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QUANTIFICATION OF ANTIFUNGAL LIPOPEPTIDE GENE EXPRESSION LEVELS IN *BACILLUS SUBTILIS* DURING ANTAGONISM AGAINST RUBBERWOOD SAPSTAIN FUNGUS

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ABSTRACT OF THE PROJECT PROPOSAL

1.	Project No.	KFRI RP 709/2015
2.	Title	Quantification of antifungal lipopeptide gene expression levels in *Bacillus subtilis* during antagonism against rubberwood sapstain fungus
3.	Investigator	Dr. Suma Arun Dev, Senior Scientist, KFRI
4.	Research Fellow	Ms. Sajitha K.L.
5.	Objectives	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*.
7.	Duration	2 Years
8.	Funding Agency	KFRI Plan Grants, Govt. of Kerala

CONTENTS

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 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). 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).

Rubberwood, *Hevea brasiliensis* Muell. Arg. belonging to the family Euphorbiaceae, is one of the tropical softwood timber species mainly cultivated in the countries like Indonesia, Malaysia, Thailand, India and Sri Lanka. *Lasiodiplodia theobromae* Pat. 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 blusih black discolouration which leads to a productivity and economic loss. Very recently, a successful biocontrol agent, *Bacillus subtilis* strain B1 isolated from the market compost was demonstrated to have high levels of antagonism towards the sapstain fungus, *Lasiodiplodia theobromae* infecting the rubberwood (Sajitha et al., 2014). For the commercial utilization of this biocontrol agent, a mass production protocol using optimal cost effective medium with regionally available coconut water was also reported using a bioreactor. The specific antifungal lipopeptides *viz.* iturin C, surfactin, fengycin A & B, bacillomycin D, bacilysin and mycobacillin produced by *B. subtilis* B1 responsible for the inhibition of sapstain fungus, *L. theobromae* were qualitatively identified using analytical technique, MALDI-TOF-MS (Sajitha et al., 2016). Biocontrol efficiency of the mass produced *B. subtilis* B1 was also demonstrated in the laboratory as well as in the field conditions (Sajitha et al., 2018). The present study aims 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

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).

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.

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 30̊C 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 Ulaganthan, 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.

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 minutes 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 minutes 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).

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 gel and the eluted products were subjected to Sanger's

dideoxy sequencing. The similarity searches for the coding sequences were carried out using the *BLAST (N)* option in the NCBI genbank (www.ncbi.nlm.nih.gov). The confirmed gene sequences obtained were used for designing the primers for RT-qPCR using the PRIMER EXPRESS 3.0.1 software (Applied Biosystems, USA).

Gene expression study using SYBR Green RT-qPCR

The RT-qPCR was carried out in a Step One Real-Time PCR System (Applied Bio Systems, CA) based on the changes in fluorescence relative to the cyclic increase in the PCR products. The detector used was the SYBR Green, which emits fluorescence while binding to a double stranded DNA. The fluorescence value was recorded at the threshold cycle (C_T). The primers designed for the RT-qPCR of fengycin, bacilysin and surfactin have been given in Table 2. The *rpsJ* gene (encoding for ribosomal protein), which is already reported as a house keeping gene and internal standard in *Bacillus subtilis* is used as the endogenous control in the present experiment (Jordan et al., 2006). Prior to quantification, primer concentrations and other parameters were optimized. Real time PCR was carried out in 20 µl reaction volume containing 10 µl Power SYBR Green PCR Master Mix 2X (Invitrogen, USA) with the internal reference dye Rox, 1 µl forward and reverse primers (10 pmol), 2 µl genomic DNA (100 ng) and 6 µl sterile millipore water. A thermal cycling programme (initial denaturation at 95˚C for 10 minutes, 40 cycles of 95˚C for 15 sec and 60 sec at 60˚C) was used. The accuracy of the reactions was analyzed using the melt curve analysis at the end of PCR programme. The final relative quantification was done based on the comparative C_T method of $2^{-\Delta\Delta CT}$ as explained (Livak and Schmittgen, 2001).

