INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
21–0918/2021/26–6–731–740
DOI: 10.17957/IJAB/15.1889
http://www.fspublishers.org

Full Length Article

Volatile Organic Compounds of some Antagonists against *Lasiodiplodia theobromae*, a Pathogen of Coconut

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Received 09 October 2021; Accepted 01 November 2021; Published 15 December 2021

Abstract

Biocontrol agents are the potential microbes and used for the control of aerial and soil-borne pathogens present in all crops. An attempt was made on identification, morphological and molecular characterization of *Lasiodiplodia theobromae*, a pathogen causing disease in nuts and leaves of coconut. A virulent isolate *Lasiodiplodia theobromae* L26 was selected based on the growth parameters and pycnidiospore production. Three efficient biocontrol agents (BCAs) namely *Trichoderma asperellum*, *Bacillus subtilis* and *Streptomyces rochei*, were selected for *in vitro* studies. Among these, *T. asperellum* showed a significantly higher percentage of inhibition (81%) in dual culture assay against L26. The inhibition was also confirmed in light microscopic observation, the mycelium of L26 was distorted, lysis of cell wall during the interaction. Volatile organic compounds (VOCs) emitted from BCAs inhibited the fungal growth of L26 by 59.61–47.03% in sealed plate method. Solid-phase microextraction GC-MS analysis revealed numerous new VOCs compounds emitted from the BCAs, whereas the dominant compound was identified as peptaibols, 2,4-di-*tert*-butylphenol, 2-piperidinone. The strength of peaks of these compounds augmented during the interaction of BCAs with L26, the peak intensity for terpenoids was the predominant class, followed by phenols and heterocyclic organic compound. Crude metabolite (75 μL) of each antagonist tested through agar well method against L26 and showed a complete inhibition. This study demonstrated the ability of BCAs to produce volatile and nonvolatile antifungal compounds, showing that there could a major mechanism involved in and that will be responsible for the successful inhibition of L26 under *in vitro*. In future combination of these three strains as commercial formulation may be a better management practices for leaf blight and malformation of nuts in coconut.

Keywords: Antifungal compounds; Biocontrol agents; GC-MS; *Lasiodiplodia theobromae*

Introduction

Philippines is the second largest country in production, which account for 32.67% of global production of coconut (Naik 2017). In India, coconut plays an important role in GDP contribution of about 15,000 crore rupees and it accounts 72% of world production. Tamil Nadu is at the top of the list in the productivity of coconut among the states in India, (CDB-Statistics-area 2018). Coconut, a versatile crop is being used for various uses, but in India, almost 70% of the coconut is used for the edible purpose. There are several biotic factors which are responsible for the drastic reduction in coconut production and productivity. The major devastating diseases occurring on coconut in Tamil Nadu are bud rot (*Phytophthora palmivora*), Tanjore wilt or basal stem end rot (*Ganoderma lucidum*), Kerala wilt (*Candidatus phytoplasma*), grey blight (*Pestalotiopsis palmarum*), leaf blight (*Lasiodiplodia theobromae*) and stem bleeding disease (*Thievolopsis paradoxa*). Among these, *L. theobromae* has become severe problem in major coconut growing districts of Tamil Nadu namely, Coimbatore, Erode, Dindigul, Tirunelveli and Kanyakumari which, causes yield losses of 10–25% (Johnson *et al*. 2014). The fungus has been act as a secondary infector as the primary factor was due to eriophyid mite (*A. guerreronis*) and this interaction was studied by Lakshmanan and Jagadeesan (2004). Management of this malady is of immense importance by exploiting the recently reported facts, suitable effective management strategies could be formulated in future from the antagonists. Several biological agents *viz*., *Trichoderma* spp., (Ali *et al*. 2020), *Aspergillus* spp. *Penicillium* spp. (Khan and Javaid 2021a, b) *Bacillus subtilis*, *Pseudomonas fluorescens* and *Streptomyces* spp. (Sharf *et al.* 2021) inhibit phytopathogens growth. It is well known that, *Trichoderma* spp., are successful biocontrol agents, which produced primary metabolites with antifungal properties to induce resistance (Mukherjee *et al*. 2012; Khan and Javaid 2020). *Macrophomina phaseolina* was inhibited by the secondary metabolites of *T. viride* and it was proved

To cite this paper: Sudha A, M Rajesh, MS Kumar, M Vijayakumar, E Sumathi (2021). Volatile organic compounds of some antagonists against *Lasiodiplodia theobromae*, a pathogen of coconut. *Intl J Agric Biol* 26:731–740

by Khan *et al.* (2021). The plant growth-promoting microbe, *Bacillus* spp. colonizing the rhizosphere and rhizoplane region, which in turn improves vigour, plant growth and reduce disease incidence. El-Tarabily *et al.* (2009) and Monteiro *et al*. (2017) reported that, actinomycetes played an important role in growth promotion when compared to all rhizosphere microbes (Ilic *et al*. 2007). VOCs produced by *T. virens* control *R. solani* under *in vitro*, are due to the presence of the antifungal compound *viz*., α-cadinane, docosane and oleic acids were detected in *T. virens*. The n-butanol extract from crude culture of *Bacillus* spp., showed effective inhibitory activity against *T. harzianum* which is a pathogen observed from mushroom (Fernandes *et al*. 2019). However, investigations on biopotential of antagonists and the combined interaction with *L. theobromae* are still lacking. So far, not much work has been done on identification of volatile compounds from the BCAs with the pathogens were to be investigated. In recent investigations the potential BCAs were identified and evaluated by the researchers in the department of plant pathology, TNAU, Coimbatore against few pathogens. By using the BCAs, the potent antagonist will be identified under *in vitro* against *L. theobromae.*

