Effect of volatile organic compounds mediated fungal growth inhibition by *Trichoderma asperellum* HbGT6-07

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Abstract

The species of *Trichoderma* are one of the most frequently used natural biocontrol agents to mitigate plant diseases and improve crop yields. In this study, sixteen *Trichoderma* spp. were isolated from soil of different regions of China. However, we identified *Trichoderma. asperellum* HbGT6-07 by initial fungal growth inhibition assay and molecular approach and also evaluated the antimicrobial effects. Tested 10% concentrated culture filtrate of *T. asperellum* HbGT6-07 inhibited 93 % of colony radial growth in *Botrytis cinerea* (B05.10) as well as 91 % of *Sclerotinia sclerotiorum* (A367).

VOCs emitted from HbGT6-07 have antimicrobial properties against *Botrytis cinerea* (B05.10) and *Sclerotinia sclerotiorum* (A367). In *in-vitro* DwD method, The *T. asperellum* HbGT6-07 volatile organic compounds (VOCs) effectively reduced colonial diameter, mycelial growth rate and sclerotia production by two virulent fungal pathogens. The GC-MS analysis identified thirty-two VOCs derived from HbGT6-07 isolates. Moreover, the hyphal fragments of the *T. asperellum* HbGT6-07 demonstrated successful mycelia growth suppression of two virulent fungal agents by competing toward the invasion on oilseed rape leaves. The above findings indicated that *T. asperellum* HbGT6-07 could attain competitive progress via volatile antifungal compound production and comprehensive mycelial growth. This study provided an outlook of using *T. asperellum* HbGT6-07 to control virulent pathogens of *B. cinerea* and *S. sclerotiorum*.

Introduction

Trichoderma is one of the most intensively studied genera of hypocrealean fungi because of its enormous agricultural, industrial, and environmental applications (Blaszczyk et al. 2014; Schuster and Schmoll 2010). This anamorphic fungal genus acts as a potential biocontrol agent (BCA) against minimum 18 genera and 29 pathogenic fungi members as well as a range of bacterial microbes since *Trichoderma lignorum* was eliminated *Rhizoclonia solani* microbial growth (Weindling 1932; Wu et al. 2017). Some species of *Trichoderma* can alleviate the pathogenic fungal infection of different soil-borne plants such as *Colletotrichum* spp. on mango (de los Santos-Villalobos et al. 2013) and sugar cane (Singh et al. 2014), *Fusarium oxysporum* on tomatoes (Segarra et al. 2010), *Alternaria solani* on tomatoes (Chowdappa et al. 2013; Fontenelle et al. 2011) and chilies (Begum et al. 2010), *Sclerotinia sclerotiorum* on common beans (Geraldine et al. 2013) and peas (Jain et al. 2015), *Penicillium expansum* on apple fruits (Batta 2004) and even *Botrytis* on onions (Elad et al. 1995); strawberries (Kovach et al. 2000) and Begonia (Horst et al. 2005). In agricultural sites, biocontrol agents based on *Trichoderma* species have been commercially occupying the majority of the fungicides in recent years and preventing soil-borne pathogens worldwide.

The mechanisms of action of *Trichoderma* spp. comprise mainly aggression and mycoparasitism, accompanied by plant-resistance, antifungal metabolite production, plant growth promotion, and immunity stimulation (Benítez et al. 2004; Harman 2006; Vos et al. 2014). The biological control ability of *Trichoderma* is different between species and isolates, and the mechanism of mycoparasitism may have little association with antagonism for the same isolates (Lopes et al. 2012), thus screening of

Trichoderma isolates for biocontrol needs to consider several factors. In recent years, attempts have been devoted to discover and establish eco-friendly methods which are harmless to plant growth and human health (Schalchli et al. 2016). A significant number of research follows bio-control based strategy where different volatile organic compounds (VOCs) emitted from microorganism use to inhibit crop diseases causing microbes and thus referred as biopesticides (Glare et al. 2012). Several bioactive compounds generated by fungi which considered as antimicrobials have been reported in the literature, including isonitrile, oligosaccharides, sesquiterpenes, polyketides, hydrogen cyanide, alkylpyrones, stemids, peptaibols, lytic enzymes, diketopiperazines, and lytic enzymes (Degenkolb et al. 2008; Heydari and Pessarakli 2010; Ownley et al. 2010). However, volatile metabolites released by *Trichoderma* spp. are considered to be active biocontrol agents (R. Hung et al. 2013; Menjivar et al. 2012; Suwannarach et al. 2013).

