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RESEARCH ARTICLE

Antifungal Volatiles from Macrobasidiomycetes Inhibits *Fusarium oxysporum* f.sp. *lycopersici*

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ABSTRACT

Several macrobasidiomycete fungi have potential biological properties naturally to combat fungal diseases. *F.oxysporum* f.sp. *lycopersici* is a soil-borne ascomycetous fungus, which causes disease in several vegetable crops. The present study aimed to explore the antifungal activity of VOCs produced by several macrobasidiomycete fungi against *F. oxysporum* f.sp. *lycopersici*. The VOCs emitted by macrobasidiomycete fungi were demonstrated by the inverted sealed plate assay against the target pathogen. Among the mushroom isolate tested *in vitro,* the VOCs exhibited from *Coprinus cinereus* inhibited 70 % mycelial growth of *F. oxysporum* f.sp. *lycopersici*, followed by *Ganoderma lucidum* (60.28 %) and *Lentinus edodes* (35.28 %). In addition to inhibition of the pathogen, the headspace volatiles emitted by the effective isolate were trapped by Tenax column and subjected to GC-MS analysis. A total of 15 and 25 VOCs were identified from *C. cinereus* and *G. lucidum*, respectively. Among them, Alfa copaene showed a peak area percentage of 7.82 (14.99 RT) in *Coprinus cinereus*. followed by 2 undecanone. Similarly, trichloromethane and 1- pentanol produced from *G. lucidum*, showed high relative abundance of 71.5 per cent at 9.84 RT and 70.3 per cent at 2.69 RT, respectively. The VOCs produced by macrobasidiomycete fungi could possess antimicrobial activities against the fungal pathogen. These volatile compounds may be explored as a novel biocontrol agent against soil borne pathogens of vegetable crop.

Keywords: *Macrobasidiomycetes; C. cinereus; VOCs; Antifungal activity.*

INTRODUCTION

Fusarium oxysporum is a common pathogen present in various environments, both in plant tissues and rhizosphere soil. It is a soil-borne pathogen widely distributed in majority of vegetable and fruit crops. *F. oxysporum* express both pathogenic and non-pathogenic mode of infection. Plant pathogenic form of infection is defined by its strict host specificity (Arie *et al*., 2007 Arie, 2010). Among the vegetable crops, tomato is infected by *Fusarium* wilt caused by *F. oxysporum* f.sp. *lycospercisi*. In India, the total area under cultivation and production of tomato has gradually decreased from 2017 to 2019 (NHB, Horticultural statistics, MOA, GOI, 2018). Over the past centuries, various attempts have been made to control plant diseases by eradication or prevention through the use of synthetic fungicides. Most of the fungicides used are found to be ineffective due to the development of fungicide resistance in the pathogen (Wuest *et al*., 1974; Gea *et al.,* 2005). Currently, biological control of plant pathogens promotes disease suppression to improve plant health (Handelsman and Stabb, 1996). The Biocontrol mechanism seems to be eco-friendly, safe, and gives long-term protection to the crops (Soylu *et al.,* 2010).

Recently, volatiles exhibited by microorganisms have been attempted to eradicate some stages of the disease during plant-pathogen interaction due to their potent antimicrobial properties (Koitabashi 2005; Fialho *et al*., 2011; Nishino *et al*., 2013). The volatile secondary metabolites from mushroom have been used to inhibit plant pathogens which possess several biological and pharmacological properties. Some mushroom also exhibit several aromatic odour, flavour, fragrance that helps in suppression of fungal pathogens (Nishino *et al*., 2013). Volatile organic compounds (VOCs) produced by *Muscodor albus*, *Trichoderma* spp., *Irpex lacteus* and *Oxyporuslate marginatus* were related to the volatiles produced by mushroom (Dennis and Webster, 1971; Strobel *et al*., 2001; Koitabashi 2005; Lee *et al*., 2009; Nishino *et al*., 2013). For instance, more than 150 VOCs were identified from wild mushroom, which possesses

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several flavor and medicinal properties (Pinho *et al*., 2008; Ouzouni *et al*., 2009). Similarly, the volatiles from fruiting bodies of *Laetiporus sulphurous* inhibited the growth of *Aspergillus flavus* in tomato paste (Petrovic *et al*., 2013). A volatile antifungal compound like 1-phenyl-3-pentanone produced from *Mycoleptodonoides aitchisonii* inhibited the growth of some phytopathogenic fungi and was reported as fungistatic activity (Nishino *et al*., 2013; Oka *et al*., 2014). Berendsen *et al*., 2013 reported that the volatile compound Octen-3-ol produced by an edible mushroom, *Agaricus bisporus* inhibits spore germination of ascomycetous fungi. The volatiles produced by *Hypsizygus marmoreus*, an edible mushroom had antifungal activity against some phytopathogenic fungi and also suppressed the mycelial growth, and spore germination of *Alternaria brassicicola* (Oka *et al*., 2015).