This present study aims to identify the symptomatology, molecular and morphological variations of *L. theobromae* in Tamil Nadu. We employed potential antagonist for screening against the pathogen. FT-IR spectra and Tensor 27 Infrared spectrometer was used for the compound identification and to ascertain the biocontrol effects of volatile blends in antagonists i*n vitro* studies were performed.

Materials and Methods

Isolation and Selection of virulent isolate

A survey was conducted in thirty districts of Tamil Nadu to find out the occurrence of both mite and fungal infested nuts to assess the percent incidence, to identify the virulent isolate. Diseased nuts collected from 30 districts of Tamil Nadu during survey were used for the isolation of *L. theobromae.* Isolation and purification of fungal pathogen was carried by hyphal tip transfer procedure (Rangaswami *et al.* 1975) and incubated at 25 ± 3ºC for 7 days (Phipps and Porter 1998). By measuring the culture radial growth, number of pycnidia and pycnidiospore production, virulent isolate of *L. theobromae,* was selected. Molecular identification of *L. theobromae* was carried out by the (CTAB) method described by Ma *et al.* (2001) and similarity matrix was developed using the Jaccard's coefficient of similarity with the data matrix (Jaccard 1998).

Preparation of media and *in vitro* antagonistic activity against plant pathogens

The medium was obtained from Himedia Laboratories (India) was used for culturing fungi (Potato agar medium), nutrient agar medium was used for culturing *B. subtilis* and starch casein agar was used for culturing *S. rochei*. In addition, the medium (starch casein agar) was added with 25 μg mL^{-1} nystatin to minimize the fungal contamination. After a week the colonies from antagonistic fungi, bacteria and *S. rochei* were maintained. Antifungal activity screening was studied using potato dextrose agar medium.

Effect on radial mycelial growth by dual culture technique

The efficacy of antagonistic organisms against the selected *L. theobromae* was tested by dual culture technique (Dennis and Webster 1971). The nine biocontrol strains were obtained from the Culture Collection Centre, Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India to carry out the study. A nine mm actively growing PDA culture disc of virulent isolate *L. theobromae* was placed onto sterilized PDA medium, previously poured into a sterilized Petridish approximately at a distance of 1.5 cm away from the periphery of the plate. Similarly, nine mm culture disc of the respective fungal antagonists *viz*., *T. asperellum* (Tv1), *T. harzianum, T. asperilloides, T. koningeopsis,* bacteria *viz.*, *Bacillus subtilis* (Bs1), *B. amyloliquefaciens, B. megatherium, B. lichiniformis* and actinobacteria *viz.*, *S. rochei* were placed onto the medium at the opposite side of the culture plate separately (Dennis and Webster 1971). A plate with pathogen alone on the periphery served as control. Three replications were performed to study the direct efficacy of the antagonist. These plates were inverted and maintained at 28 ± 2°C, for five days. Percent inhibition was calculated using the formula:

$$PI=C-T/C\times100$$

Where PI = percent inhibition; C = radial growth of the pathogen in the control plate; T = radial growth of the pathogen in treatment.

During the study of interaction of antagonists with *L. theobromae* culture was placed on slide and stain by lectophenol cotton blue, observed by Digital light microscope at 400X to study the mode of action.

Inverted plate assay

Inverted bioassay method was carried out to know the efficacy of antifungal volatiles described by Garbeva *et al.* (2014). In this method both the test pathogens and antagonists were inoculated in same plates. To expose the pathogen to the volatile secreted by the antagonists, the test pathogen inoculated plate was placed over the three antagonists, and sealed; these setups were incubated at 28 ± 2°C. The growth of hyphae was marked at regular interval and inhibition of pathogen growth was calculated in percentage by comparing with control plate.

$$\text{Inhibition of fungal growth} = 100 \text{ X } (1-(G_e-G_a))$$

G_e- mycelial growth of pathogen in the presence of the antagonists

G_a- mycelial growth of pathogen in the absence of the antagonists.

Preparation of inoculum and fermentation

The isolated and antifungal tested strains of pathogen, BCAs were taken for this study for inoculums production and fermentation. Yeast molasses broth for *T. asperellum*, King'B broth for *B. subtilis*, Starch casein agar medium for *S. rochei* and Potato dextrose broth for *L. theobromae* were used for growing the strain for 10 days in 28°C.The well grown spore suspension was prepared in distilled water for about 10 days. These inoculated broths of bacteria alone were kept in a shaker at 120 rpm at 28°C for 3–5 days as stocks.