Volatile organic compounds (VOCs) produced by the species *Trichoderma* are of significant concern for the content of antifungal effects. VOCs have low molecular mass, higher steam pressure, low polarity, low melting points and quickly evaporate lipophilic substances at 25°C (room temperature) (Schulz-Bohm et al. 2017). Moreover, VOCs are chemically diverse and include aromatics, lactones, amines, alcohols, ketones, thiols, esters, cyclohexenes, terpenes, mono- and sesquiterpenes (Korpi et al. 2009; Schenkel et al. 2015). More than 300 distinct VOCs have been reported in the fungi, mostly known to be developed by different species of *Trichoderma* (Rahnama 2016; Siddiquee et al. 2012). Although VOCs are a tiny part of the overall compounds formed by *Trichoderma* spp. their unique characteristics enable antibiotic action against fungal pathogens (Lee et al. 2016; Nieto-Jacobo et al. 2017).

Soil VOCs are potential indicators of microbial community structure and community shifts (McNeal and Herbert 2009). However, many studies have shown that several plant and fungal VOCs have potent physiological effects where they act in signalling, communication, antagonism and inter- and intra-specific association. In recent years, more attention has been paid to VOC-mediated impacts, their ecological and biological importance and their effect in the growth of soil ecosystems (Bitas et al. 2013; D. T. Hung et al. 2015; Peñuelas et al. 2014). VOCs vary enormously in structure and composition where a single compound can influence multiple aspects of the growth and development of an organism. For instance, dimethyl disulfide, developed by plants and microbes, has numerous roles as an insect attractant, plant systemic resistance elicitor, and pathogenic fungus suppressor (Crespo et al. 2012; Kai et al. 2007). Microbial VOCs mixtures play a role in the development and control of symbiotic associations and the distribution of saprophytic, mycorrhizal and pathogenic species in the soil (Müller et al. 2013; Rigamonte et al. 2010).

The purpose of the study was to identify potential bioactive isolates of *Trichoderma* and to explain that VOCs have become a significant factor in the advancement of growing plants that could be applied directly as biocontrol agents. Finally, we used gas chromatography-mass spectrometry (GC – MS) analysis to classify the isolate-generated volatile status to evaluate the metabolites that were essential for the antifungal impacts.

Materials And Methods

Fungal isolates and culture conditions

Trichoderma isolates were isolated from agriculture soil in different locations of China (Supplementary Table S1). Soil specimens were put in clean containers, delivered to the laboratory and held at 4 °C until they were used. The sample were prepared 10^{-4} serial dilutions in sterilised distilled water (SDW) and 500 µL sample (diluted) was spread on the potato dextrose agar (PDA) media plates and incubated at 20 ± 2 °C for 72 h. The cultivation plates were checked frequently, and each visualised colony was known to be one colony-forming unit (CFU). Various fungal colonies were sub-cultured to PDA plates following the counting of CFU. For this analysis, *B. cinerea* strains B05.10 and one isolate (A367) of *S. sclerotiorum* were taken. Originally isolated B05.10 and A367 was obtained from grapes (Büttner et al. 1994), and eggplant (Magioli and Mansur 2005). The experimental cultures of the isolates were developed by shifting the mycelia to PDA plates, and incubated at 20 °C between 5-10 days under the 12-h dark-light regime.

Assay of inhibition of *B. cinerea* and *S. sclerotiorum* growth by *Trichoderma* spp. through dual culture method

For dual cultures, isolates of *Trichoderma* were screened for inhibition against *B. cinerea* B05.10 and *S. sclerotiorum* A367. The microbes and *Trichoderma* were cultivated for 5-6 days at a temperature of 20 ± 2 °C on PDA plates. Mycelium agar plug (MAPs, 5 mm in diameter) of the target necrotrophic fungus (*B. cinerea* and *S. sclerotium*) collected from the periphery and inserted onto new PDA dishes. After 2 days incubation of *Trichoderma* spp. the fungal discs were moved aseptically in the center of the target fungi plate and were kept at 25 °C with intermittent light-dark conditions for 10 days and monitored frequently. Triplicates were used in each study, and after 10 days of constant growth of *B. cinerea* and *S. sclerotium* colonies, the degree of the invasion was estimated and the control (pure cultures of *B. cinerea* and *S. sclerotium*) was compared. The fungal growth inhibition zone formula was estimated by $I = (C-T)/C \times 100$, where C is the growth of mycelium in the control plate, T is the test species growth of mycelium in the inserted plate, and I is the mycelial growth inhibition (Wonglom et al. 2019). Each experiment was done three times, with three replications each time. Finally, the plates where *Trichoderma* colony completely covered by *B. cinerea* or *S. sclerotium* colony surface was selected as a super isolate.

