Antagonistic Activity of *Trichoderma cerinum* Gu1 VOCs Against Fungal Phytopathogens and Pant growth Promotion

Ekta Khare, Upma Singh, Department of Microbiology, School of Life Sciences and Biotechnology, Chhatrapati Shahu Ji Maharaj University, Kanpur-208024, UP India. *Email: ektakhare@csjmu.ac.in, ekhare.cnu@gmail.com

Abstract

Many *Trichoderma* species are used as biological fungicides and biofertilizers to enhance crop growth. The biocontrol activity may result from space competition, mycoparasitism, or an antibiosis involving a chemical diffusate. This study demonstrated that in addition to mycoparasitism, VOCs play a significant role in *Trichoderma cerinum* and *T. harzianum* ability to combat pathogenic fungi. Our findings showed that *T. cerinum* Gur1 non-volatile and volatile compounds performed better than *T. harzianum* H1 in inhibiting *Rhizoctonia solani* and *Fusarium* sp. Microscopic study showed mycoparasitism and VOCs are involved in inhibition of pathogens. GC-MS analyses demonstrated that a chemically varied set of volatile chemicals were synthesised by *T. cerinum* Gur1. Extracted VOCs improved the growth of *Arabidopsis thaliana* Col-0 lateral roots and shoot. Our research revealed the importance of *T. cerinum* VOCs in the biocontrol of pathogens, as well as how they affect plant growth and may function as signalling molecules that activate pathways connected to plant growth hormones.

Keywords: Biocontrol, Mycoparasitism, *Trichoderma cerinum*, Volatile organic compounds

Introduction

Soil-borne plant pathogens can be a significant barrier to the growth of marketable yield in the majority of agricultural settings. In compared to pathogens that attack the plant's above-ground growing region, they are also the most difficult to manage and control (Bruchl 1987). According to Agrios (2005), plant diseases are to blame for annual agricultural losses totaling more than $200 billion USD. To control plant disease, resistant plants and chemicals are frequently utilized. Disease takes a huge toll on crop productivity and consistently causes crop damage. Chemicals have been in use to combat phytopathogens for a very long period. Fungicide and fumigant applications, which are frequently used in greater quantities than herbicides and insecticides in agricultural production, can have profound consequences on the environment and consumers. Chemical techniques are not cost-effective in the long run since they hurt the environment, pollute the air, leave dangerous residues, and, with repeated use, might cause the development of resistant strains of the target organisms (Naseby et al. 2000). Numerous soilborne fungi, including some strains of *Rhizoctonia solani* and *Fusarium oxysporum*, are harmful to plants and challenging to eradicate. However, for regulating soilborne fungus growth, biological techniques might be a dependable substitute for chemical ones. It would be ideal if agricultural applications of synthetic pesticides decreased or stopped altogether.

Trichoderma species are well known among biocontrol microorganisms for their capacity to suppress fungus from numerous other genera, including some that are harmful to agricultural plants. *Trichoderma* is able to do this because it produces extracellular lytic enzymes, toxic non-volatile and volatile metabolites, high levels of competitive saprophyte activity, a rapid rate of reproduction, among other things, and it induces systemic resistance in host plants (Halifu et al. 2019). Workers review a lot of research on the mechanisms underlying their defences against phytopathogens. While there is still much to learn about how volatile chemicals affect fungi infections. For their positive impacts on plant growth, including the synthesis of antibiotics, and for their capacity to outcompete other fungi and harmful bacteria, *Trichoderma* species have received substantial research (Harman et al. 2004). *Trichoderma* fungus also have a direct impact on plant growth, increasing plant biomass above ground and causing adventitious roots to develop below ground (Windham et al. 1986; Contreras-Cornejo et al. 2009).