This study focuses on identifying the volatile organic compounds (VOCs) produced by several macrobasidiomycete fungi and their potential inhibitory activity towards *F. oxysporum* f.sp. *lycospercisi* .

MATERIAL AND METHODS

2.1. Macrobasidiomycetes fungi

Pure cultures of *Ganoderma lucidum, Auricularia auricular, Lentinus edodes, Coprinus cinereus, Pleurotus florida* and *Calocybe indica* were obtained from the culture collection center at the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.

2.2. Isolation of pathogen

The virulent strain of *Fusarium oxysporum* was isolated from wilt infected necrotic tissue of stem. The infected stem was first cut into small pieces, washed with sterile water followed by disinfection with 0.1 % sodium hypochlorite, repeatedly washed with sterile water and dried on sterile filter paper. The dried fragments were cut lengthwise and placed on the Petri plate containing potato dextrose agar (PDA) medium and incubated for 4-5 days at 28 °C. The fungus was confirmed for the presence of spores, subcultured on the fresh Petri plate for morphological and molecular confirmation.

2.3. Antagonistic screening of macrobasidiomycetes volatiles

The antifungal activity of volatile phase of fungus towards mycelial growth of pathogen was carried out by inverted sealed plate method. A *Macrobasidiomycete* fungus obtained was inoculated by transferring 5 mm mycelial disc onto the Petri dish containing PDA medium. Likewise, a mycelia disc (5 mm) was excised from the 7 days old fungal culture and placed onto the PDA in a second Petri dish. The Petri dish with fungal culture was invertedly placed over the pathogen inoculated plate to test the antagonistic activity of volatiles from macrobasidiomycete fungal mycelia. At this point, the Petri dish was sealed with cling film and incubated at room temperature. The plates without macrobasidiomycete fungi served as control. The experiment was replicated four times. The colony diameter of pathogenic fungi was observed constantly until 90 mm of growth reached. The inhibition of mycelial growth of pathogenic fungi (mm) was designed by using the formula.

$$\text{Growth inhibition (\%)} = \frac{\text{(Fungal growth on control plate - Fungal growth on treated plate)}}{\text{(Fungal growth on control plate)}} \times 100$$

2.4. Trapping of volatiles and detection of biomolecules

The VOC emitted by effective macrobasidiomycete fungi were profiled by growing onto the PDA medium in the flask. Afterward, the headspace volatiles collected through a specialized Tenax column was analyzed using GC-MS fitted with an automated thermal desorber (ATD), and Turbo Matrix 150 (Perkin Elmer, USA). When the Tenax column was exposed to heat to 200 °C with a coiled heater around the tube, desorbed volatiles from heated Tenax were transferred into the injection port of the gas chromatograph through the stainless needle within 20 sec. At that point, the Tenax tube was removed from the injection port and the GC analysis was initiated. The conditions for GC-MS analysis were as follows: 10:1 split, Helium gas carrier at 20 psi, oven temperature of 50 to 250 ºC at 10 ºC per minute increment, electron impact spectra at 70 eV, and positive ion mode. The analysis was performed using 30 m × 250 µm capillary column coated with 5% phenyl methyl siloxane. The VOCs detected were compared with mass spectra available in NIST mass spectral library database. Volatile compounds that found 100% similarity were shortlisted as tentative compounds of interest.

2.5. Statistical analysis

The design of the experiment and statistical analysis was performed using SPSS. The comparative analysis of volatile compounds was followed using heatmapper online (http://www.heatmapper.ca/). The metabolic pathway enrichment analysis involved in macrobasidiomycete fungi was analyzed through Metabo Analyst 4.0 (http://www.metabanalyst.ca).

RESULTS AND DISCUSSION

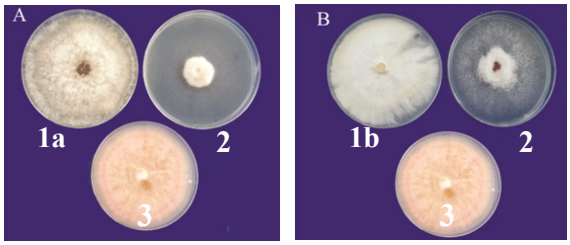
Fusarium sp. was obtained from wilt infected necrotic tissue of stem. Based on the morphological characteristics and nucleotide sequence of 18S rRNA, *Fusarium sp.* was confirmed as *Fusarium*

oxysporum f.sp. *lycopercisi*, which showed 100 per cent identity. The sequence was submitted to the NCBI database with accession no: MN088389.