Biological characterization of isolates HbGT6-07

According to the protocol of Samuels et al. 2002, the phenotypic and cultural properties of *Trichoderma asperellum* HbGT6-07 isolates were analyzed in numerous media viz. PDA, SDA, CDA, CPA, MM, MMN, MYA, YMEA, YPG, and YSS (Samuels et al. 2002). Mycelial discs of developing isolates HbGT6-07 were inoculated at the edge of the Petri plates that included earlier in this section-mentioned media and incubated at 25 ± 2 °C for a week. Colony radius was calculated at 24, 48, and 72 h intervals. The test was replicated three times, and the tests for each isolate were averaged. External characteristics including the presence of pigments, green conidia, odor and colony appearance are also noted. Morphological

findings from mycelial growth on PDA plates have been reported. Thus every feature was calculated in 3% KOH for each isolate from the water after preliminary soaking.

Genomic DNA extraction, PCR amplification and phylogenetic tree analysis

Isolates HbGT6-07 with the strongest inhibition against *B. cinerea* and *S. sclerotiorum* growth was further identified through the analysis of its 5.8S rDNA sequence. Genomic DNA were extracted by cetyltrimethylammonium bromide (CTAB) method (M Kamaruzzaman et al. 2018) and DNA was dissolved in 50 µl TE buffer fluid to create DNA suspension and quantified using fluorescence of ethidium bromide (Raeder and Broda 1985). Then PCR amplification was done by using the universal primers ITS1 (3'-TCCGTAGGTGAACCTGCGG-5') and ITS4 (3'-TCCTCCGCTTATTGATATGC-5'). A total volume of 25 µl of reaction mixture was used for PCR amplification. Every reaction comprises 0.2X PCR buffer, 0.16 mM MgCl₂, 0.01 µM ITS1 (forward primer), 0.01 µM (reverse primer), 0.144 mM dNTP, 0.5 mU / µl Taq polymerase, 2.00 µl template DNA and 14.95 µl PCR water, respectively. The initial denaturation of these reactions was 90s at 95 °C, followed by 30 cycles of 1 min at 95 °C, 30s at 55 °C and 1.5 min at 72 °C, with a final extension of 10 min at 72 °C and a final hold of 4 °C. The PCR band were visualized using a 1% agarose gel. PCR products were cloned in *E. coli* DH5α with vector pMD18-T (You et al. 2016) and sequenced by Wuhan Tianyi Huiyuan Biological Technology Co., Ltd, Hubei, China. The obtained sequence was submitted to GeneBank to get the accession number. Multiple sequence alignments and comparisons with reference strain for each of the genes were performed through the aid of CLUSTALW and Neighbour-joining method was used to constructed phylogenetic tree topologies by performing bootstrap values of 1000 data sets using MEGA7.0 (Molecular Evolutionary Genetic Analysis) tools. The corresponding sequence accession numbers are listed in Supplementary Table S2 for constructing phylogenetic tree analyses. The sequence was deposited to GeneBank under the mentioned accession number: MH280010.

Preparation of the Culture Filtrate (CF) of *Trichoderma asperellum* HbGT6-07

Two blocks of a 7-day-old HbGT6-07 mycelia agar plug (5 mm diameter) was inoculated in a 250 mL conical flasks containing 150 mL of sterilized potato dextrose broth (PDB) and cultured in an electrical shaker for 7 days at 150 rpm and 22 °C. Then, the fermented outcome was centrifuged at room temperature at 10,000 rpm for 10 min to remove the mycelium debris. Then, the obtained supernatant was collected after the pass through a 0.22 µm membrane filter (Millipore Sigma, USA).

The HbGT6-07 CF influenced the growth and morphology of *B. cinerea* and *S. sclerotiorum*

B. cinerea and *S. sclerotiorum* were separately inoculated on PDA and cultured at 20 °C. Purified CF of HbGT6-07 were used in PDA plate to evaluate the effects on the mycelial radial growth of *B. cinerea* *S. sclerotiorum*. In the experiment, the select concentrations of the CF (10%, 6%, 2%, and 1% (V/V)) were mixed with PDA, while sterile dd water was mixed with PDA used as a control (0%). treatment. One three-days-old mycelial agar plug (5 mm in diameter) of *B. cinerea* was put in the middle of each petri dish and incubated at 20 C. Similarly, *S. sclerotiorum* mycelial agar plug as used. Each treatment was done with

three replications. Five days after incubation, the colony diameter of each dish was calculated in two reverse directions, and the growth inhibition percentage (% GI) of HbGT6-07 was calculated using the formula $\% \text{ GI} = [(\text{mean of colony diameter in control} - \text{mean of colony diameter in treatment}) / \text{mean of colony diameter in control}] \times 100$ (Hao et al. 2020). This experiment was repeated three times.