Lipopeptide	Primer name	Sequence (5'-3')	Annealing temp.	Product size (bp)	Reference
Bacilysin	BACF BACR	CAGCTCATGGGAATGCTTTT CTCGGTCCTGAAGGGACAAG	60	500	Mora et al., 2011
Bacillomycin	BACCI F BACCI R	GAAGGACACGGCAGAGAGTC CGCTGATGACTGTTCATGCT	59.3	875	Ramarathnam et al., 2007
Fengycin	FEND1F FEND1R	TTTGGCAGCAGGAGAAGTT GCTGTCCGTTCTGCTTTTTC	62	964	Ramarathnam et al., 2007
Mycosubtilin	FENF-F FENF-R	GACATGTATCCGTTCTAGAAGATTG ATCGGCCATTCAGCATCTC	59	550	Leclere et al., 2005
Iturin	ITUDI F ITUDI R	GATGCGATCTCCTTGGATGT ATCGTCATGTGCTGCTTGAG	59	650	Ramarathnam, 2007
Surfactin	P17- F P18- R	ATGAAGATTTACGGAATTTA TTATAAAAGCTCTTCGTACG	49	675	Hsieh et al., 2004

Table 1 Primer and PCR informations

Lipopeptide	Primer name	Sequence (5'-3')
Bacilysin	BacRT F	CAGGCCGTACATCGGTGTATC
	BacRT R	AGGCCGGATTTGCAGCTAT
Fengycin	FenRT F	GCCCGAGGGCCACAA
	FenRT R	AGCCAGGTGGCGAATGTC
Surfactin	SurRT F	GCCGTGCATCCCTGATCT
	SurRT R	CGGCCGGAGTGAGAATGAGAAATGT

Table 2 Primer informations of RT-qPCR

Statistical analysis

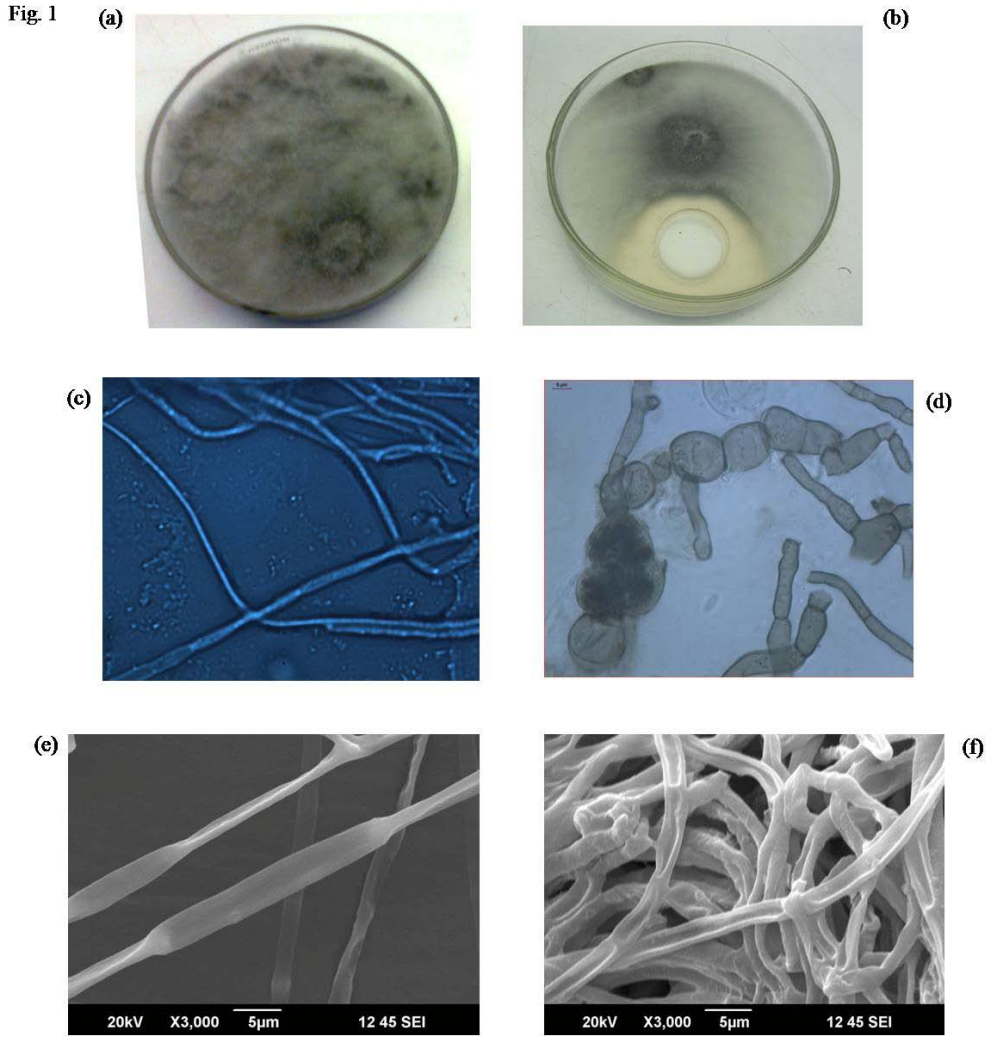
The fold change in gene expression of three lipopeptides (surfactin, fengycin and bacilysin) in six consecutive days obtained from RT-qPCR were statistically analyzed through analysis of variance, followed by Duncan's multiple range test ($P< 0.05$), using SPSS version17 (SPSS Inc., Chicago, Illinois, U.S.A).