3.1. Antifungal activity of macrobasidiomycetes volatiles against Fusarium

Several macrobasidiomycetes are known to produce wide variety of antimicrobial substances naturally that protect from large spectrum of phytopathogenic microorganism (Spiteller, 2008). In this study, the efficacy of volatiles exhibited from mycelia of several macrobasidiomycete fungi against *F. oxysporum f*.sp. *lycopercisi* through inverted plate method are displayed in Fig. 1.

Fig 1. Effect of volatiles produced by macrobasidiomycete fungi against *F. oxysporum* f.sp. *lycopercisi*



A) *C. cinereus*(1a) B) *G. lucidum*(1b); FOL inhibited(2); FOL Control (3)

We found that the volatiles produced by mycelia of *Coprinus cinereus* significantly inhibited 70 % mycelial growth of *F. oxysporum* f.sp. *lycopercisi,* followed by *G. lucidum*(60.28 %) and *L. edodes* (35.28 %), over control (Fig. 2).

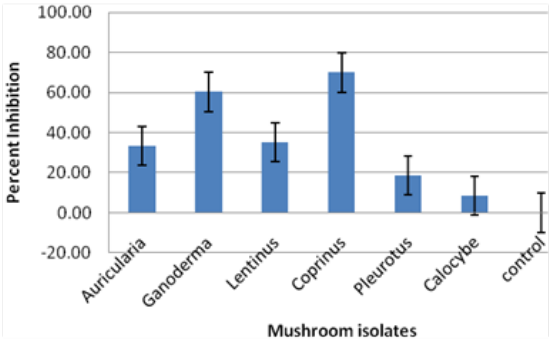


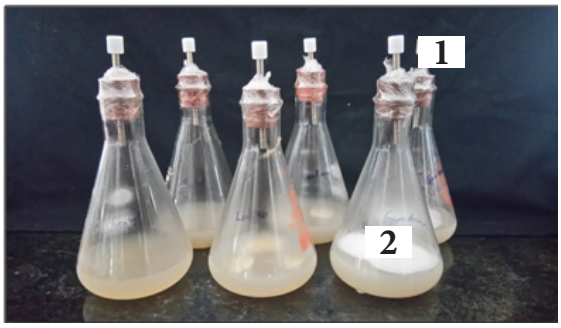
Fig 2. Inhibition potential of macrobasidiomycetes volatiles against *F.oxysporum* f.sp.*lycopercisi*

Apart from the inhibition of mycelia growth, there was also morphological differences in the mycelia of tested fungi. The mycelia are stunted, puffy appearance with no spore germination, which completely differentiated from control. Similarly, *M. aitchisonii* is an edible mushroom, which highly suppressed the growth of several phytopathogenic fungi *viz., Alternaria alternata, A. brassicicola, A. brassicae, Colletotrichum orbiculare* and *Corynespora cassiicola* (Nishino *et al*., 2013).

Previous studies have been reported that the basidiomycetes fungi have potent antimicrobial activities against wide range of phytopathogenic fungi (Combet *et al*., 2006; Noble *et al*., 2009 and Berendsen *et al*., 2013). The higher inhibitory percentages were reported for all three species of wood-rotting basidiomycetes (*Coriolopsis gallica, Megacollybia platyphylla* and *Lentinus arcularius*) against *Fusarium solani* and *Sclerotinia sclerotiorum*. These fungal pathogens are more susceptible to the antagonistic activity of the volatiles exhibited by the basidiomycetes (Petre *et al*., 2017). Futher more, Sangeetha (2018) also demonstrated a similar result on the effect of headspace volatiles produced by Chinese caterpillar mushroom (*Ophiocordyceps sinensis*) that strongly inhibited the mycelia growth of *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *cubense*. Volatile compounds extracted from ectomycorrhizal fungus such as *Russulaa anthracina*, *R. chloroides* and *R. senecis* were shown to inhibit the of conidial germination of a phytopathogenic fungus, *Alternaria brassicicola* (Osaki oka, 2019). In conjunction with the available findings, *Coprinus cinereus*. and *G. lucidum* could play as an alternative agent for controlling the disease caused by fungus.

3.2. Isolation and trapping of headspace volatiles

To demonstrate the antifungal compounds produced by *Coprinus cinereus*. and *G. lucidum*, headspace volatiles are trapped by a special Tenax GC trapping technique. The effective macrobasidiomycete fungi were cultured on PDA medium into the conical flask. A specialized tenax column was fitted into the flask with the aid of rubber cork (Fig. 3).