The application of mycelial hyphal fragments to inhibit the necrotic diseases lesion

Rapeseed oil plants seeds were planted in plastic pots with organic culture mix including 2% -5% N + P_2O_5 + K_2O (N:P₂O₅:K₂O = 1:1:1, w:w:w:w). The pots were placed in a chamber for plant growth where water was needed. The plants were softened to one seedling per pot at the multiple-true-leaf level (45-d old). Isolates HbGT6-07, B05.10 and A367 were individually cultured at 20 °C below 12 h light-dark intervals on CF-PDA for 5 d, and the subsequent mycelial volumes of every isolates were obtained and mixed in PDB to create hyphal fragments (HF) filtrates at a density of roughly 3×10^6 HF per mL (Supplementary Fig. S1). HbGT6-07 HF mixture was combined with B05.10 HF suspension comprising hyphal segments (HFB05.10) at density proportions 6.6:3.3, 5:5 and 3.3:6.6, or coupled with A367 HF mixture also containing hyphal segments (HFA367) at the similar size ratio.

Every one of such HF mixtures were considered as inoculum in double isolates. They were lifted on the upper portion of the 5mm diameter size of filter paper discs (FPD) positioned on rapeseed leaves to assist strengthen the inoculum, 25 μL culture on each FPD, single FPD around each leaf, and five plants per inoculum (a total of 12 to 15 leaves per inoculum). $\text{HFS}^{\text{HbGT6-07}}$ acts as negative control, $\text{HF}^{\text{B05.10}}$ or HF^{A367} alone used as a positive control. Most of the plants were kept in a humid cabinet at 20 °C for three days in 12-h light-dark conditions. The leaf lesion diameter was estimated on the growth of leaves around each FPD. The following formula was used to calculate the biocontrol efficacy by the treatment of HbGT6-07 ($\text{BE}^{\text{HbGT6-07 HF}_s}$) hyphal fragments:

$$\text{BE}^{\text{HbGT6-07 HF}_s} = \{(\text{AD}^{\text{Positive control}} - \text{AD}^{\text{Treatment HF}_s}) / \text{AD}^{\text{Positive control}}\} \times 100\%$$

[Where $\text{AD}^{\text{Positive control}}$ is the average length of the leaf lesions in the treatment with $\text{HF}^{\text{B05.10}}$ alone or HF^{A367} by itself as an inoculum, while $\text{AD}^{\text{Treatment HF}_s}$ is the average length of the leaf lesions in the $\text{HF}^{\text{HbGT6-07}}$ treatment with $\text{HF}^{\text{B05.10}}$ or A367 treatment at the stated HF ratio]. Repeated the test three more times.

Effect of mixed culture on sclerotia production

Sterilized carrots were used as a carrier for sclerotia reduction experiment. Briefly, about 75 g carrots were cut into pieces (1.5-2.5 cm in size) and sterilized at 121°C for 30 min in a 250 mL conical flask. Five actively growing MAPs of B05.10 or A367 were placed inside the conical flask used as control. The mycelial mixtures (5 MAPs of HbGT6-07 + 5 MAPs B05.10) or (5 MAPs of HbGT6-07 + 5 MAPs of A367) were used in two separate treatments. Six conical flasks of each treatment were inoculated at 20°C for 15

d. The average number of sclerotia and weight per flask were calculated. This experiment was repeated twice.

Antifungal volatiles production by *Trichoderma asperellum* HbGT6-07

In order to check the antifungal activity of the *T. asperellum* HbGT6-07 volatiles, Dish-within-Dish (DwD) sets method was performed where includes composed of one pair of the dish, a small inside dish (6 cm in diameter) and a big outside dish (16 cm in diameter). For this study, *B. cinerea* isolate B05.10 was selected as a fungal target to evaluate the antimicrobial efficacy of the volatiles generated by HbGT6-07. There were three DwD sets for the three treatments. AWG in a 250 mL Erlenmeyer flask containing 100 g AWG was inoculated with four MAPs of HbGT6-07 or B05.10 or A367 (20°C). In the case of B05.10, blank AWG (50 g) alone in the internal dish and B05.10 on AWG (50 g) in the external dish are defined as the first DwD set and considered as negative regulation. In the case of B05.10, blank AWG (50 g) individually in the inner dish and B05.10 on AWG (50 g) in the outer dish are known as the first DwD set and considered as a negative control. The third DwD set was B05.10 in both plates, it was known as B05.10/B05.10, that were chosen to remove the effect of HbGT6-07 O₂ consumption and/or CO₂ development on the radial formation of B05.10 in the outer plates in the inside plates. The three DwD groups were first developed by examining the inner dishes, where the B05.10 was left empty, inoculated with HbGT6-07 (one MAP each plate), or seeded with B05.10 (one MAP for each plate), and put in an incubator at 20 °C below 12 h light-dark intervals for 20 d. Next, B05.10, one MAP for every dish at a length of 5.5 cm from the internal dish was seeded on the external dishes (Supplementary Fig. S2). Specific techniques have been employed to establish the DwD sets against *S. sclerotiorum* A367 to recognise antimicrobial behaviour of HbGT6-07 volatile components. The resulting filtrates were incubated at 20 °C under 12 h light-dark conditions for 5 d and used as the volatile origin in the subsequent DiD sets (inner dish/outer dish): fresh AWG / A367 (a negative control), HbGT6-07/A367 AWG culture, and A367 AWG culture (another negative control). Simultaneously with the loading of the fresh AWG, the AWG culture of HbGT6-07 and the AWG culture of A367 in the internal dishes, A367 MAPs from a 3-day PDA culture (20 °C) were incubated in the external dishes. The DwD sets were covered to parafilm and incubated for 20 d (20 °C). The DwD sets were covered separately with parafilms (Parafilm M, Chicago, USA) and held at 20 °C for the next 20 d. Diameters of the B05.10 or A367 colony were calculated in each inner dish. Meanwhile, the regular growth rate, colony length, and sclerotia generated in the B05.10 or A367 colony were recorded and measured in each outer platter. The procedure was replicated once in each repetition, with three replicates per test.