Extraction and FT-IR and GC-MS analysis of the antifungal metabolite

Antifungal metabolites from 7 days old cultures of BCAs were extracted from cell pellets with methanol and also from culture filtrates in ethyl acetate. The extract was dried at 40°C using a rotary evaporator and it was suspended in 5 mL of 1% HPLC grade methanol (Intana *et al*. 2005; Vinale *et al*. 2006). FT-IR spectra were recorded on Bruker, Tensor 27 Infrared spectrometer. GC-MS analysis was performed by using FTIR and GC-MS analysis is used for the analysis of volatile compounds in crude ethyl acetate extract of axenic and co-culture of BCAs along with *L. theobromae* was carriedout by trapping them in Tenax TA™ coated stainless steel desorbing columns. The crude antibiotics of the effective BCAs were analyzed for the detection of active biomolecules responsible for the suppression of pathogens through GC-MS (GC Clarus 500 Perkin Elmer). Using database searches on the NIST version 2005 MS data library and comparing the spectrum obtained through GC/MS, the compounds present in the crude sample were identified.

Screening of antifungal metabolite against *L. theobromae*

The diluted metabolite from the antagonists was further tested for their antagonistic property against the fungal pathogen using the agar well diffusion assay. 25, 50, 75 and 100 μL crude metabolites of the BCAs were placed inside the PDA wells. The mycelia of the test pathogen were inoculated in with an agar plug of 9 mm dia. in each plate in the center of the Petridish. The percentage inhibition was studied after 5 days by calculating the inhibition percentage.

Statistical analysis

The data observed from the above study were analysed using the statistical tool for agricultural Research (STAR 2.0.1). For comparing the treatment means Tukey's honest significance difference test was used, and the significant level of treatment was calculated by the magnitude of the F value ($P < 0.05$). Plant growth parameters were calculated by the mean ± standard deviation using GraphPad Prism (v. 6.0) and further comparisons was conducted using DMRT at $P < 0.05$ (XLSTAT).

Results

Survey, isolation and selection of virulent isolate

A survey was conducted in various districts of Tamil Nadu to assess the occurrence of *L. theobromae* in eriophyid infested coconut. The results of the report revealed that the incidence of *L. theobromae* was varied from 30.60 to 88.12 (PDI) in all districts of Tamil Nadu. Maximum occurrence of the disease was recorded in Vizhupuram (PDI- 88.12), followed by Attur, Salem district (Fig. 1).

The organism grown on PDA and produced a light gray, fluffy and aerial mycelium. The culture became dark in colour in advanced stages. Mycelium was inundated or superficial, branched, septate and brown. Dark brown flask shaped, ostiolate pycnidia appeared in 7-day-old cultures. The ostiole was circular and arranged at the apex of an elongated neck through which pycnidiospore extruded. Pycnidia varied in size, 125–180 μm × 80–145 μm. Pycnidiospores were at first hyaline globose to oval and unicellular, but became brown and 1-septate with age and measured 20.5–30.0 μm × 11.0–13.5 μm. Based on characteristics of the pycnidia and pycnidiospores, the fungus was identified as *Botryodiplodia theobromae* (Pat) syn. *Lasiodiplodia theobromae* Pat. Griffon & Maubl (Mullen *et al*. 1991; Woodward *et al*. 2005). Maximum No. of pycnidia (90 per plate) with more matured pycnidiospores (65.3) were produced by the isolate L26. The isolate L26 produced highest numbers of pycnidiospore within a short period and it would have the capacity to infect healthy nuts. From this study the isolate L26 was selected for entire studies (Fig. 1). From a total of thirty isolates collected from different places in Tamil Nadu, all the isolates confirmed as *L. theobromae* by PCR technique (Fig. 2). All the thirty could get an amplification size of approximately 560 bp.

***In vitro* effect of BCAs on L26 isolate**

The antagonistic assay was performed using dual plate technique and the radial mycelial growth of the pathogen was monitored. The BCAs, *T. asperellum*, *B. subtilis* and *S. rochei* inhibited the mycelial growth of L26 and the percent inhibitions were 67.11, 58.49 and 57.48 respectively, the clear inhibition zone exhibited the antagonistic activity after four days of incubation (Fig. 3). Likewise, other antagonist also inhibited the mycelial growth of the pathogen from 37 to 46%. The spore inhibition percentage also increased from 73.75 to 58.95 (Table 1 and 2). Based on this antagonistic potential the three antagonists were taken for further study. The antagonists also caused extensive hyphal coiling, lysis and thinning respectively and less dense hyphal network compared to control. Light microscopy of the fungal mycelia revealed that distortions, damages and also shrinkage of L26 isolate in the treated plates. In addition, the hyphae were parasitized by spores of the antagonist. In control plate they were no such changes were detected (Fig. 4).