3. RESULTS

Agar well diffusion assay

The observations were made on the inhibitory effect of the culture fitrate on *L. theobromae* from the first day onwards. Very slight inhibition was noted in the first two days of the culture filtrate. From third day onwards clear inhibition was observed and the highest inhibition was noted on the sixth day. No further increase in fungal inhibition was noted from the seventh day to the fifteenth day of culture filtrate. The fungal mycelia from pure culture (Fig. 1a) were thin, clear and slender under light microscope (Fig. 1c) while fungal mycelia from the zone of inhibition of the agar well diffusion assay (Fig. 1b) showed swelling and distortions (Fig. 1d). In the scanning electron microscope, the distortion of the fungal mycelia from the inhibition zone was more clear and the shrinkage due to plasmolysis (Figs. 1e & 1f), leading to the death of the fungus were observed.

Figure 1. Pure and agar well diffusion assay of *Lasiodiplodia theobromae* under light and SEM microscope



a)- Pure culture of *Lasiodiplodia theobromae* isolated from infected rubberwood pieces
(b)- *B. subtilis* B1 culture filtrate showing effective inhibition of *L. theobromae*
(c)- Thin slender hyphae of normal *L. theobromae* under light microscope
(d)- Distorted fungal hyphae from the zone of inhibition under light microscope
(e)- Clear normal mycelia under scanning electron microscope
(f)- Plasmolysed and shrunken fungal mycelia from the zone of inhibition under scanning electron microscope

PCR amplification of lipopetide biosynthetic genes from genomic DNA

All the mentioned lipopeptidic genes like *bacA* (for bacilysin synthase), *fenB* (fengycin synthatase), *srfA* (surfactin synthatase), *ituA* (iturin A synthatase), *bamD* (bacillomycin synthatase D) and *fenF* (for malonyl co-enzyme A transacylase for mycosubtilin biosynthesis) were amplified in the *B. subtilis* B1 genome showing the presence of all these genes in the *B. subtilis* B1 strain.