Gas chromatography/Mass spectrometry (GC-MS) analyses

Using the GC-MS experiment, the chemical elements of the HbGT6-07 VOCs have been quantified. *T. asperellum* HbGT6-07 was cultivated in a 250 ml Erlenmeyer sterile flask including 100 mL PDA with 6 pcs MAPs. The mycelial cultures were placed in a 25 °C incubator for 7 days. An initial experiment revealed that the emission of VOCs has achieved the plateau under favorable environments in 7 days. The HbGT6-07 VOCs were obtained in the flask for 20 min at 40 °C via solid-phase micro-extraction

(SPME) fiber assemblies (Superco, PA, USA) (Md Kamaruzzaman et al. 2020). The fiber (2 cm, 50/30 μ m divinylbenzene-DVD) was placed straightly into the TRACE™ GC Ultra (TRACE-DSQ II) (Thermo Electron Corporation, USA) GC intel splitless mode. The desorption time was 5 min and the deported compounds were segregated on a DB-5 MS capillary column (30 m $\times$ 0.25 μ m $\times$ 0.25 mm) through the following operating program. Initially, the oven temperature was kept at 50 °C for 3 min. The column temperatures were slowly raised at 10 °C/min from 50 °C to 180 °C and increased to 240 °C at 4 °C/min, then retained for 5 min. Total running time was 30 min. Helium gas (99.99%) as used as a carrier with a flow rate of 1.0 mL/min. The ionising power was set at 70 eV with an acquiring range of 50 to 800 m/z and 1 scan/s scan rate. The temperature of the ion source was 230 °C, and the transfer axis was adjusted at 280 °C. Data acquisition and processing were performed with Thermo Scientific Mass Frontier software system. Based on the comparison of their comparable retention time and their mass spectra, the chemical components were classified with those in the NIST07 database (National Institute of Standard and Technology). Individual peak compositions as a relative percentage of total peak area were recorded. In the meantime, it also obtained and classified the VOCs released from non-inoculated sterilised AWG. Finally, the VOCs appearing in the SPME extract from the non-inoculated PDA were removed during computation.

Statistical analysis

Data analysis was conducted in the SAS program (SAS ver. 8.0, NC, USA) using the ANOVA technique. Data from different treatments were measured using the $P = 0.01$ or 0.05 standard multiple-range test by Duncan. Data linked to independent control with the Student's t-test ($P < 0.01$ or 0.05).

Results

Isolation and screening of isolates HbGT6-07

A total of 16 *Trichoderma* isolates were isolated from various agricultural field in a different province of China. The initial screening for effective *Trichoderma* isolates revealed the percentage of inhibition range at 3 DAI 69.52–81.43% and at 6 DAI for *B. cinerea* B05.10 (Supplementary Fig. S3 (A), Table S3). On the other hand, the percentage of inhibition range at 3 DAI 69.52–81.43% and at 6 DAI for *S. sclerotiorum* A367 (Supplementary Fig. S3 (B), Table S3). Among them, *T. asperellum* HbGT6-07 potentially reduced the growth of *B. cinerea* B05.10 and *S. sclerotiorum* A367, respectively. Besides the inhibition of fungal growth, *T. asperellum* HbGT6-07 suppressed the conidial production of *B. cinerea* B05.10 and *S. sclerotiorum* A367 in the dual culture assay plates.

