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Biofumigation with the endophytic fungus *Nodulisporium* spp. CMU-UPE34 to control postharvest decay of citrus fruit

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ABSTRACT

Forty-six fungal endophytes were isolated from *Lagerstroemia loudoni*. Only one fungus, identified as *Nodulisporium* spp. CMU-UPE34, produced antifungal volatile compounds. It produced 31 volatile compounds, primarily composed of alcohols, acids, esters and monoterpene. The most abundant volatile compound was eucalyptol. *In vitro* tests showed that volatile compounds produced by *Nodulisporium* spp. CMU-UPE34 inhibited or killed 12 different plant pathogens. *In vivo* mycofumigation with jasmine rice grain cultures of *Nodulisporium* spp. CMU-UPE34 controlled green mold decay on *Citrus limon* caused by *Penicillium digitatum*, blue mold decay of *Citrus aurantifolia* and *Citrus reticulata* caused by *Penicillium expansum*. *Nodulisporium* spp. CMU-UPE34 has potential as a biofumigant for controlling postharvest disease.

1. Introduction

Endophytic fungi are characterized by their ability to asymptomatically colonize living plant tissues (Bacon and White, 2000). The prospect that endophytes produce novel bioactive products stems from the idea that some endophytes may have coevolved with their respective higher plant, and as a result may produce certain phytochemicals characteristic of their hosts (Tan and Zou, 2001; Strobel and Daisy, 2003). Thus, the search for novel endophytic microbes is ongoing, accompanied by an examination of the bioactivities of their natural products encompassing such uses as antibiotics, antiviral compounds, anticancer agents, antioxidants, insecticides, antidiabetic agents and immunosuppressive compounds (Stinson et al., 2003; Strobel and Daisy, 2003; Dai et al., 2006; Mercier et al., 2007; Wu et al., 2010). Several endophytic fungi producing volatile antifungal substances have been previously reported (Dennis and Webster, 1971; Strobel et al., 2001; Ezra et al., 2004). Mycofumigation is the use of antimicrobial volatiles produced by fungi for the control of other organisms (Stinson et al., 2003). For this reason, most of the research on the development of *Muscodor albus* as a biocontrol agent has focused on strain CZ-620 (Mercier et al., 2007) and strain MFC2 (Worapong and Strobel, 2009). These strains have been shown to be an effective biofumigant against fungal fruit decay (Mercier and Jiménez, 2004; Mercier and Smilanick, 2005) and grain smut fungi (Strobel et al., 2001; Goates and Mercier, 2009). The volatile mixture produced by strain CZ-620 was also shown to have some nematicidal and insecticidal activities (Lacey and Neven, 2006; Riga et al., 2008). In addition, *Phomopsis* spp., *Nodulisporium* spp., *Hypoxylon* spp. and *Oxyporus latemarginatus* have been rather than were reported to produce volatile compounds that control fruit decay (Lee et al., 2009; Park et al., 2010; Tomsheck et al., 2010; Singh et al., 2011).

In this study, we aimed to evaluate the ability of *Nodulisporium* spp. CMU-UPE34 was isolated from stem of *Lagerstroemia loudoni* as a potential biological agent to control both *in vitro* and *in vivo* *Penicillium digitatum* and *Penicillium expansum*, which causes devastating citrus fruit decay. The volatile compounds of *Nodulisporium* spp. CMU-UPE34 were identified by gas chromatograph and mass spectrometer (GC/MS). In addition, a suitable solid medium for inoculum production was examined. Knowledge gained is expected to be used to develop *Nodulisporium* spp. CMU-UPE34 as a biocontrol agent for mycofumigation, which may replace the toxic fungicides that are currently used.

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2. Materials and methods

2.1. Isolation of endophytic fungi

Samples of leaf and stem tissues of *L. loudoni* Teijsm. & Binn. were collected from natural forest in The University of Phayao, Phayao Province, Thailand (19°1′39.21″N, 99°53′43.43″E, temperature 26–31 °C, humidity 80–100%), in June 2011. Five branches (2 cm diam) and 10 leaves from a plant were collected. The samples were then taken to the laboratory and processed within 24 h. Samples were washed in running tap water for 15 min. The samples were cut into pieces (leaf, 5 mm × 5 mm and stem, 5 mm long). All pieces were surface sterilized in 75% ethanol for 30 s, 2% sodium hypochlorite for 3 min and 95% ethanol for 30 s under a laminar flow hood (Nuangmek et al., 2008). The sterilized samples were placed in Petri dishes containing 2% malt extract agar, 0.05% streptomycin sulfate and 0.03% rose bengal (Bussaban et al., 2001). Petri dishes were sealed with Parafilm M® and incubated at room temperature (25 ± 2 °C) for one week. The fungi growing out from the samples were aseptically transferred to potato dextrose agar (PDA). Each pure isolate was kept on PDA slants.