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

The antagonistic ability of *B. subtilis* B1 against *L. theobromae* is evident from the observed zone of inhibition between *B. subtilis* B1 and *L. theobromae* (Figs.1a & 1b). The concentration of the RNA isolated from *B. subtilis* B1 dual cultured with *L. theobromae*, was decreased from 1st to 6th day. RT-PCR was carried out to confirm the transcripts of antifungal lipopeptides (*viz*. bacilysin, fengycin, bacillomycin, mycosubtilin, surfactin and iturin) during antagonism and gave rise to specific amplified products of 500bp, 964bp, 875bp, 550bp, 675bp and 650bp for these lipopetide genes respectively (Fig. 2). NCBI-BLAST homology searches were carried out to confirm the coding sequences of these respective polypeptide genes. The confirmed sequences of bacilysin, fengycin and surfactin after homology searches were used for further primer designing and real time quantification of the gene expression levels.

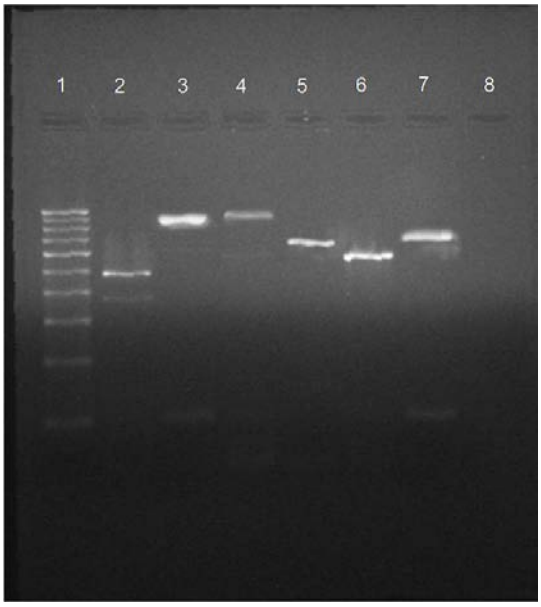
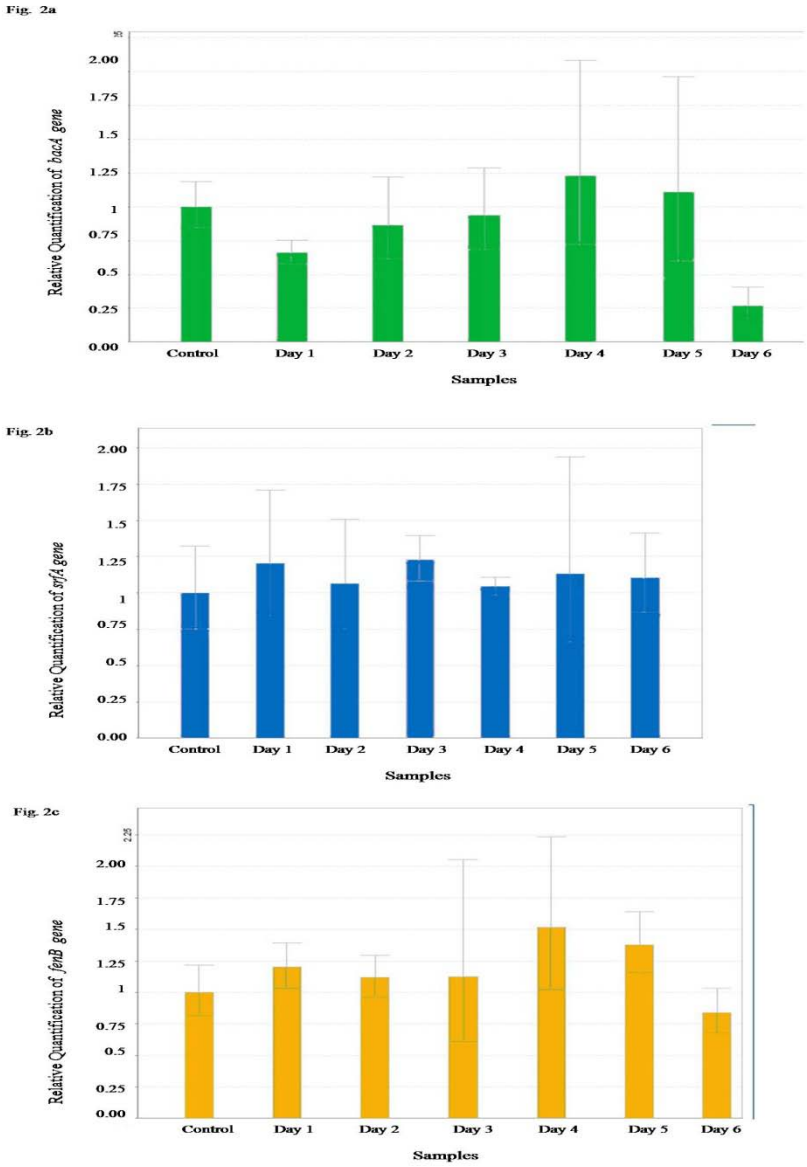