Trichoderma sp. are also the most studied and used class of biocontrol agents due to their widespread presence, simplicity in isolation and identification, and safety for both plants and animals. However, the distribution of biocontrol fungi, such as *Trichoderma* spp., is not constant in the soil of various geographical areas. In deficient soils, frequent application is required. Crops

grown in soil that is rich in biocontrol agents may not have access to the favourable environmental conditions needed for their activity, and they may not be protected from fungus pathogens. Understanding the biocontrol mechanism of a certain pathogen is therefore important in order to use *Trichoderma* to combat disease. Studies on the role of volatile compounds (VOCs) are not as common as those on non-volatile compounds with biocontrol potential. The presence of air-filled pores is a crucial feature of most soils. In the liquid and gaseous phases of soil, volatile molecules can travel farther and faster than diffusible ones (Effmert et al. 2012; Insam and Seewald 2010). This promotes interactions between soil microorganisms. In order to communicate and compete among physically distant soil microorganisms, volatiles are crucial (Effmert et al. 2012; Kai et al. 2009). According to an intriguing body of research, bacterial volatiles frequently cause plants to develop systemic resistance (Kishimoto et al. 2007; Ryu et al. 2003). On the other hand, despite the fact that fungi are known to create a huge number of volatile organic compounds (VOCs), little research has been done on how these VOCs relate to plant disease or growth promotion. Therefore the objective of present investigation is (i) to screen *Trichoderma* strains for antagonistic activity against various fungal phytopathogens (*Fusarium oxysporum* f. sp. *lactucae*, *F. oxysporum* f. sp. *ciceri*, *F. solani*, *F. graminearum*, and *Rhizoctonia solani*), (ii) to screen and determine the role of volatile and non-volatile metabolites of *Trichoderma* against phytopathogens, and (iii) to check the effect of VOCs on *Arabidopsis thaliana* Columbia-0.

Materials and Methods

Fungal antagonists

Trichoderma cerinum Gur1 and *T. harzianum* H1 were procured from the Department of Microbiology, CSJM University, Kanpur, India. Strains were grown and maintained on potato dextrose agar (PDA) at 28º C and 4º C respectively.

Fungal phytopathogen

The phytopathogens *Fusarium oxysporum* f. sp. *lactucae, F. oxysporum* f. sp.*ciceri, F. solani, F. graminearum,* and *Rhizoctonia solani* were procured from the Department of Microbiology, CSJM University, Kanpur, India. Fungal phytopathogens were grown and maintained on PDA media at 28º C and 4º C respectively.

Antagonistic activity

Direct confrontation plate assay

A mycelial disc (6 mm dia.) was transferred to a PDA plate from the periphery of a pathogen culture that had been growing on PDA for six days. The antagonist's (*Trichoderma* sp.) mycelia disc was then positioned six centimetres distant from the pathogens disc. The control was maintained without inoculating the antagonists. Radial growth reduction was estimated after 4 days incubation period in relation to growth of the control as follows:

$$\frac{C - T}{C} \times 100 = \% \text{ Inhibition of radial mycelial growth}$$

Where, C = radial growth measurement of the pathogen in control

T = radial growth of the pathogen in the presence of antagonistic fungi

Three duplicates of the experiment were carried out. From the zone of inhibition, pathogen mycelium was removed for inspection under a compound microscope.

Indirect confrontation plate (VOC) *assay*

In this procedure, the antagonist and the pathogen are inoculated in two different PDA dishes. The chosen antagonist is then placed at the bottom of two dishes, and the pathogen is placed at the top, creating an assembly. To prevent the loss of any volatile compounds, two plates were taped together using paraffin (Jayaswal et al. 1993). The cultural circumstances were the same as those in the aforementioned direct confrontation testing. In the control treatment, an inoculum of 6 mm of sterile PDA media was used in place of the antagonist. Every 24 hours, mycelial growth was assessed by measuring the diameter of the pathogenic fungus's mycelia colony, and the results were displayed as a percentage of growth inhibition. After 4 days deformities in pathogen hyphae was analysed by compound microscope examination.