Colony morphology and molecular identification of isolates HbGT6-07

Morphological investigations revealed that the front of the novel isolate HbGT6-07 colony after five days of culture was dark green (colour of spore), and the tail was white (colour of mycelium). The mycelial growth phenotype was rough, and bright green spores started developing at significant levels in the middle of the colonies. Evaluation of the growth and sporulation performance of isolate HbGT6-07 was

done at ten different solid media. This study showed that PDA, CPA and SDA is the best mycelial growth media to isolate HbGT6-07 (Fig. S4). MM agar media showed minimum growth against the HbGT6-07 isolates. Also, to find out the optimum as well as the best temperature for the growth of the pathogen, the isolate HbGT6-07 was grown at different temperatures on potato dextrose agar medium. After 10 DAI, the average mycelial growth, colony diameter, and the number of spores per dish were recorded. From the analysis presented in Supplementary Fig. S5, it can be concluded that the growth of the fungi was better at the temperature range of 20°C, 25°C and 30°C. Maximum average dry weight was observed at 20-30°C.

Sequencing reactions performed with ITS1/ITS4 primer pairs which amplified the fragments of -600 bp (Supplementary Fig. S6). Following an evolutionary analysis of the ITS sequences, NCBI BLAST showed that this new strain exhibits the maximum similarity with *T. asperellum* T-17 (KC884774) followed by *T. hamatum* (KC884761), *T. koningiopsis* (KC884790), *T. atroviride* (KC884770), *T. hatzianum* (KC884786), and *T. saturnisporum* (KC884818), with *Protocrea pallida* (NR_111329) used as an outgroup (Fig. 1). We identified the strain to be *T. asperellum* along with the morphological characteristic and called it HbGT6-07.

The HbGT6-07 CF Influenced the Growth of *B. cinerea* and *S. sclerotiorum*

B. cinerea and *S. sclerotiorum* isolates were cultured on potato dextrose agar (PDA) medleyed with CF of HbGT6-07, and PDA plus dd H₂O was used as the control. In case of *B. cinerea*, the radial colony size on PDA that amended 1% (V/V) CF were significantly lesser as compared with colonies grown on control treatment. The inhibition percentages of HbGT6-07 CF to *B. cinerea* significantly ($P < 0.01$) increased with an increase in concentration of HbGT6-07 CF (Figure 2, A). Among of the applied concentrations (10%, 6%, 2%, and 1% (V/V) of CF, CF at a concentration of 10% showed 93% growth inhibition to *B. cinerea*, and an inhibition rate of 29% was recorded when treated with culture filtrate at a concentration of 1%. On the other hand, the % GI of HbGT6-07 CF to *S. sclerotiorum* significantly ($P < 0.01$) increased with an increase in concentration of HbGT6-07 CF (Figure 2, B). Concentration of 10% showed 91% growth inhibition to *S. sclerotiorum*, and an inhibition rate of 17% was found when treated with CF at a concentration of 1%.

Efficacy of the VOCs of HbGT6-07 in suppression of *B. cinerea* and *S. sclerotiorum*

The dish within dish method was used to detect the antifungal activity of HbGT6-07 against *B. cinerea* and *S. sclerotiorum* through the production of antifungal volatiles. In the double dish sets of blank AWG/B05.10 and B05/B05 (inner/outer dishes), isolate B05.10 in the external dishes grew and produce sclerotia on PDA with average colony diameters bigger than 14.5 cm (Fig. 3 A, B). Notably, mycelial growth reduction was observed during the treatments. Initially, all outside B05.10 culture grew normally (average 12 mm) up to 4 days but at 5th day HbGT6-07/B05.10 treatment reduced growth rate about 1 mm while AWG and B05.10/B05.10 treatment growth rate 10 and 8 mm/day, respectively (Fig. 3 C). B05.10 colonies sclerotia were obtained in the with average sclerotia yields up to 33 and 28 sclerotia per dish, respectively in blank AWG/B05 and B05/B05. In comparison, in the DwD sets of HbGT6-07/B05.10,

isolate B05.10 grew in the outer plates, but developed limited colonies with an average colony size of 4.1 cm without apparent sporulation or sclerotia (Fig. 3 D).

The DwD sets of HbGT6-07/A367 demonstrated a related antifungal impact of volatiles from the AWG cultures of HbGT6-07 on mycelial growth and sclerotial development by *S. sclerotiorum* A367. In the DwD sets of blank AWG/A367 and A367/A367, isolate A367 in the outer plates developed rapidly, colonised the whole outer plates at 20 °C for 15 d incubation, and generated sclerotia with yield potential up to 53 and 42 sclerotia per dish. Besides, in the HbGT6-07/A367 DwD sets, isolate A367 in the outer dishes developed gradually, forming modest colonies with an average size of 7.6 cm after 20 °C incubation (Fig. 4 A-D).