Figure 2. RT-PCR amplification of biosynthetic genes of *Bacillus subtilis* B1 corresponding to bacilysin (lane 2), bacillomycin (lane 3), fengycin (lane 4), iturin (lane 5), mycosubtilin (lane 6) and surfactin (lane 7) along with 100bp DNA ladder (lane 1)

Gene expression study using SYBR Green real-time PCR

The relative expressions of *bacA, srfA* and *fenB* genes of *B. subtilis* B1 during the inhibition of stain fungus *L. theobromae* in dual culture are shown in Figure 3. The *bacA* gene was suppressed during the first three days of inhibition and a 1.2 and 1.1 fold increases were observed in the fourth and fifth days respectively. A sudden decline of expression was noted in the sixth day. The *fenB* gene for fengycin biosynthesis was expressed in all the five days and highest gene expression was observed in the fourth day of dual culture. As noticed in *bacA* gene, a decline in *fenB* gene expression was observed in the sixth day. The *srfA* gene for surfactin biosynthesis showed a steady expression level till 6^{th} day of the co-inhibition. In the melt curve analysis, single peak was observed for all the real time PCR amplifications and no amplification was observed in negative controls.

Figure 3. Gene expression study of three *B. subtilis* B1 lipopeptide genes (a) *bacA* gene for bacilysin biosynthesis (b) *srfA* gene for surfactin biosynthesis (c) *fenB* gene for fengycin biosynthesis in six consecutive days of *B. subtilis* B1 and *L.theobromae* dual culture



(a) Bacilysin gene is expressed more in the fourth and fifth days of dual culture and is declined in sixth day (b) Surfactin gene is expressed throughout the dual culture and no decline noticed (c) Fengycin gene is expressed throughout the dual culture and is expressed more in fourth and fifth day. Decline in expression is noted in day 6

Statistical analysis

Significant differences in gene expressions were observed for bacilysin and surfactin in all the six days and for fengycin from 4^{th}-6^{th} days of dual culture at ($p<0.05$). The F and significance (Sig.) value of the expression of three genes are given in Table 3.

Table 3

ANOVA table for testing the significance of gene expressions of surfactin, bacilysin and fengycin in six consecutive days

ANOVA between groups			
Tests	**df**	**F**	**Sig.**
Surfactin	6	222.000	.000
Bacilysin	6	3.090E3	.000
Fengycin	6	1.620E3	.000

df - degrees of freedom, F- F ratio, Sig.- significance probability

4. DISCUSSION

The inhibition of fungal growth by an antagonistic bacterium is generally accomplished through plasmolysis, shrinkage and lysis of the fungal mycelium due to active molecules produced by the bacterial biocontrol agent (Hashem and Alamri 2009; Ashwini and Srividya 2014). The fungal mycelia from the zone of inhibition showed swelling and distortions while the control mycelia were thin, clear and slender under the light microscope (Figs. 1c & 1d). The scanning electron microscopic image of the fungal mycelia from the inhibition zone displayed plasmolysis and shrinkage of the fungal hyphae due to the action of antifungal lipopeptides which may have led to the death of the fungus (Figs. 1e & 1f). Similar observations during antagonism of *Bacillus* sp. against *Curvularia lunata*, *Colletotrichum gloeosporioides* and yeast cells against *Lasiodiplodia theobromae*, were earlier reported (Basha and Ulaganathan 2002; Hashem and Alamri 2009; Ashwini and Srividya 2014).