Biocontrol characteristics

Cellulase activity

On basal salt media supplemented with 2% carboxy methylcellulose (CMC), the Trichoderma sp. 6 mm disc was cultivated for 48 hours at 28ºC (Hankin and Anagnostaksis 1975). Congo red solution (0.1%) was poured into the Petri plates, and after 5 minutes, it was removed. Following a 1M NaCl solution wash, the plates were left to stand for 15–20 minutes. When the cellulose had been used by the enzyme, a clean zone was seen around the colony. The experiment was carried out three times. The following formula was used to determine cellulase activity index (CAI):

$$CAI = \frac{\text{Colony diameter + Halozone diameter}}{\text{Colony Diameter}}$$

Protease activity

Trichoderma sp. disc (6 mm) was placed in the centre of protease media plates and then incubate at 28ºC for 72 h. Exo protease activity was shown by the halo's formation (Brown and Foster 1970). The experiment was carried out three times. The protease activity index (PAI) was determined as follows:

$$PAI = \frac{\text{Colony diameter + Halozone diameter}}{\text{Colony Diameter}}$$

Collection of volatile compounds

This technique involved stacking two dishes, one PDA plate with Trichoderma spp. and another with 3 g of sterilised activated charcoal. After inoculation, the plates were firmly sealed and incubated at 28°C for a further 7 days. Three duplicates of each experiment were carried out. After the incubation, the activated charcoal was removed and extracted all trapped volatile chemicals with 5 ml of ethyl acetate. Gas chromatography-mass spectrometry (GC-MS) was used to analyse the extracted volatile components from selected *T. cerinum* strain Gur1. The GC-MS was performed at IIT, Kanpur.

Antagonistic activity of extracted volatile compound

To further confirm the effect of VOC, the extracted product was used with slight modification of VOC assay. Briefly, 6 mm disc of pathogen was placed in the center of PDA plate. At the edge of the PDA plate, a sterilised PCR tube (without cap) was positioned vertically. 75 µl of VOC extract were added to the PCR tube before the plate was covered with a lid and parafilm was used to seal it. As a control, a PCR tube containing 75 µl of ethylacetate was used. The plate was then incubated at 28°C for 4 days and observed for the growth and effect on hyphae was analyzed by compound microscopy. The experiment was performed in triplicate.

Effect of extracted VOC on *Arabidopsis* plant

To surface-sterilize *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) seeds, ethanol (70%) was used for 5 minutes, followed by sodium hypochlorite (2%) and a number of sterile distilled water washes. After that, seeds were placed on plant growth media (PGM) (Engelke et al. 1987) and stratified for 2 to 3 days at 4°C in the dark. Seedlings that were seven days old were arranged in a queue on the second PGM plate (8 per plate). In the plate's margin, a sterilised PCR tube (without a cap) was positioned vertically. 75 µl of extract were put into the PCR tube, which was then covered with a lid and paraffin-sealed. After that, the plate was incubated for 4 days at 24°C. The experiment was carried out three times.

Results

Antagonistic activity

Direct confrontation plate assay

T. cerinum Gur1 showed better result against most of the selected fungal phytopathogens over *T. harzianum* H1 (Table 1, Fig. 1). *T. cerinum* showed better antagonistic activity against *F. oxysporm* f. sp. *ciceri* (44.8%), *F. oxysporum* f. sp. *lactucae* (40%), *F. solani* (36%) and *R. solani* (42%).

There was 70%, and 40% inhibition of *F. graminearum* by *T. harzianum* and *T. cerinum* strains respectively. Pathogens' hyphae displayed lysis, deformation, and swelling in compound photomicrographs of fungal hyphae collected from the inhibitory zone. It was also possible to see the accumulation of hyphal cytoplasm that led to the development of empty cells, illustrating the function of antifungal metabolites (Fig. 1).

Indirect confrontation plate assay

T. cerinum and *T. harzianum* showed (44%) and (19%) inhibition of *F. graminearum.* In comparison to *T. harzianum* H1, *T.cerinum* Gur1 volatile compounds showed better antagonistic activity against *F.oxysporum f. sp. ciceri* (44.84%), *F.oxysporum* f.sp. *lactucae* (11.11%), *F.solani* (45 %) and *R. solani* (20 %) (Table 1, Fig. 2). Compound photomicrograph showed the shrinkage and reduction of cytoplasm (Fig. 2).

Biocontrol characteristics

Both *T. cerinum* Gur1 and *T. harzianum* H1 showed cellulase activity index of 2.12 and 2.48, respectively. Protease activity index of 1.4 and 1.57 was observed for *T. cerinum* Gur1 and *T. harzianum* H1.