Table 1: BCAs with their accession Numbers

Isolate No.	BCAs	Accession number deposited in NCBI
1	*Streptomyces rochei*	MT122809
2	*T. asperilloides*	Y848322
3	*T. koningeopsis*	MF423101
4	*T. harzianum*	KX533990
5	*T. asperellum*	KX533985
6	*B. subtilis*	KF718836
7	*B. amyloliquefaciens*	WODE00000000
8	*B. lichiniformis*	MG241257
9	*B. megatherium*	CP032527.2

Table 2: *In vitro* effect of antagonists against radial mycelial growth and spore germination of L26

Sr. No.	Antagonists	Radial mycelial growth (mm*)	% growth inhibition over control	(%) spore germination *	(%) spore inhibition over control
1	*Trichoderma asperellum*	28.30 (38.22)^{a}	67.11	23.25 (35.20)^{a}	73.75
2	*Bacillus subtilis*	36.00 (42.70)^{b}	58.49	32.70 (46.66)^{b}	63.45
3	*Streptomyces rochei*	36.90 (43.22)^{c}	57.48	36.83 (40.80)^{c}	58.95
4	*Bacillus amyloliquefaciens*	47.90 (43.79)^{c}	46.36	46.11 (42.77)^{cde}	49.73
5	*Trichoderma harzianum*	49.80 (44.88)^{cd}	44.23	53.47 (46.99)^{cd}	41.71
6	*Bacillus lichiniformis*	51.10 (45.63)^{cd}	48.78	48.85 (44.34)^{de}	46.75
7	*Trichoderma koningeopsis*	52.00 (46.14)^{cd}	41.77	49.87 (43.18)^{cde}	45.63
8	*Trichoderma asperilloides*	54.00 (47.29)^{cd}	39.53	54.52 (47.60)^{cde}	40.56
9	*Bacillus megatherium*	55.80 (48.33)^{cd}	37.51	52.89 (46.66)^{e}	42.34
10	control	89.30 (71.47)^{e}		91.73 (73.30)^{de}	
SEd (0.01)			4.23		

*Mean of three replications.
Values in parantheses are arc sine transformed values.
In a row, means followed by a common letter are not significantly different at 5% level by DMRT

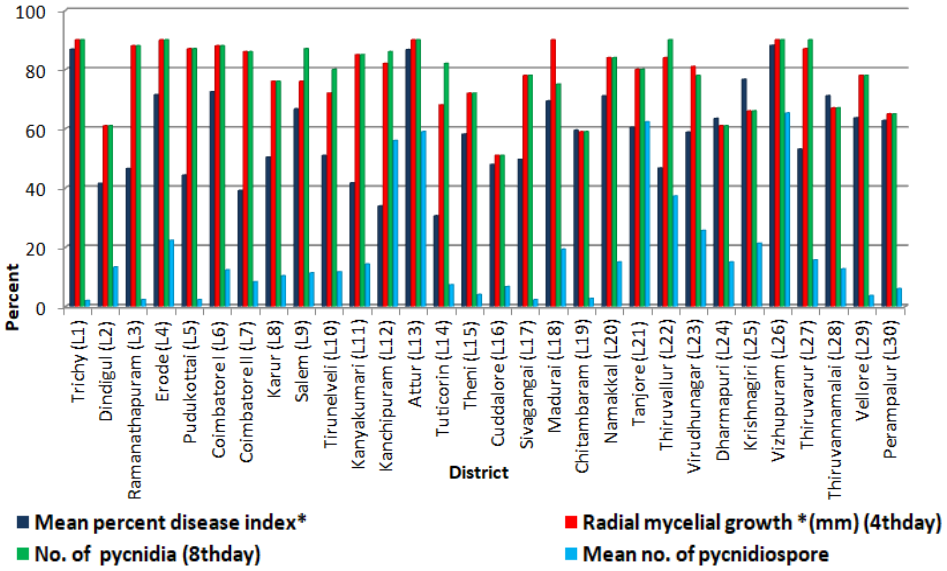

Fig. 1: Prevalence and morphological variation of *L. theobromae*

Antifungal volatiles test-inverted bioassay

Antifungal volatiles from the antagonists were studied against L26 using inverted bioassay method. It was noticed that the presence of volatile compounds adversely affect the mycelial growth of L26 on the 3rd day. There was reduction in percent mycelial growth (59.61, 53.20, 47.03%) in all BCAs compared to control. As support to these results, there was no pycnidia formation in all three test plates where as in control there was increased or more number of pycnidia on day of seventh. The *S. rochei* explored plates had sparse mycelial growth without pycnidia production and there was no inhibition percentage in mycelial growth but reduction in spore formation. The effectiveness of the volatile compounds on the mycelial growth and pycnidia production of the test pathogen proved the suppressing ability of the antagonist under *in vitro* (Fig. 5).

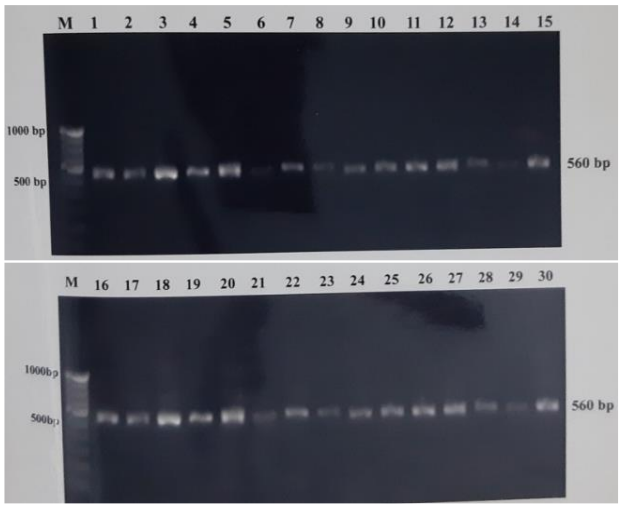

Fig. 2: Agarose gel electrophoresis showing ITS1, 5.8S I and ITS4 ribosomal DNA PCR product of *L. theobromae*