Bacillus spp. generate diverse group of antimicrobial lipopeptides which differ in modes of action, biochemical properties, molecular mass, range of activity and genetic origin (Velho et al., 2011). The process of antibiosis has been defined as the interaction that involves antimicrobial lipopeptides produced by the microorganism that has a direct effect on another microorganism (Weller, 1988; Handelsman and Parke, 1989; Weller and Thomashow, 1993). Several species of *Bacillus* are known to use antibiosis as their primary mode of action in suppressing fungal infection (Romero et al., 2007). In the present study, the dual culture of biocontrol agent, *B. subtilis* and sapstain fungus (*L. theobromae*) displayed inhibition of the sapstain fungus from the first day of dual culture onwards with highest inhibition of the stain fungus in the sixth day. No further growth of stain fungus was observed beyond 6 days, even when the dual culture was kept for a period of one month. Gene expression levels of three antifungal lipopeptides were quantified using RT-qPCR during six consecutive co-culture days. Single peaks in melt curve analysis and absence of amplification in negative controls explained the specificity and efficiency of the reactions. The use of real time PCR in the gene expression analysis of induced resistance caused by the antimicrobial peptides were reported previously in various crop plants (Cruz and Buttner, 2008; Velho et al., 2011; Tripathi et al., 2013; Waewthongrak et al., 2014). .

A gradual decrease in RNA observed in the present study could be explained based on the degradation of stable RNAs in the stationary phase. rRNAs and tRNAs are the stable RNAs which accounts for 98 per cent of cellular RNA (Bremer and Dennis, 1996). Although, they are quite stable during exponential phase of bacterial growth, their degradation is associated with starvation during stationary phase (Deutscher, 2003). One important molecular change reported to the cells in prolonged stationary phase is the decline in its protein synthetic capacity. During starvation, these genes are reported to express several survival genes including lipopeptidic genes in order to overcome stress due to depletion of nutrients and other environmental factors (Arunasri et al., 2014). The enzymatic activity for the biosynthesis of the lipopeptides produced during this time was also reported to disappear within few hours (Katz and Demain, 1977). The antifungal metabolite production of *B. subtilis* was reported in the late exponential phase and stationary phases (Katz and Demain1977; Dunlap et al., 2011). The metabolite production of *B. subtilis* in yeast extract glucose broth was effective in the seventh and fourteenth day culture filtrate (Feio et al., 2004). It is reported that among antifungal lipopeptides, surfactin is produced during the late exponential phase while fengycins, bacillomycins, mycobacillins and iturins are produced during the early and late stationary phases of *B. subtilis* culture (Cosby et al*.,* 1998; Jacques et al., 1999).

The three lipopeptide biosynthetic genes experimented in this study were displayed different levels of activity during inhibition of the sapstain fungus. The gene for bacilysin biosynthesis was not induced for three days and then subsequently shown good amount of expression (Fig. 2a). Bacilysin is reported to regulate the cell division, protein and cell wall synthesis of pathogens and in turn control the diseases*.* Even though, bacilysin is an antifungal lipopeptide, the biosynthetic gene regulation can be associated with the survival and defence strategy of the pathogen or related to quorum sensing associated with population density of the bacteria (Conway et al., 2012). The expression level of this gene was less than that of the control cDNA from the monocultured *B. subtilis* during initial days of dual culture. An increase in the expression level of *bacA* gene in the fourth and fifth days apparently indicates the specific role of bacilysin lipopeptide in the inhibition of sapstain fungus. Gene expression is directly related to the mRNA produced in the bacteria and the rapid fall in gene expression is

due to the mRNA decay which serves continuously to adjust the message to the needs of cell for specific protein (Condon, 2003; Deutscher, 2006).

