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Survival of a novel endophytic fungus *Phomopsis liquidambari* B3 in the indole-contaminated soil detected by real-time PCR and its effects on the indigenous microbial community

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ABSTRACT

The recently isolated fungal strain *Phomopsis liquidambari* B3 can degrade high concentrations of indole, indicating its potential for the bioremediation of indole-contaminated soil. In this study, a specific real-time PCR was developed to detect the survival of *P. liquidambari* B3 in soil. Subsequently, degradation activity of strain B3 and its effects on indigenous microbial community were analyzed. Results showed the amount of *P. liquidambari* B3 genomic DNA increased to a maximum 5.67 log (pg g^{-1} dry soil) 10 days after inoculation of 5.04 log (pg g^{-1} dry soil), and then gradually decreased with time and after 40 days it was below the detection limit. By the end of the experiment (day 40), bioaugmented microsoms showed a 93.7% decrease in indole, while the values for biostimulated and control microcosms were much lower. Higher microbial biomass and enzyme activities were observed in bioaugmented soil. Denaturing gradient gel electrophoresis analysis showed bioaugmentation increased richness of resident microbial community. These results indicate that *P. liquidambari* B3 is effective for the remediation of indole-contaminated soil and also provides valuable information about the behavior of the inoculant population during bioremediation, which could be directly used in the risk assessment of inoculant population and optimization of bioremediation process.

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1. Introduction

Indole and its derivatives form a class of toxic recalcitrant N-heterocyclic compounds which are now considered pollutants, since they are released into the environment through coaltar, sewage, coking and dye-stuff wastewater (Kamath and Vaidyanathan 1990; Hong et al. 2010; Chen et al. 2013b). Indole has been reported to cause acute pulmonary edema, emphysema (Carlson et al. 1972), hemoglobinuria, and hemolysis (Hammond et al. 1980) in cattle and goats. Plant tissues exposed to indole showed low pigmentation, presumably due to the inhibition of anthraquinone biosynthesis (Yin et al. 2005). Indole also showed cytotoxic properties in yeasts and bacteria (Kamath and Vaidyanathan 1990; Chimerel et al. 2012; Kim et al. 2013). Liu et al. (1994) reported that indole (10 mg l^{-1}) was lost from the sediment slurries for 27 days under denitrifying conditions. Madsen et al. (1988) found that indole (50 mg l^{-1}) could persist in digested sludge for 45 days under methanogenic conditions. Therefore, accelerating the removal of these compounds is very important from the environmental point of view.

Bioaugmentation is one of the most common approaches for the *in situ* bioremediation of accidental chemical spills and chronically contaminated sites worldwide (Mrozik and Piotrowska-Seget 2010; Tyagi et al. 2011). However, many factors, such as strain selection, contaminant type, microbial ecology, environmental constraints, and culture introduction methods may lead to the failure of bioaugmentation approaches (Avrahami and Bohannan 2007; Longa et al. 2009). The key to success is the survival of the microorganisms that are introduced into the complex ecosystems (Pujol et al. 2006). Because it is an exogenous agent, the environmental impact of the introduced microorganism needs to be evaluated to assess the risk to indigenous microorganisms.

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Table 1
Fungal isolates used to screen to specificity of the primers.

Species	Host	Isolate code	GenBank accession no.
Phomopsis liquidambari	*Bischofia polycarpa*	B3	JQ945200.1
Phomopsis liquidambari	*Liquidambar formosana*	P4	FJ478124.1
Phomopsis eucommiae	*Eucommia ulmoides*	P1	AY601921.1
Phomopsis eucommicola	*Eucommia ulmoides*	P2	AY578071.1
Phomopsis loropetali	*Liquidambar formosana*	P3	AY601917.1
Phomopsis sp. NY302	*Eucommia ulmoides*	NY5	GU462142
Phomopsis sp. SY435	*Eucommia ulmoides*	SY11	GU462143
Phomopsis sp. LG389	*Eucommia ulmoides*	LG20	GU462144
Phomopsis sp. LV286	*Eucommia ulmoides*	LV34	GU462146
Phomopsis sp. BJ188	*Eucommia ulmoides*	BJ40	GU462147
Phomopsis sp. XA242	*Eucommia ulmoides*	XA56	GU462148
Phomopsis sp. NJ6	*Eucommia ulmoides*	NJ57	GU462149
Phomopsis sp. ALX17	*Atractylodes lancea*	LLX 0123	KC172081
Alternaria alternata	*Atractylodes lancea*	LLX 0102	KC134318
Guignardia vaccinii	*Atractylodes lancea*	LLX 0104	KC172070
Cercospora zebrina	*Atractylodes lancea*	LLX 0105	KC172066
Septoria lycopersici	*Atractylodes lancea*	LLX 0108	KC134319
Villosiclava virens	*Atractylodes lancea*	LLX 0112	KC172069
Pseudocercospora marginalis	*Atractylodes lancea*	LLX 0113	KC172077
Colletotrichum jasminigenum	*Atractylodes lancea*	LLX 0118	KC172075
Rhizoctonia bataticola	*Atractylodes lancea*	LLX 0119	KC172071
Leptospora rubella	*Atractylodes lancea*	LLX 0120	KC172076
Sarocladium strictum	*Atractylodes lancea*	LLX 0121	KC172080
Acremonium alternatum	*Atractylodes lancea*	LLX 0122	KC172079
Stemphylium solani	*Atractylodes lancea*	LLX 0124	KC172065
Cladosporium cladosporioides	*Atractylodes lancea*	LLX 0125	KC172067
Fusarium solani	*Atractylodes lancea*	LLX 0127	KC202941
Nectria ipomoeae	*Atractylodes lancea*	LLX 0129	KC202945
Chaetomium globosum	*Atractylodes lancea*	LLX 0134	KC202936
Paraphoma chrysanthemicola	*Atractylodes lancea*	LLX 0135	KC202946
Pseudallescheria boydii	*Atractylodes lancea*	LLX 0141	KC202949
Edenia gomezpompae	*Atractylodes lancea*	LLX 0142	KC202950