Biocontrol of disease suppression by *T. asperellum* HbGT6-07

The findings of the antimicrobial analysis on detached rapeseed leaves indicated that HbGT6-07 hyphal mixtures were successful in suppressing lesion extension responsible by *B. cinerea* and *S. sclerotiorum* (Fig. 5). In the negative control, the hyphal mixtures of HbGT6-07 alone as inoculum, no noticeable exposure or mild infection with development of small leaf lesions (< 0.5 mm in size) were recorded on the leaves at 3 d post-inoculation (dpi) under 20°C. Furthermore, the serious outbreak was reported on the leaf tissue in the positive control with hyphal components of B05.10 alone as inoculum, and broad necrotic leaf lesions were developed with an average lesion size of up to 23 mm. The hyphal fragments of HbGT6-07 and B05.10 isolates with three biocontrol treatments at the ratios of 33.33:66.67, 50:50 and 66.67:33.33 (HbGT6-07: B05.10) as inoculum, the diameter of leaf lesion were reduced by 43%, 65% and 97%, respectively, associated with the treatment of positive control (Fig. 5 left, Supplementary Fig. S7 A).

Equal inhibitory action on rape seed leaves was detected from available hyphal fragments of HbGT6-07 toward invasion with *S. sclerotiorum* A367. While the positive treatment group with the available hyphal mixtures of A367 alone as inoculum responsible for more prominent leaf lesions with 27 mm of average lesion diameter at 3 dpi (20°C), the biological control action with viable hyphal fragments of HbGT6-07 and A367 at the ratios of 33.33:66.67, 50:50 and 66.67:33.33 (HbGT6-07:A367) as inoculum caused small leaf lesions with average lesion diameters of 16, 9 and 0.6 mm, respectively. The effectiveness of biocontrol strategy of these three treatments was as high as 40%, 66% and 97% respectively, compared to positive control treatment (Fig. 5 right, Supplementary Fig. S7 B).

Reduction of sclerotia formation under mixed culture condition

The carrot blocks were the perfect substrates for sclerotia production by *B. cinerea* and *S. sclerotiorum*. In the mixed culture condition, the number of sclerotia production and the weight of sclerotia were significantly reduced in the co-cultured by the action of *Trichoderma* isolates HbGT6-07 (Fig. 6 A). Results from this mixed culture assay exhibited that the isolate HbGT6-07 was significantly ($P < 0.01$) reduced the sclerotia production turned into zero as compared to control for B05.10 and A367, respectively (Fig. 6 B). Moreover, the dry weight of sclerotia data also revealed that isolates HbGT6-07 caused complete inhibition of sclerotia production (Fig. 6 C).

GC/MS profiling of *T. asperellum* HbGT6-07 VOCs

The mass spectra structural information of the VOCs was evaluated via the data in the NIST Mass Spectral Search Program (version 2.2). Results of GC-MS analysis identified 32 compounds in the *T. asperellum* HbGT6-07. The molecular weight (MW), name of the compound (NoC), chemical formula (CF), retention time (RT) and relative peak area (RPA) were given in Table 1 and illustrated in Fig. 7. These compounds were into classes of alkane (R-H), alcohols (R-OH), aldehydes (R-CHO), alkene (R=), amines (R-NH₂), benzene (R-C₆H₆), and ketone (R-CO) (Fig. 8). 2-Ethylhexanal (C₈H₁₆O) seemed to be the enormous compound with 18.8% relative peak area (RPA), accompanied by Octan-3-one (C₈H₁₆O) with the RPA value of 11.2 % and Octan-2-one (C₈H₁₆O) with the RPA value of 5.6%. The other 18 molecules, such as 1-octen-3-ol, were less common, with the RPA levels between 0.9% to 4.2%.

Discussion

One of the essential concern of food production is plant disease management. Different combined schemes can be used to breed excellent disease-resistant cultivars, use crop rotation to prevent heavy building up pathogens, change seeds from diseased to disease-free, flexible planting dates, maintain proper moisture in the field, and use pesticides to control plant pathogens. However, the most common method for controlling diseases is the use of pesticides. While a large variety of pests and insects can be targeted by synthetic pesticides, they can cause significant harm to our climate. Overuse of pesticides can seriously affect public health. Thus, use of pesticides to monitor diseases and target precision becomes a major concern.

