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Quantification of antifungal lipopeptide gene expression levels in *Bacillus subtilis* B1 during antagonism against sapstain fungus on rubberwood

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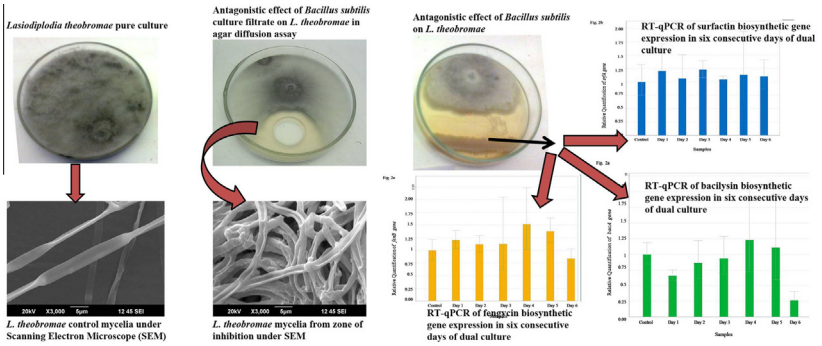
HIGHLIGHTS

- Antibiosis plays an important role in *L. theobromae* inhibition.
- Surfactin lipopeptide helps in the easy movement and spreading of *B. subtilis.*
- Constantly expressed fengycin lipopeptide controls the sapstain growth.
- Bacilysin gene expressed in fourth and fifth days compliments sapstain inhibition.

GRAPHICAL ABSTRACT



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ABSTRACT

The aesthetic value of the rubberwood is lost due to the bluish black discolouration caused by sapstain fungus, *Lasiodiplodia theobromae*, which leads to an economic loss in the wood industry. In our earlier study, *Bacillus subtilis* B1 has been identified as the potential biocontrol agent against *L. theobromae*, the dominant sapstain fungus infecting the rubberwood. *Bacillus subtilis* is known for its biocontrol activity against a wide range of fungal pathogens by various means including the action of non-ribosomal antifungal lipopeptides *viz.* iturin, surfactin and fengycin. The present study aims to characterize and quantify the gene expression levels of these antifungal lipopeptidic genes using RT-qPCR during inhibition process in the dual culture. Among the three lipopeptide genes, fengycin biosynthetic gene was constantly expressed in high amounts throughout the antagonism. The gene for surfactin biosynthesis was also expressed all through and may have helped in the growth and spreading of *B. subtilis* B1. Bacilysin biosynthetic gene expressed only in the fourth and fifth days dual culture might have complemented the action of fengycin in inhibiting the sapstain fungus.

1. Introduction

Microorganisms with characteristic biocontrol potential generally produce one or more compounds with antibiotic activity (Hjeljord and Tronsmo, 1998; Islam et al., 2005; Leclere et al., 2005; Shahraki et al., 2009). All these antimicrobial compounds need not be expressed during biocontrol process simultaneously. The specific antibiotics involved in the antagonistic reaction will be triggered sequentially in the presence of the pathogen (Patterson and Bolis, 1997). Several species of *Bacillus* are known to use antibiosis as their primary mode of action for suppressing fungal infection (Romero et al., 2007). *Bacillus subtilis*, the endospore forming bacterium is reported to produce various antimicrobial antibiotics with 4–5 per cent of the bacterial genome devoted

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for the biosynthesis of these antibiotics (Stein, 2005). The techniques like polymerase chain reaction (PCR), reverse phase high performance liquid chromatography (RP-HPLC), electro spray ionization-mass spectrometry (ESI-MS), matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS), etc. are generally used for the identification of specific antibiotics produced by the biocontrol agents.

Quantification of the antibiotic gene expression levels has been performed using real time PCR technology coupled with reverse transcription (Murphy et al., 1990; Winer et al., 1999; Livak and Schmittgen, 2001; Pantelides et al., 2009; Almoneafy et al., 2014; Jia et al., 2015). Real Time PCR has been considered as one of the best techniques to analyze bacterial gene expression levels during biocontrol processes (Bustin, 2002; Sharkey et al., 2004). The increased expression of an endochitinase gene of the mycoparasite *Stachybotrys elegans* against the pathogen *Rhizoctonia solani* was demonstrated using RT-qPCR (Morissette et al., 2003). Similarly, suppression in the gene expressions of *Pseudomonas aeruginosa* in a co-culture with biocontrol agent *Roseobacter denitrificans* was also identified using the technique (Conway et al., 2012). *B. subtilis* is the routinely practiced biocontrol agent against many fungal diseases in agriculture crops and are known to produce three main families of antifungal lipopeptides *viz*. iturin, surfactin and fengycin (Stein, 2005; Kim et al., 2010; Pathak and Keharia, 2014). The production of these antibiotics by *B. subtilis* has been identified by many techniques like PCR, RP-HPLC, ESI-MS, MALDI-TOF-MS, etc. The gene expression study of four *Bacillus* sps. against *Ralstonia solanacearum*, the causative agent of tomato bacterial wilt was carried out using real time PCR (Almoneafy et al., 2014; Xiong et al., 2015).