1.Inoculation of macrobasidiomycete fungi on PDA medium 2.Tenax column fitted into flask for trapping volatiles

Fig 3. Trapping of headspace volatiles from effective macrobasidiomycetes

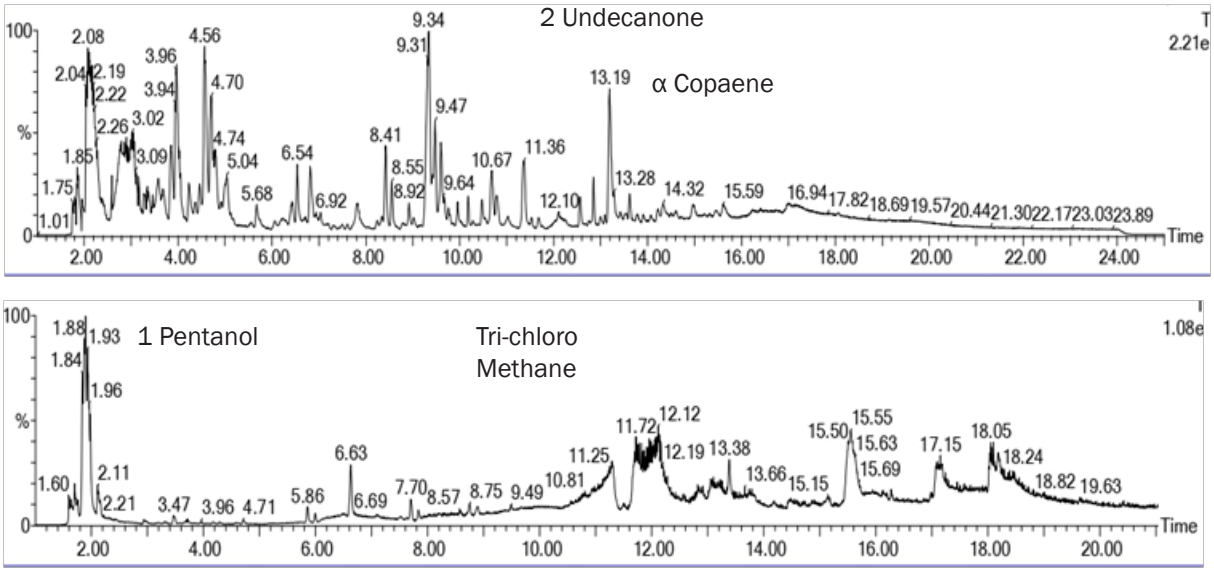
The headspace volatiles produced by mycelia of effective macrobasidiomycete fungi were trapped from the initiation of mycelia growth over a period of 7 days. Afterward, the volatiles trapped by Tenax column is taken away from flask, fitted to the column holder for GC-MS analysis and the antifungal

biomolecules were detected. The tenax trapping technique is a simple technology, highly efficient for analysis of headspace volatiles within a short time (Tsugita *et al*., 1979). Tenax is a porous polymer used for quantitative trapping of floral volatilomes (Dudareva and Pichersky 2006; Maiti *et al*., 2019).

3.3. Identification of antifungal biomolecules

The GC-MS analysis indicated that the volatiles produced by *Coprinus cinereus*., *G. lucidum* and *L. edodes* produced diverse volatile compounds, including aldehydes, ketones, alcohol, esters, fatty acids etc.,

Fig 4. GC-MS chromatogram of VOCs from (a) *Coprinus cinereus* and (b) *G. lucidum*



The non-inoculated control were also analysed to eradicate the contaminent produced from column. No differences were observed between control and effective isolates. Based on 90 % similarity of mass spectra, a total of 15 VOCs were shortlisted from *Coprinus cinereus* (Fig. 4a) and 29 VOCs from *G. lucidum* (Fig. 4b). Among these compounds, Alfa copaene showed a peak area percentage of 7.82 (14.99 RT) in *Coprinus cinereus* followed by 2 undecanone. Antifungal volatile compounds like trichloromethane and 1- pentanol showed high relative abundance (71.5 per cent at 9.84 RT and 70.3 per cent at 2.69 RT, respectively) in case of *G. lucidum*.