Endophytic fungi live within the inner tissues of plants without causing visible disease symptoms (Aly et al. 2010). In recent years, an increasing number of studies have shifted from examining the ecological functions of endophytic fungi *in vivo* toward *in vitro* experiments (Borges et al. 2009; Chen et al. 2013a; Russell et al. 2011). The recently characterized novel fungal endophyte strain *Phomopsis liquidambari* B3, which was isolated from the inner bark of the stem of *Bischofia polycarpa*, could decompose 99.1% of indole at high concentration (100 mg l^{-1}) within 60 h (Chen et al. 2013b). By contrast, other indole-degrading strains, such as *Aspergillus niger*, is unable to tolerate indole concentrations above 50 mg l^{-1} (Kamath and Vaidyanathan 1990). Hong et al. (2010) also faced to the similar problem in the research of bacterial community degradation of indole: the initial concentration of indole was only 40 mg l^{-1}. The high indole-degrading capacity of *P. liquidambari* B3 has established it as a promising candidate for the bioremediation of soils contaminated with high levels of indole. However, the fate and activity of this exogenous strain in soil and its impact on indigenous microbial populations during bioaugmentation processes remain unclear.

Therefore, the objectives of present study were to develop a real-time PCR assay to analyze the survival of endophytic *P. liquidambari* in soil, and assess its effect on the indigenous microbial community in indole-contaminated soil. Our study is the first to use the real-time PCR assay to monitor the population dynamic of *P. liquidambari*, and to investigate the microbial communities in indole-contaminant soil using denaturing gradient gel electrophoresis (DGGE).

2. Materials and methods

2.1. Fungal isolates and plasmid

Endophytic *P. liquidambari* B3 was isolated from the inner bark of the stem of *B. polycarpa* (Chen et al. 2011). *P. liquidambari* B3 and all the fungal isolates utilized in our study are listed in Table 1. All fungal isolates were stored on potato dextrose agar slants at 4 °C at Nanjing Normal University, Jiangsu Key Laboratory for Microbes and Functional Genomics, China.

Plasmid pEGFP-C1 (Vector pUC19 with the enhanced green fluorescent protein insert; GenBank accession number U55763.1) and corresponding primers EGFP1f (TGGATTGCACGCAGGTTCTCCGGCC)/EGFP1r (CACCCAGCCGGCCACAGTCGATGAAT) were kindly provided by Dr. L. Lu (Nanjing Normal University), for use as internal standard to enable normalization of DNA extraction efficiencies between soil samples.

2.2. Design of PCR primers for P. liquidambari B3

A collection of *Phomopsis* ITS regions obtained from National Center for Biotechnology Information's (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov/) were aligned using the ClustalX program (Thompson et al. 1997). Based on this alignment, a primer set (Bf1: 5′-CTGGCCCCCTCGGGGTCCCTGG-3′; Br1: 5′-TTTCAGGGCCTGCCCTTTTACAGGC-3′) was designed to contain specific parts of the *P. liquidambari* sequence. The specificity of the primer pair Bf1/Br1 was tested against genomic DNA isolated from 31 other fungal isolates using conventional PCR assays. To confirm amplifications of the target regions, the PCR product was cloned into pMD 19-T vector (Takara) for DNA sequencing.

2.3. Real-time PCR reactions

Real-time PCR amplifications were performed on the StepOne Real-time PCR systems (Applied Biosystems) using SYBR Green I fluorescent dye. The reaction mixture contained 10 μl of SYBR Premix *Ex Taq* (dNTP, *Ex Taq* polymerase, SYBR Green I, and *Ex Taq* reaction buffer; Takara), 1 μl of each primer (10 mmol l^{-1}), 0.4 μl ROX Reference Dye (50×), 1 μl template DNA, and made up to a final volume of 20 μl with sterile ultra-pure water. The thermal cycling

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