2.2. Identification of endophytic fungi and screening in vitro for fumigation activity of endophytic fungi

The endophytic fungi were identified according to their macro- and microscopic structures (Ellis, 1971; Carmichael et al., 1980; Sutton, 1980; von Arx, 1981; Ellis and Ellis, 1985; Barnett and Hunter, 1998; Hyde et al., 2000). All identified endophytic fungi were maintained in cryovials containing 20% glycerol at −20 °C and deposited in the Sustainable Development of Biological Resources Laboratory, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand. All of the endophytic fungi isolated from *L. loudoni* were initially screened by dual culture volatile assay for volatile antimicrobial activity against *Rhizoctonia solani*. Fungal endophytes were inoculated on one site of a two-compartment plastic plate (92 × 16 mm) and incubated for three days at 25 ± 2 °C. An agar plug of *R. solani* was then placed on the other compartment before sealing with Parafilm M® and incubated for one week at 25 ± 2 °C. Colony diameter of *R. solani* was measured after seven days and the percentage of inhibition was calculated. To assess viability of the pathogen, the mycelia plugs of *R. solani* were then transferred to fresh PDA and incubated for seven days. The experiment was repeated twice with five replicates.

2.3. Phylogenetic study of volatile-producing fungi

A volatile-producing fungus (*Nodulisporium* spp. UPE-CMU34) was subcultured onto PDA and incubated for 10 days. Mycelium was harvested, freeze dried and ground into a fine powder with a pestle and mortar. Genomic DNA was extracted by a modified SDS-CTAB method (Bussaban et al., 2005). The internal transcribed spacer (ITS) regions 1 and 2, including 5.8S rDNA were separately amplified in a 25 μL reaction on a GeneAmp 9700 thermal cycler (Applied Biosystems) under these reaction conditions: 1 μL of template DNA extraction, 0.2 mM each of dNTP, 0.2 μL of FastTaq (Applied Biosystems), 0.2 mM each of primers, 2.5 μL of the supplied 10X PCR buffer with $MgCl_2$ and sterile water to bring the volume to 25 μL. The ITS regions were amplified by using ITS4 and ITS5 primers (White et al., 1990). Amplification of ITS regions was for 30 cycles (initial denaturation at 95 °C for 2 min, denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min). PCR products were analyzed by electrophoresis in 1% agarose gels in TAE buffer (20 mM Tris-acetate, 1 mM EDTA, pH 8.0) and viewed by staining with ethidium bromide. PCR products were purified using PCR clean-up Gel extraction NucleoSpin® Extract II purification Kit (Macherey-Nagel, Germany) following the manufacturer's protocol. The purified PCR products were directly sequenced. Sequencing reactions were performed and sequences determined automatically in a genetic analyzer (1ST Base, Malaysia) using the PCR primers mentioned above. Sequences obtained in this study were compared to those from GenBank database using the BLAST software on the NCBI website: (http://www.ncbi.nlm.nih.gov/BLAST/). A multiple sequence alignment was carried out using the alignment subroutines in Clustal X (Thompson et al., 1997). The data was analyzed to determine the phylogenetic relationship based on distance and parsimony criteria phylogenetic tree was inferred with PAUP beta 10 software program, versions 4.0 (Swofford, 2002).

2.4. In vitro assay for fumigation activity of Nodulisporium spp. CMU-UPE34

Nodulisporium spp. CMU-UPE34 was studied by dual culture volatile assays against 12 plant pathogenic fungi, *Alternaria porri*, *Alternaria solani*, *Colletotrichum capsici*, *Colletotrichum musae*, *Colletotrichum gloeosporioides*, *Fusarium oxysporium*, *Fusarium solani*, *Nigrospora oryzae*, *Penicillium digitatum*, *P. expansum*, *R. solani* and *Sclerotium rolfsii*. All of the pathogens were isolated from host plants and pathogenicity was confirmed by Koch's postulates technique. Before testing, all pathogens were separately grown on PDA for two weeks. The pathogen cultures not exposed to the volatiles of *Nodulisporium* spp. CMU-UPE34 were used as control treatments. Colony diameters of pathogens were measured after seven days and the percentage of inhibition was calculated. To assess viability of the pathogen, mycelial plugs of the exposed pathogen were then transferred to fresh PDA and incubated for seven days. The experiment was repeated twice with five replicates.

2.5. Qualitative analysis of Nodulisporium spp. CMU-UPE34 by GC/MS

Analyses of volatiles produced by *Nodulisporium* spp. CMU-UPE34 grown for one week at 25 ± 2 °C on PDA were carried out according to the protocols described earlier by Strobel et al. (2001). A baked solid phase microextraction (SPME) fiber (Supelco) consisting of 50/30 divinylbenzene/carboxen on polydimethylsiloxane on a stable flex fiber was exposed for 45 min to the vapour phase of head space of the grown culture. The fiber was inserted into the splitless injection port of a gas chromatograph GC 2010 (Shimadzu, Japan) equipped with a 30 m × 0.25 mm I.D. DB-Wax capillary column with a film thickness of 0.25 μm. The column was subjected to a thermal program as follows: 40 °C for 2 min increased to 200 at 5 °C min^{-1}. Ultra high purity helium was used as a carrier gas with an initial column head pressure of 60 kPa. The fiber was conditioned at 250 °C for 57 min under a flow of helium gas prior to trapping the volatiles. A 30 s injection time was used to introduce the adsorbed volatiles into the GC. The gas chromatograph was interfaced to a mass spectrometer MS-QP 2010 Plus (Shimadzu, Japan) mass selective detector mass spectrometer operating at unit resolution. Data acquisition and processing were performed on the software system. The compounds produced by CMU-UPE34 were tentatively identified through library comparison with the NIST database, hence all chemical compounds described in this report use the NIST database chemical terminology.