Lasiodiplodia theobromae is the dominant sapstain fungus infecting the felled rubberwood in tropical countries (Punithalingam, 1980; Florence, 1996). Even though this fungal infection does not cause much damage to the wood structure, the aesthetic value of the wood is lost due to the bluish black discolouration which leads to a productivity and economic loss. The antifungal lipopeptides of iturin family were qualitatively identified in *B. subtilis* against rubberwood sapstain fungus in our previous experiment using analytical techniques. Aim of the present study is to characterize and quantify the gene expression levels of the antifungal lipopeptide genes using RT-qPCR in *B. subtilis* B1 during the inhibition of rubberwood sapstain fungus, *L. theobromae*.

2. Materials and methods

2.1. B. subtilis B1 isolation, identification and antagonism

Protocols for the isolation of the bacterial biocontrol agent, morphological/ biochemical/ molecular identification of *Bacillus subtilis* B1 as well as its antagonisitic potential have been standardised and performed in our laboratory previously (Sajitha et al., 2014). The biocontrol bacterium, *Bacillus subtilis* subsp. subtilis was isolated from compost available in market by serial dilution and plating on to nutrient agar plates (Johnson and Curl, 1972). Various morphological characters, staining techniques along with the biochemical tests (Aneja, 2001) were conducted. The PCR amplification of 16S rRNA gene encoding region was conducted using universal bacterial primers and sequencing of the amplified region was done by Sanger's dideoxy method. Sequenced product was deposited in NCBI genbank (Accession No. KU556155.1) and phylogenetic analysis was carried out for species and subspecies identification.

2.2. Dual culture

The dual culture of rubberwood sapstain fungus (*L. theobromae*) as well as the biocontrol agent (*B. subtilis* B1) was performed according to Johnson and Curl (1972). *B. subtilis* B1 was streaked on one side of the potato dextrose agar (PDA) plate and a 6 mm width disc of actively growing *L. theobromae* was simultaneously inoculated on the opposite side of the plate and incubated at 28 °C. *L. theobromae* culture grown in PDA was used as control.

2.3. Agar well diffusion assay

A modified method was used to assess the inhibitory effect of the *B. subtilis* B1 culture filtrate by culturing it in the antibiotic production medium, Landy broth at 30 °C for 15 days (Hassan et al., 2010). Each day, the culture was filtered through 0.22 μm membrane and the antagonism was tested against *L. theobromae* by agar well diffusion method (Sen et al., 1995). Culture filtrate was added to the well bored on one side of PDA plate while the sapstain fungus *L. theobromae* was inoculated on the opposite side. Plates were incubated for one week and observations were made. Triplicates were maintained for each experiment. The mycelial portion from the inhibition zone was viewed under the light microscope (Basha and Ulaganathan, 2002). Scanning electron microscopic (SEM) study of the fungal mycelium from the zone of inhibition was carried out to reveal the structural changes during antagonism, induced by the culture filtrate of *B. subtilis* B1.

2.4. PCR amplification of lipopeptide biosynthetic genes from genomic DNA

The *Bacillus subtilis* B1 genomic DNA was isolated from the monoculture using the SDS method (Ausubel et al., 1994). The concentration and purity of the isolated DNA was determined by measuring the absorbance at 260/280 nm (Nano Drop 1000, Thermo Scientific, USA). The genomic DNA was amplified using specific primers for the reported antifungal lipopeptide biosynthetic genes for iturin A, fengycin, surfactin, bacillomycin, mycosubtilin and bacilysin (Table 1). DNA-PCR amplification was carried out as per standard protocols with an initial denaturation step at 94 °C for 5 min followed by 35 cycles of denaturation (94 °C for 1 min), primer annealing (different annealing temperatures for 1 min) and extension (72 °C for 2 min) and a final extension of 10 min at 72 °C (Table 1). After 35 cycles of PCR amplification, the PCR products were resolved on 1.5 per cent agarose gel and the eluted products were subjected to Sanger's dideoxy sequencing. The similarity searches for the sequences were carried out using the *BLAST (N)* option in the NCBI genbank (www.ncbi.nlm.nih.gov).

2.5. Reverse transcriptase PCR (RT-PCR) for characterization of antifungal lipopeptide biosynthetic genes

For identifying and confirming the expressed antifungal lipopeptide genes during antagonism, the total RNA was isolated from the *B. subtilis* B1 dual cultured with *L. theobromae* having a visible inhibition zone using RNeasy mini kit (Qiagen, USA) according to the manufacturer's instructions. The bacterial RNA from first day to sixth day (highest visible inhibition of *L. theobromae*) of dual culture was isolated. The concentration and purity of the isolated RNA was determined by measuring the absorbance at 260/280 nm (NanoDrop 1000, Thermo Scientific, USA). The total mRNA was used as the template for the cDNA synthesis using first strand cDNA synthesis kit (Fermentas, Lifesciences). The concentration and purity of the synthesized cDNA was determined by measuring the absorbance at 260/280 nm (NanoDrop 1000, Thermo Scientific, USA). The synthesized cDNA was used as the template for the Reverse Trascriptase PCR. To characterize the genes, the cDNA was amplified using the specific primers with the same PCR conditions standardized for the genomic DNA (Table 1). The PCR products were resolved on 1.5 per cent agarose