidation processes (AOPs), is another method to degrade ferulic acid (Legrini et al., 1993). The main disadvantage of AOPs was their high cost (Gernjak et al., 2003). Biodegradation

*Abbreviations:* MSM, mineral salt medium; FADase, ferulic acid decarboxylase; 3,4-PCD, protocatechuate 3,4-dioxygenase; LiP, lignin peroxidase; MnP, manganese peroxidase; SS, sterile soils; nSS, non-sterile soils.

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usually depends upon the oxidative activities of microorganisms and has played an important role in eliminating environmental pollutants (Fuchs et al., 2011). Nevertheless, microorganisms have not been used frequently to degrade ferulic acid because high concentrations of ferulic acid inhibited the growth of most microbes, although they had the metabolic capability to use it (Mendonça et al., 2004). Therefore, screening appropriate microorganisms for environmental ferulic acid degradation will benefit natural ecology bioremediation.

As a large and novel microbial resource, endophytic fungi have been detected within the tissues of healthy host plants, and their ecological functions have attracted increasing attention. Studies have suggested that endophytic fungi could convert their endogenous roles and adopt a saprobic lifestyle following the senescence of a plant (Promputtha et al., 2010). Müller et al. (2001) and Korkama-Rajala et al. (2008) reported that the decomposition of litter to obtain nutrients was an important strategy for allowing endophytic fungi survive as saprotrophs, and this strategy was associated with their capacity to secrete extracellular degrading enzymes such as laccase, LiP and MnP (Promputtha et al., 2010; Rodríguez et al., 2004). These lignocellulosic enzymes not only decompose lignin but also oxidize phenolic and methoxyphenolic compounds, decarboxylate them and attack their methoxyl groups through demethylation (Rodríguez et al., 2004). Therefore, endophytic fungi should have a great potential to degrade aromatic pollutants.

*Phomopsis liquidambari* is one type of broad-spectrum endophytic fungus that is isolated from the inner bark of the *Bischofia polycarpam* stem (Chen et al., 2011). The presence of exogenous litter significantly induced *P. liquidambari* to produce lignocellulosic enzymes including laccase and LiP (Chen et al., 2013b). Phenolic compounds are general biochemical indicators of lignin decomposition, and their concentrations in soil were associated with the speed of litter decomposition. A previous study found that inoculation of *P. liquidambari* significantly reduced soil phenolic compounds produced by litter decomposition (Chen et al., 2013a). Further study indicated that *P. liquidambari* could effectively degrade 4-hydroxybenzoic acid (Chen et al., 2011). Thus, *P. liquidambari* may have the ability to degrade methoxyphenolics such as ferulic acid, although its structure is more complex than 4-hydroxybenzoic acid.

Therefore, the aims of this study were to investigate the ability of *P. liquidambari* to degrade ferulic acid and the metabolic mechanism of degradation and to investigate the dynamics of the degrading enzyme activities and their gene transcription levels during degradation. Moreover, the important roles of laccase have also been evaluated in ferulic acid degradation. In order to show that endophytic fungus has a great potential to degrade methoxyphenolic pollutant, ferulic acid, and also provide a basis for the widespread application of endophytic fungi in ferulic acid-contaminated soil environment.

## 2. Methods

### 2.1. Experimental materials and fungal inoculum preparation

Ferulic acid, 4-vinyl guaiacol, vanillin, vanillic acid, and protocatechuic acid (purity $\geqslant$ 97%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile was purchased from Honeywell Burdick & Jackson Company (Morristown, NJ, USA). HPLC-grade acetic acid and dichloromethane were purchased from Hanbon Sci. Co. Ltd. (Jiangsu, China).

Soil samples (Ferri-Udic Argosols) were collected from the top layer (0–15 cm) of an uncultivated field located in Nanjing (Jiangsu, China), eastern China, and no ferulic acid was detected. Soil physicochemical properties: Sand (2–0.02 mm), 32.42%; Silt (0.02–0.002 mm), 42.24%; Clay (<0.002 mm) 25.34%; Organic matter, 19.2 g $kg^{-1}$; Total nitrogen, 0.85 g $kg^{-1}$; Total phosphorus 0.43 g $kg^{-1}$; Total potassium, 8.23 g $kg^{-1}$; pH (1:2.5, w:v), 7.30. The soil had a moisture content of 15.67% (w:w).