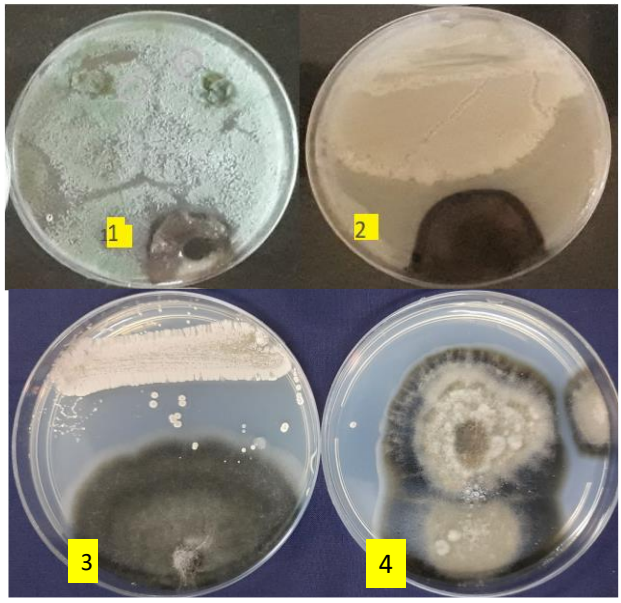

Fig. 3: *In vitro* effect of *T. asperellum*, *B. subtilis* and *S. rochei* against *L. theobromae*
1: *T. asperellum vs.* L26; 2: *B. subtilis vs.* L26; 3: *S. rochei vs.* L26; 4: Control (L26)

Volatilome pattern associated with antagonists with L26 isolate

The vo latile compounds from the antagonists had an inhibitory effect on L26. Totally, 138 mVOCs were identified, with typical mass spectra and a broad range of molecular weights, ranging 51–492 g mol^{-1}. The deconvoluted mVOCs belonged to eighteen classes, the most dominant class is the Benzene derivatives, followed by Heterocyclic organic compound, Terpenoid and Pyrone derivatives and the trend is strong in the interaction of antagonists (Tv, Bs, Sr), with L26 isolate.

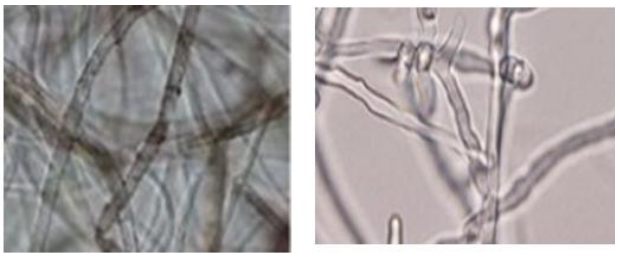

Fig. 4: Light microscopic image of L26 taken at the interaction zone with the antagonists
a. Image of L26 without interaction; **b**. Hyphal septum malformation and branch deformation during the interaction with antagonists

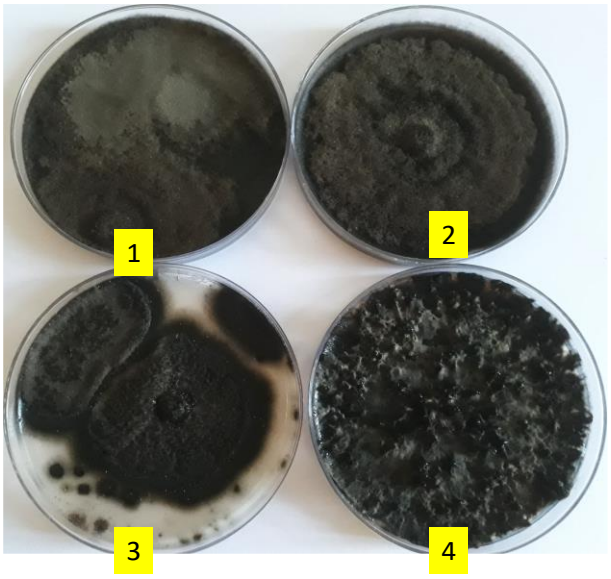

Fig. 5: Effect of volatile metabolites of antagonists on pycnidia formation of L26 isolate
1: *T. asperellum vs.* L26; 2: *B. subtilis vs.* L26; 3: *S. rochei vs.* L26; 4: Control (L26)

GC-MS results revealed that antagonists (Tv) emitted terpenoid compounds (*β*-caryophyllene (7.667%) with peak areas, followed by isoamyl alcohol (3-methyl-1-butanol (15.764%) and benzene derivatives (1, 2-benzenedicarboxylic acid (22.877%), 2-butoxy-2-oxoethyl butyl ester (24.655%). The antagonists *B. subtilis* also emitted benzene derivatives (benzothiazole (5.192%), cyclic lipopeptides (surfactin A (9.687%), saturated hydrocarbons (alkanes (4.064%) and *S. rochei* emitted few organic compounds *viz*., hexahydro-pyrrolo[1,2-a] pyrazine-1,4-dione (7.655%), 2-piperidinone (22.876%), hexahydro-3-(phenylmethyl (15.664%). The pathogen *L. theobromae*, produces, cyclohexenes and cyclohexenones (4R, 5R)-4,5-dihydroxy-3-methylcyclohex-2-enone, theobroxide), jasmonates (methyl jasmonate, (11R)-11-hydroxy-jasmonic acid). The intensity of peaks of these compounds increased during the interaction with antagonists with *L. theobromae* the peak intensity for terpenoids (*β-caryophyllene*) was the predominant class, followed by phenols (Ketoconazole) and Heterocyclic organic compound