Based on the availability of nutrients and moisture conditions, *B. subtilis* remains localized, produces biofilms or inhabits huge area by swarming movement. The swarming and sliding movement of *B. subtilis* is due to the biosurfactant properties of surfactin, which increases the dampness of surface (Ghelardi et al., 2012). Surfactin is one of the most commonly characterized biosurfactant with tremendous surface and membrane active properties along with better emulsifying and foaming properties (Vater et al., 2002). *B. subtilis* is a known surfactin producer, which plays an important role in decreasing the surface tension and increasing the mobility of the producing bacteria. Surfactin is not primarily considered as an antifungal agent. But there are reports on the adherence of surfactin molecules to form pores on the membranes of fungi and causes membrane solubilisation or disruption (Ongena and Jacques, 2008). The *srfA* gene for surfactin biosynthesis was consistently expressed in all the six days of co-culture (Fig. 2b). The high expression level of surfactin in the first day may be due to its involvement in the growth and spread of *B. subtilis* B1 for inhibiting the sapstain fungus in synergy with the other two lipopeptides.

Among the antifungal lipopeptides, fengycins are reported as the most effective with high fungicidal, bactericidal and insecticidal properties (Kim et al., 2004). Even in low concentrations, fengycin readily interacts with lipid layers and retain the potential to alter cell membrane structure, permeability, solubilise the membranes and form pores on them (Deleu et al. 2005). Fengycins are strong fungitoxins especially against filamentous fungi and are less haemolytic (Vanittanakom et al., 1986; Hofemeister et al., 2004). Fengycin, potent antifungal antibiotic is observed to express throughout the inhibition process of sapstain fungus. Even though its expression was observed from the first day, the highest expression was detected in the fourth day. Cawoy et al. (2014) reported the ability of *Bacillus* strains to sense the signals emitted from the pathogens to enhance the production of lipopeptides like iturins and fengycins. A signal transfer mediated induction of antibiotic production in some marine bacteria was also reported (Nair, 2012). Fengycin is able to overcome *L. theobromae* defence mechanism and its gene expression is observed from the first day of dual culture. The gene

expression level of fengycin was high when compared to bacilysin and surfactin. Since fengycin gene is expressed from the first day of dual culture and being the highly expressed antibiotic, it could be the main inhibitor of sapstain fungus *L. theobromae.* The combined action of fengycin and surfactin in *B. subtilis* to elicit induced systemic resistance in controlling the green mold decay on citrus fruits by *Penicillium digitatum* was reported formerly (Ongena et al., 2007; Sangwanich et al., 2013). Similarly synergistic actions of all the three lipopeptides in the fourth and fifth days of dual culture are responsible for the effective inhibition of sapstain fungus (*L. theobromae*) in rubberwood.

5. SUMMARY AND CONCLUSIONS

The aesthetic value of the rubberwood is lost due to the blusih black discolouration caused by sapstain fungus, *Lasiodiplodia theobromae*, the dominant sapstain fungus infecting the rubberwood which leads to economic loss in the wood industry. In our earlier study, *Bacillus subtilis* B1 has been identified as the potential biocontrol agent against *L. theobromae*,. *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*. 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.

Bacillus subtilis B1 is a potent biocontrol agent against the rubberwood sapstain fungus, *L. theobromae*. The RT-qPCR of the antifungal lipopeptide biosynthetic genes in *B. subtilis* from the first day to sixth day of dual culture with effective inhibition clearly suggested the synergistic role of these lipopolypeptides during the antagonistic process. Among the three lipopeptide genes, fengycin biosynthesis gene was constantly expressed in high amounts throughout the antagonism. The gene for surfactin biosynthesis was also expressed the whole time and may have helped in the growth and spreading of *B. subtilis* rather than in controlling the sapstain growth. 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.

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