The endophytic fungus *P. liquidambari* was previously isolated from the inner bark of the stem of *Bischofia polycarpam* (Chen et al., 2011) and stored at 4 °C on PDA medium (200 g $L^{-1}$ potato extract, 20 g $L^{-1}$ glucose and 20 g $L^{-1}$ agar, pH 7.0). The fungal strain was activated in 50 mL PDB medium (200 g $L^{-1}$ potato extract, 20 g $L^{-1}$ glucose, pH 7.0) for 48 h at 28 °C, 180 rpm in an orbital shaker. Fungal mycelia were collected and washed twice with distilled water in order to eliminate most of the available nutrients from PDB, and then diluted to 50 mL mineral salt medium (MSM, 2 g $L^{-1}$ $NaNO_3$, 1 g $L^{-1}$ $K_2HPO_4$, 0.5 g $L^{-1}$ KCl, 0.5 g $L^{-1}$ $MgSO_4$ and 0.01 g $L^{-1}$ $FeSO_4$) in 100-mL Erlenmeyer conical flasks. The dilution was incubated for 2 h at 28 °C in an orbital shaker (180 rpm) to further consumption of remaining available nutrients adhered on mycelia surface and then used as inoculums.

### 2.2. Identification of fungal tolerance concentration of ferulic acid for degradation

Two-milliliter inoculums containing fungal mycelia at a concentration of 1.2 mg $L^{-1}$ were added to 50 mL of MSM with 50–400 mg $L^{-1}$ ferulic acid as the sole carbon source. The cultures were incubated at 28 °C in a shaker rotating at 180 rpm for 48 h. The fermented broth and fungal mycelia were respectively collected by filtering to detect the remaining ferulic acid concentration and fungal biomass (dry weight). Cultures were grown in triplicate for each treatment. Controls without fungal addition were processed in an identical manner.

An Agilent 1290 Infinity with UV detector (Agilent, Palo Alto, CA, USA) and Agilent ChemStation Software were used for detecting ferulic acid concentration. An Agilent C18 column, 250 mm × 4.6 mm, with a particle size of 5 μm was used for HPLC. The mobile phase contained acetonitrile and 1.3% acetic acid at a ratio of 17:83 (v:v). The flow rate, injection volume, detection wavelength and column temperature were 0.6 mL $min^{-1}$, 20 μL, 260 nm and 25 °C, respectively.

### 2.3. The degradation of ferulic acid

Ferulic acid (200 mg $L^{-1}$) was used as the sole carbon source for evaluating the biodegradation ability of *P. liquidambari* because the fungal biomass and degradation rate were relatively high at this concentration. Fungal inoculums and culture conditions were consistent with those described in Section 2.2. Controls without fungal mycelia were handled in an identical manner. To determine whether exogenously added glucose could affect the ability of *P. liquidambari* to degrade ferulic acid, 100 mg $L^{-1}$ glucose was added to the 200 mg $L^{-1}$ ferulic acid test medium to analyze the effect of available carbon on ferulic acid degradation.

The cultures with ferulic acid as the sole carbon source were grown in twelve replicates, and the glucose test cultures were grown in triplicate for each sampling point. At 4 h intervals during 48 h of incubation, three ferulic acid-only cultures and three glucose test cultures were used to detect the remaining ferulic acid concentration and fungal biomass. The other ferulic acid-only cultures were used to analyze fungal metabolic products, degrading enzyme activities and their gene transcription.

### 2.4. Detection of metabolic products and pathway

4-Vinyl guaiacol and vanillin in the 30-mL subsamples (incubation for 32 h) of fermentation broth were extracted and identified by Agilent-5975 GC–MS (Agilent, Santa Clara, CA, USA). A 30-mL aliquot of fermentation broth was extracted with 20 mL of