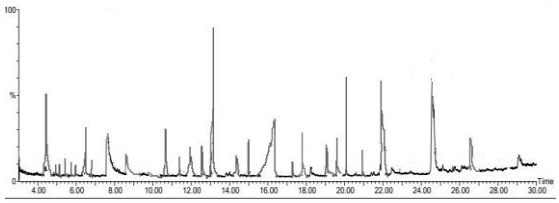

Fig. 6a Identification and profiling of volatile metabolites of *T.asperellum* by HS-SPME-GC-MS.jpg

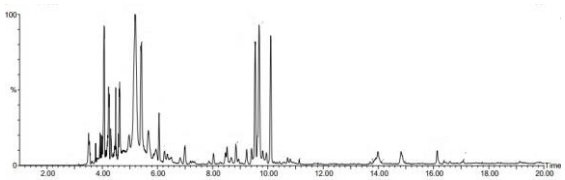

Fig.6b.Identification and profiling of volatile metabolites of *B.subtilis* by HS-SPME-GC-MS.jpg

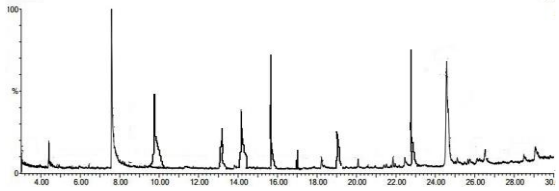

Fig.6c. Identification and profiling of volatile metabolites of *S.rochei* by HS-SPME-GC-MS.jpg

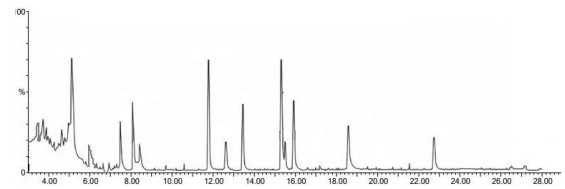

Fig.6d.Identification and profiling of volatile metabolites of L26 by HS-SPME-GC-MS.jpg

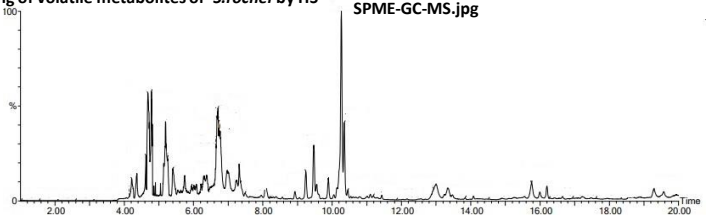

Fig.6e. Identification and profiling of volatile metabolites of Ta+Bs+Sr+Lt by HS-SPME-GC-MS.jpg

Fig. 6: Identification and profiling of volatile metabolites of antagonists and their interactions by HS-SPME-GC-MS

(2-piperidine) were increased on their interaction (Fig. 6a–e). However, the peak intensity of 5.19% corresponding to pyrrolo-quinoline derivatives decreased in the interaction of L26 with antagonists (Fig. 8).

Screening of antifungal metabolite against *L. theobromae*

In agar well diffusion method the metabolites of the three antagonists, showed good antifungal activity against the test pathogen. The dilution from 25 μL to 75 μL of antimicrobial extract was poured in three replications with a control. The 25 and 50 μL of the crude extract from *T. asperellum* inhibited 85.12 and 97.32% of mycelial growth of L26. There is absolute reduction in mycelial growth at 75 μL concentration. The extract from *B. subtilis* at the concentration of 25 and 50 μL showed 86.03 and 95.93 percent mycelial growth. The antagonist *S. rochei* inhibited the mycelia of the pathogen at different concentrations of 25, 50 and 75 μL were 84.09, 93.38 and 100%. At the concentration of 75 μL of the extract showed that, complete inhibition in treated compared to control. There was no mycelial growth was observed in 75 μL of crude extract treated plates inoculated with L26 (Fig. 7).

Discussion

For many years, the etiology of the destructive symptom in coconut was deserted by researchers and in spite of the value of this disease, only *L. theobromae* had been reported as a pathogen to coconut. It causes heavy losses in coconut being a very destructive fungal pathogen worldwide. For the proper identification, distribution, yield loss of the pathogen, the survey was conducted and isolates were collected and validated. Based on the survey report all the isolates were causing the same symptom in coconut with a difference in level of incidence. The pathogen was identified on morphological and molecular basis and identified as *L. theobromae.* The most frequently described morphological characteristics include mycelial growth, pycnidia and pycnidiospore production (Machado *et al*. 2014; Linaldeddu *et al*. 2015). Similarly, Ashokkumar *et al.* (2018) reported that all the isolates showed variation within the morphological characters and the pycnidiospore estimate changes from 14.3 × 7.69 μm (LT-CL2) to 25.59 × 13.31 μm (LT-CL5). Latha *et al*. (2013) reported that, isolates from Coimbatore (TNAU), showed dark grey colonies and found to be more virulent as it produced maximum pycnidia (63) in 90 mm Petridish. The virulent was distinguished based on the pathogenicity and production of pycnidiospores under *in vitro*.