Fig 5. Heatmap of VOCs from *Coprinus cinereus*, *G. lucidum* and *L. edodes* indicating the highest efficiency in the compound (Alpha copaene and 2 undecanone in *Coprinus* sp and Trichloromethane, 1 pentanol in *G. lucidum*)

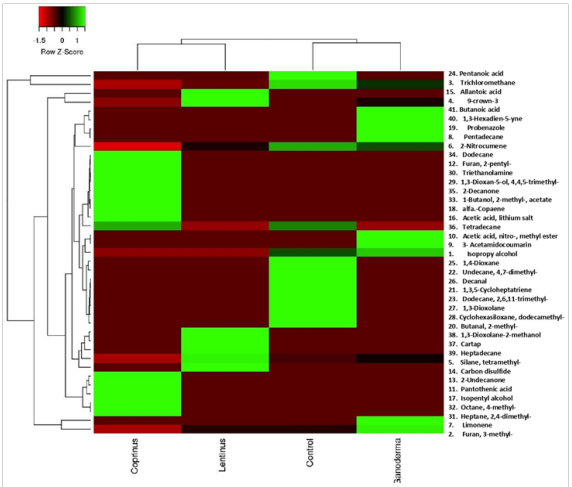
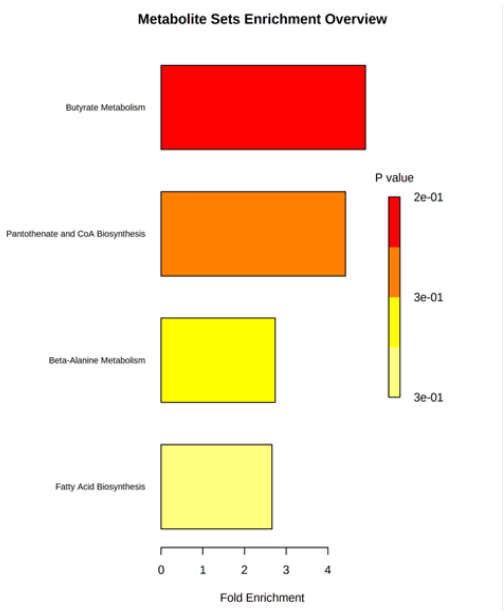


Fig 6. Over representation analysis of selected VOCs from *Coprinus cinereus, G. lucidum* and *L. edodes*



A clear disparity observed between the VOCs of *Coprinus cinereus G. lucidum* and *L. edodes* were shown with a heatmap (Fig. 5). The most important VOCs copaene, was identified in Chinese herb (Meliaazedarach) and also from *Coprinus cinereus* (Melliou *et al*., 2007; Yang *et al*., 2011). Eugenio *et al*. (2015) reported that VOCs like pentanol, undecanone, nonanol, octanol, pentadecanoic acid produced by raspberry fruit, helps in fruit resistance to mold disease. Several findings strongly supported the result and these VOCs could possess antifungal properties against broad spectrum of pathogens.

3.4. Volatile metabolite set enrichment

The over-representation analysis (ORA) was performed to identify the volatile pathway involved in *Coprinus cinereus*, *G. lucidum* and *L. edodes*. ORA was implemented using hypergeometric test to evaluate whether a particular volatile metabolite set was represented more than expected time by chance within the given compound list. The p-value was highest for butyrate metabolism, followed by pentothenate and COA biosynthesis and least for beta-alanine and fatty acid metabolism (Fig. 6). The metabolite enrichment pathway analysis confirms that the volatile compounds produced by *C. cinereus, G. lucidum* and *L. edodes*, highly metabolize in fatty acid biosynthesis, butyrate metabolism and CoA biosynthesis pathway.

CONCLUSION

In this study, a total of 6 species of basidiomycetes were screened for volatile production with antagonistic activities against fungal pathogens. As a result, mycelia of *C. cinereus* and *G. lucidum* showed maximum inhibition, suggesting that antifungal volatile organic compounds in macrobasidiomycete fungi could be produced widely in nature. Some macrobasidiomycetes with excellent aromatic odor may have a potential role as biocontrol agents. More than 50 VOCs were detected in each of the mushroom isolates. Based on the similarity percentage of mass spectra obtained, a total of 4 antifungal volatile compounds were shortlisted which could be responsible for the suppression of the growth of fungal pathogens. Alfa copaene and 2 undecanone are important VOCs produced by *C. cinereus* and Tricholoromethane, 1 pentanol are exhibited in case of *G. lucidum*.

To our knowledge, this is the first report on antifungal volatile compounds of *C. cinereus* which possesses antifungal activity against *F. oxysporum* f.sp. *lycopercisi* and could be effective alternatives for controlling plant diseases. Further studies are required to determine the effects of synthetic VOCs produced by *C. cinereus* against fungal pathogen; the volatile signal between the *C. cinereus* on interaction with pathogen could be exploited and also to develop suitable organic-based formulation against soil-borne pathogens of vegetable crops.

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