Antagonistic activity of extracted volatile compound

VOC extract of *T.cerinum* showed better result over *T. harzianum* against all the tested phytopathogens. There was 10% and 16%, inhibition of *F. graminearum* by VOC extract *T.harzianum* and *T. cerinum*, respectively. VOC extract of *T. cerinum* and *T. harzianum* showed 9.09% and 7.95% inhibition of *F. solani*. In case of *F. oxysporum* f. sp. *ciceri* VOC extract of *T. cerinum* showed 11 %. VOC extract of *T. cerinum* showed 12.67% inhibition of *Rhizoctonia solani* and 6.43% against *F. oxysporum f. sp. lactucae* (Table 1). The change in hyphal morphology and presence of large vacuoles was visible in compound micrographs of pathogens hyphae.

Effect of extracted VOC on *Arabidopsis* plant

Volatile compounds extract of both *Trichoderma* sp. showed enhancement in root and shoot length of *Arabidopsis* seedling. Maximum enhancement was showed by *T.ceranium* over control. There was 10% and 3.3% enhancement in root length by *T. ceranium* Gur1 and *T. harzianum* H1, respectively. *T. ceranium* and *T. harzianum* showed 22% and 2.5% enhancement in shoot length over control (Table 2). In comparison to control, volatile compounds also increased development of lateral roots (Fig. 3).

Gas Chromatography Mass Spectrometry

The volatile compounds detected in the culture samples constitute members of the compound classes of alkanes, alcohols, ketones, pyrones (lactones), furanes, monoterpenes, and sesquiterpenes. Primarily, hydrocarbons, fatty acids, alcohol and benzene derivatives were identified from *T. cerinum* Gur1 including heptanes, octane, 1,1-dimethoxy-2-methyl-propane, 4-hydroxy-2-butenoic acid (methyl ester), cyclopentane and other compounds that were found among the volatile metabolites (Fig. 4).

Discussion

Exploiting the advantageous traits of soil microorganisms is now of interest due to the growing demand for sustainable alternatives to agrochemicals and inorganic fertilisers in food production. Despite extensive research on non-volatile compounds toxic to plant pathogenic microorganisms over the years (Gu et al. 2007; Strobel et al. 2001; Wan et al. 2008; Zou et al. 2007), only a small number of research have concentrated on volatile substances produced by microorganisms against plant pathogens in the last 10-15 years. *Trichoderma cerinum* Gur1 and *T. harzianum* H1 were chosen for this study due to their antagonistic and growth-promoting properties (Khare and Kumar 2018). *Trichoderma* sp. are typical soil fungi, according to Verma et al. (2007). Our findings indicated that *Rhizoctonia solani* and *Fusarium* sp. were inhibited by both non-volatile and volatile substances of *T. cerinum* and *T. harzianum*. According to Contreras-Cornejo et al. (2009), certain *Trichoderma* species are known to parasitize plant diseases such *Fusarium oxysporum*, *Phytophthora capsici*, and *Rhizoctonia solani*. A microscopic analysis revealed that pathogen

inhibition is mediated through mycoparasitism and VOCs. Enzymes are responsible for controlling mycoparasitism. Chitinase-1, 3 glucanase, and proteases were said to be involved in the *Trichoderma*-mediated biological control by Harman (2000). The *Trichoderma* sp. strains that were employed in the investigation produced exoenzymes.

Numerous studies have examined the effect of VOCs in preventing the growth of phytopathogens (Campos et al. 2010). The degree of inhibition relies on the specific bacteria-fungus or fungus-fungus interaction (Garbeva et al. 2014b; Kai et al. 2007, 2009; Vespermann et al. 2007). Sensitivity to volatiles might significantly vary amongst fungal species. According to several independent investigations, *Pythium* species (omycetes) are substantially more susceptible to bacterial volatiles than *F. solani*, which is not greatly influenced by them (Effmert et al. 2012; Garbeva et al. 2014 a, b; Kai et al. 2009). The GC-MS analysis of *T. cerinum* Gur1 VOCs revealed the presence of benzene derivatives, fatty acids, alcohol, and hydrocarbons. Siddiquee et al. (2012) detected the normal saturated hydrocarbons (C7–C30), cyclohexane, cyclopentane, fatty acids, alcohols, esters, sulfur-containing compounds, simple pyrane and benzene derivatives from the culture of *T. harzianum*.