Initially nine BCAs were tested against the fungus, based on the inhibition percentage the three BCAs were selected and they were having highest percentage of inhibition in dual plate assay. It is known that secondary metabolite production, could be strongly influenced the culture growth of the pathogen (Miao *et al*. 2006). The Different volatile organic compounds from *Trichoderma* have been profiled with antimicrobial properties and it is

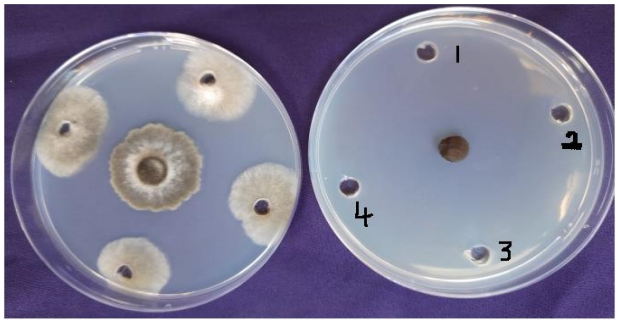

Fig. 7: Effect of extracted metabolites of the antagonists on the radial growth of L26

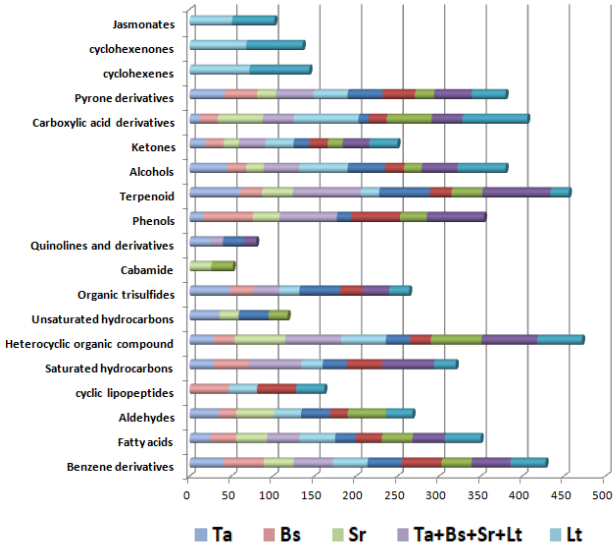

Fig. 8: Compound classes present in axenic and co-culture

specific to different species and strains of *Trichoderma* (Dennis and Webster 1971). A *Bacillus* spp. TN79 derived from Thua Nao, suppress the growth of *L. theobromae* (Chukeatirote *et al*. 2018). Liu *et al*. (2019) reported that, *S. sclerotiorum* and *R. solani* isolated from soybean root were inhibited by *Streptomyces* spp. NEAU-S7GS2. Furthermore, Srivastava *et al*. (2015) described that mycelial formation and percent disease progress of *S. sclerotiorum* was suppressed with *S. rochei* strain SM3 at an approximate rate of 74%. Similarly, Yu *et al*. (2020) and Martinez *et al*. (2020) reported that *S. triticiradicis* and *S. lydicus* caused inhibitory effects against pathogenic fungi from 30 to 63%. Hydrolytic enzymes such as chitinases or glucanases or proteases and may be the production of antibiotics against the fungal cell wall of *L. theobromae* was the cause for the zone of inhibition formed by the ultrafiltered crude extracts of BCAs in light microscopic observations (Jain and Jain 2007; Yan *et al*. 2008; Oskay 2009).

The volatile compounds have an indirect effect, without any contact with between antagonists and pathogen. In this study, there was reduction in percent mycelial growth compared to control and the hyphae regain their development once the antagonists were removed from their atmosphere. This evidence demonstrates that volatile compounds from BCAs are having fungicidal effect and similar results were obtained for sclerotia germination, in the presence of T. *reesi*, *T. harzianum*, and *T. longibrachiatum* EF5 the sclerotial germination were reduced in *S. rolfsii* isolates (Sridharan *et al*. 2020).