A. thaliana Col-0 root, shoot, and lateral root development were improved by volatile chemicals from both strains. The best result was seen with VOCs from *T. cerinum* Gur1. According to Contreras-Cornejo et al. (2009), long-term exposure to *Trichoderma viride* VOCs had growth-promoting effects as demonstrated by an increase in fresh weight, root mass, and chlorophyll concentration in leaves. It was hypothesised that *T. virens*-associated augmentation of biomass and lateral growth development in *Arabidopsis* is caused by auxin-related chemicals that *T. virens* produces and subsequently diffuses across the media (Lee et al. 2015). We hypothesise that *T. cerinum* volatile metabolites contribute to the growth-promoting action and may function as signalling molecules that activate pathways linked to plant growth hormones, as indicated by the development of lateral roots. The volatile chemicals of fungal cultures have received some study over time (Dennis and Webster 1971; Lee et al. 2016; Strobel et al. 2001). However, their impact on plant pathogenic fungi has just lately been highlighted (Lee et al. 2016). Plant pathogen-related VOC research is still in its infancy; therefore advancements are required for agriculture science and crop development.

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Table 1. Antagonistic activity of *Trichoderma* sp. against fungal phytopathogens during direct confrontation plate assay (non-volatile compounds), indirect confrontation plate assay (volatile organic compounds) and extracted VOCs.

		Inhibition %				
		F. graminearum	***F. oxysporum* f. sp. *lactucae***	***F. oxysporum* f. sp. *ciceri***	***F. solani***	***R. solani***
Trichoderma cerinum	Non-volatile compounds	40	40	44.81	36	42
	Volatile compounds	44	11.11	44.87	4.5	20
	VOCs extract	10	6.43	11	9.09	12.67
Trichoderma harzianum	Non-volatile compounds	70	20	40.62	25	33
	Volatile compounds	19	5.56	40.62	3.81	13
	VOCs extract	16	3.76	5.56	7.95	7.89

Table 2. Effect of extracted VOC on *Arabidopsis thaliana* Col-0.

	Root Length (mm)	Shoot Length (mm)
Control	$2.42^{a} \pm 0.02$	$3.58^{a} \pm 0.03$
Trichoderma cerinum	$2.67^{c} \pm 0.03$	$4.39^{d} \pm 0.04$
Trichoderma harzianum	$2.50^{b} \pm 0.02$	$3.67^{b} \pm 0.05$

Results are the mean of 10 replicates ± SD. In columns, values with the same letters are not significantly different (P < 0.05 Duncan test).

Figure 1. Effect of *Trichoderma* strains on fungal phytopathogens growth.
(a) Direct plate confrontation assay
(b) Compound photomicrograph of pathogen hyphae showing deformities.

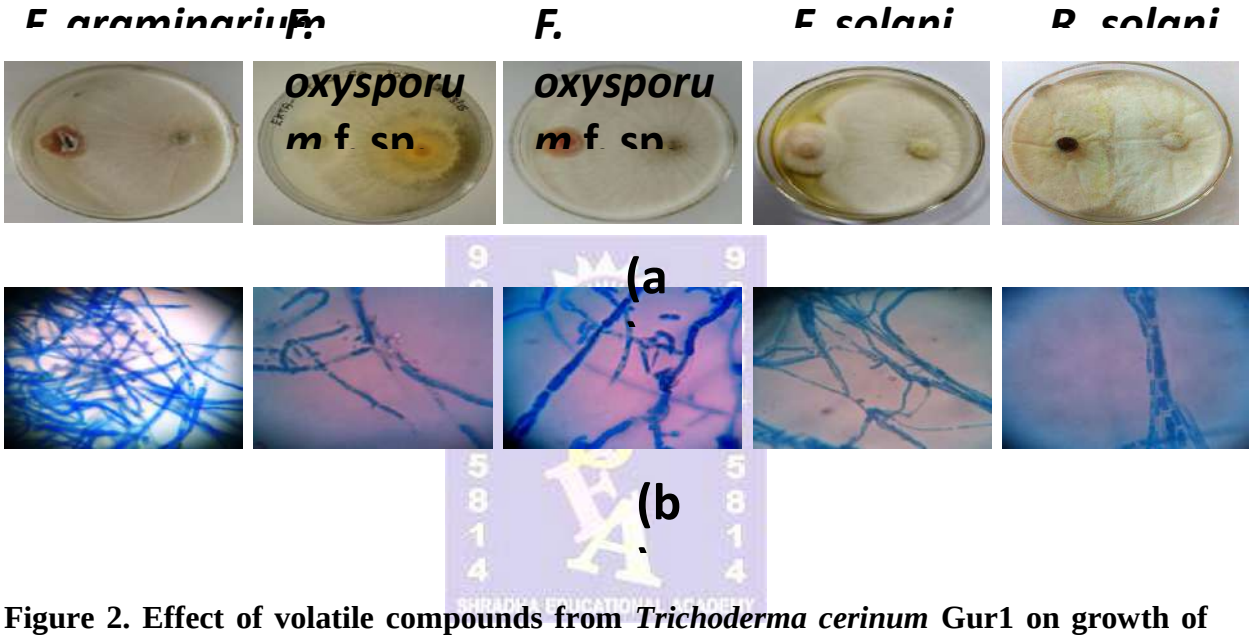


Figure 2. Effect of volatile compounds from *Trichoderma cerinum* Gur1 on growth of *Fusarium* sp. and *Rhizoctonia solani* and deformaties in pathogen hyphae.

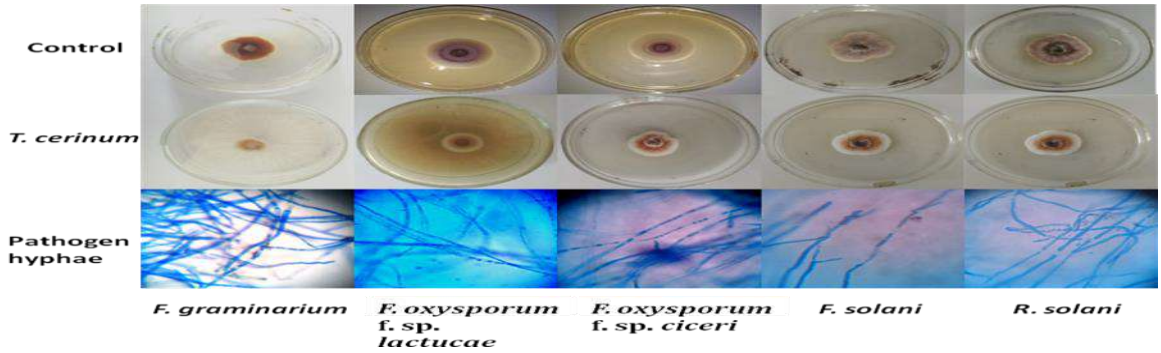
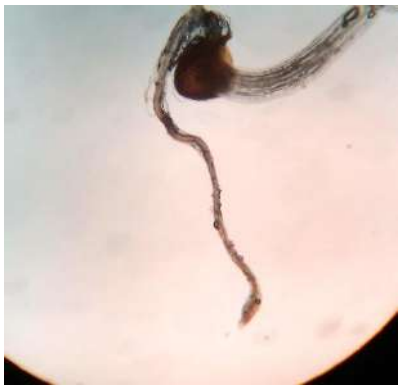
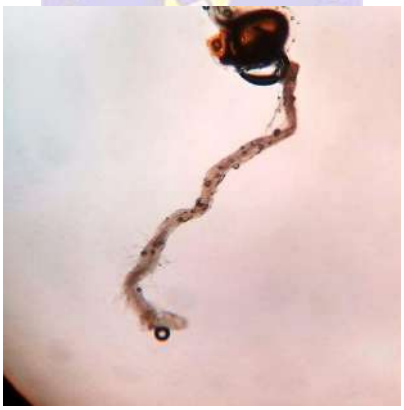


Figure 3. Effect of VOCs on root development of *Arabidopsis thaliana* Col-0.



Control

Trichoderma cerinum



Trichoderma harzianum

Figure 4. GC-MS of extracted VOCs from *Trichoderma cerinum* Gur1.