In our study, we identified mVOCs with antimicrobial properties from axenic culture of *T. asperellum* through GC-MS-TD such as, peptaibols (aspereline), limonene; *β*-eudesmol and 1, 3-octadiene. Wilkins *et al*. (2003) reported that the metabolite delivered by *T. viride* (2-propanol, 3-methyl furan, 1-pentanol, 2-hexanone), *T. atroviride* (pentanones, octanones, *etc*.), *T. harzianum* (cyclohexane, alcohols, esters *etc*.) showed hindrance in growth of pathogens and 141 compounds counting a few obscure sesquiterpenes, diterpenes and tetraterpenes from *Trichoderma* spp. (Lee *et al*. 2016). The VOCs emitted from *B. subtilis* was 2, 4-di-*tert*-butylphenol, also coincides with the results of Ongena and Jacques (2008); Wang *et al*. (2007) and Yoshida *et al*. (2001). The large amount antimicrobial activity is attributed to iturin and fengycin was reported by Robacker *et al*. (1998); Kai *et al*. (2009) and Caulier *et al*. (2019). From *Bacillus* spp. *Streptomyces* spp. are regarded as noteworthy sources in generation of secondary metabolites and these antimicrobial compounds could play parts in securing plants against distinctive pathogens (Miyada *et al*. 2017). In our study, *S. rochei* emitted 2-piperidinone and pyrrolo[1,2-a] pyrazine-1,4-dione and few other VOCs. From our investigation, it is observed that the pathogen *L. theobromae* emitted cyclohexenes and cyclohexenones (4R,5R)-4,5-Dihydroxy-3-methylcyclohex-2-enone, Theobroxide), jasmonates (Methyl jasmonate, (11R)-11-Hydroxy-jasmonic corrosive) and leads to the restraint of the defense pathway of the plant host, encouraging the disease process (Tsukada *et al*. 2010; Chanclud and Morel 2016). Few known secondary metabolites, such as jasmonic acid and 3-ICA, phytotoxin scytalone were identified from *Lasiodiplodia* species (Felix *et al*. 2018).

Remarkably, our study suggests that several biochemical pathways be drawn in in the construction of VOC in microbes while they relate with one another. These include cis-calamenene related sesquiterpenoids biosynthesis, phenolic malonylglucosides biosynthesis with Terpenophenolic Biosynthesis pathways.

During the interaction experiments, we pragmatic a boost in the expression of volatile compounds. This upregulation of mVOCs might be owed to the existence of diverse genus in the similar culture media. Signal molecules, accountable for the intra- and interspecies communication, are activated throughout these relations. This activation may perhaps be a consequence of more than a few volatile and non-volatile metabolites unconfined during the early development stage by together the microbes. Those

metabolites were formed from any of the microorganism's *viz*., BCAs, *L. theobromae* and this verdict was in lineup with the result of Karuppiah and coworkers (2019). Toffolatti *et al*. (2021) described that terpenoids act as specific or universal pathogen inhibitors. Similarly, in our study during the interaction of BCAs with L26, production of the class terpenenoids was found to be increased and this is one of the responses to assail in several plant-pathogen binomials. As well, phenols (Ketoconazole), act as signaling cursor, organization of arbuscular mycorrhizal symbioses and that can act as agents in plant defense (Mandal *et al*. 2010) and it is evident from our study during the interaction. Mahmoud and his coworkers also obvious our results that 2-piperidine, a heterocyclic organic compound disrupt the interaction shut between enteric pathogens and these derivatives have a good inhibition capacity against most tested pathogenic bacterial and fungal species due to their potential antimicrobial and fungicidal properties (Mahmoud *et al*. 2018). Pyrrolo-quinoline derivatives level is reduced in the coculturing experiments and in assessing redox cycling these derivatives has more than 100 times efficient than polyphenolic compounds, ascorbic acid, isoflavonoids potentials (Stites *et al*. 2000) and this might be a result of a few unstable and non-volatile metabolites discharged amid the beginning stage by both the microorganisms. Those metabolites were created from either of the microorganisms *viz*., *T. asperellum, B. subtilis, S. rochei, L. theobromae* nor interaction of those and this finding was in line with the findings of related proteins, secondary metabolites and plant growth promoting compounds to essentially improve the plant development and assurance against plant pathogens. However, no reports have illustrated the effects of co-cultivating the two most agriculturally important bacteria and fungi along with the pathogen (*T. asperellum*, *B. subtilis*, *S. rochei* and *L. theobromae*) on the metabolite production. In this paper, we have tended to this address a few co-cultivation technologies come about in improved action for various secondary metabolites, but frequently not for all.

Conclusion

Pycnidial producing fungi, *L. theobromae* proved to be a virulent pathogen in coconut and act as secondary pathogen next to eriophyid mite. The BCAs, proved to be effective against *L. theobromae* by producing mVOCs by inhibiting the pycnidiospore production. The compounds emitted from these BCAs can be deliberate further for additional properties, like growth promotion, medicinal properties, nutrient mobilization *etc*. Consequently, further it needs to be confirmed by developing a formulation from these biomolecules along with BCAs for more antimicrobial activity. Hence, an active bundling with these substances may be a great alternative to manage the incidence in coconut caused by the dreadful *L. theobromae*. Further, the scale-up of this co-cultivation technology within the fermentor and field study will encourage the use in farming sector.

Acknowledgments

We greatly thank the colleagues of the department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore for their support and providing access to the facilities needed for preparation of all the cultures. Additionally, we thank the colleagues from department of Microbiology, for their support and providing access to the facilities needed for the microbiological assays using GCMS.

Author Contributions

AS, MR and MS conceived the study. AS performed the experiments and analyzed the data. MR coordinated the experiments and helped to draft the manuscript. MS coordinated the experiments related to eriophyid mite. AS, MR and MS drafted the manuscript and wrote R scripts and analyzed the data. All the authors were contributed to the chemical analysis and authentic reference standard measurements. All authors have read and approved the manuscript before submission.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Availability

All datasets presented in this study are included in the article/supplementary material

Funding Source

Not applicable

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