Some species of the genus *Trichoderma* are deliberated as potential biological control agents (BCAs), and the modes of action include mycoparasitism, antibiosis, competition, enzyme activity and induced plant defense and active VOCs production (Sood et al. 2020). VOCs derived from microbes can induce the genes responsible for plant defense mechanism and prevent the infections/diseases caused by pathogens (Cordovez et al. 2017). *Trichoderma* has considerable activity against many pathogenic fungi, e.g. *Fusarium* a wide range of environmental conditions (Zhang et al. 2014). Moreover, they have been studied extensively for their beneficial role as biofertilizers and in pest management (Gupta et al. 2014). In recent years, role of VOCs as natural BCAs have been studied extensively for many reasons (Conboy et al. 2020; Kaddes et al. 2019; Tahir et al. 2017). Firstly, they offer cost effective methods to control pest by the farmers. Secondly, they reduce the use of chemicals in the agriculture fields. Third, synergistic role of multiple VOCs can target a wide range of pathogens. Fourth, these volatile chemicals promote plant growth and offer high yield. Many microbial VOCs with plant antimicrobial activity (AMA) have been identified in previous studies (Schulz-Bohm et al. 2017; Tilocca et al. 2020). They promote plant growth by protecting from pathogen attack through cost effective and environmentally friendly approach. Although a high number of *Trichoderma* genera and strains are known to date, the VOC profiles of only a minimal number of fungi have been studied so far (Guo et al. 2019; Quintana-Rodriguez et al. 2018).

Volatile compounds produced by organisms called *Trichoderma* have interactions between plants and microorganisms. These spontaneously produced VOCs boost up plant biomass and compete with the growth of infectious pathogens. All microbial VOCs work synergistically as a complex mixture where environmental condition such as nutrient content, composition, humidity, temperature etc. influence the production and mechanism of action (Tilocca et al. 2020). In this study, in order to determine the most successful isolate against *B. cinerea* B05.10 and *S. sclerotiorum* A367, we isolated sixteen *Trichoderma* strains collected from rhizosphere soil. Isolate HbGT6-07 significantly reduced the radial growth of the microbes and was capable of entirely overgrowing mycelia of plant microbes. ITS rDNA region was amplified with specific primers ITS1 and ITS4. The primers provided amplified products with a size of ~600 bp. This finding is in accordance with many researchers who effectively achieved a ~600 bp segment in *Trichoderma* after amplification of the 5.8-rDNA region (Castrillo et al. 2016; Chakraborty et al. 2010). The results acquired from BLAST query sites allowed us to identify with at least 99% homology the various species-level isolates. The result generated from the evolutionary analysis is compatible with earlier inquiries concerning the topology of *Trichoderma* phylogeny (Filizola et al. 2019).

As potential biocontrol agents that can reduce the effects of multiple plant diseases, VOCs have recently been proposed (Baiyee et al. 2019; Wonglom et al. 2019) (Blom et al. 2011; Cortes Barco et al. 2010). The volatile antifungal molecules found in a conidial suspension from *Trichoderma* isolate HbGT6-07 are groups of the following chemical components, alcohols, aldehyde, ketone, alkane, alkene, amines, benzene. 2-Ethylhexanal (C₈H₁₆O) from the aldehyde group were contribute the highest antifungal activity (4.89%) (Wonglom et al. 2020). Octan-2-one and Octan-3-one are in a ketone displayed in the VOCs by about 3.05% (Table 1), having different antimicrobial potential such as antifungal efficacy (Fernando et al. 2005). Though fatty acids are less efficient than certain substances and chemical fungicides, antimicrobial activity has been reported (Pohl et al. 2011). In agriculture, microbial VOCs were used to fumigate foodstuffs and regulate microorganisms throughout plants. Nonetheless, single VOCs have declined to have an adverse impact in several of these experiments, while blends have been successful in mediating stimulation of plant growth and development (R. Hung et al. 2015; Naznin et al. 2013).

VOCs from microbial species have shown to be able to induce protective reactions against microbial infection and to cause systemic resistance (Naznin et al. 2014). For example, compounds such as 6-*amyl*- α -pyrone, 1-octen-3-ol, methyl benzoate and *m*-cresol induce systemic pathogen tolerance by disrupting the signalling pathways for salicylic and jasmonic acid (Naznin et al. 2013; Vinale et al. 2008). Consequently, limited information is available about plant genes in acting to VOCs released by pathogens (Naznin et al. 2013; Vinale et al. 2008). Earlier research suggested that plants revealed to the volatile stage of limonene, 3-methylbutanal and undecane induced substantial effects on plant diameter and chlorophyll contents (R. Hung et al. 2015) and we found that our isolate released 3-Methylbutan-1-ol, 2-Methylbutanol, limonene, camphor, β -cedrene and α -bergamotene, called as natural volatiles microbial (Fiedler et al. 2001; Jeleń et al. 2014). Although these molecules have been reported to be ubiquitous, however, they are not expected to be the factor of our obtained growth stimulation. Though low concentration of 2-Ethylhexanal promotes the growth of *Arabidopsis*, high concentration lessens plant

growth (Blom et al. 2011; Splivallo et al. 2007). Our studied isolate HbGT6-07 also produced 2-Ethylhexanal.

In this study, we found that the *Trichoderma* isolate HbGT6-07 effectively suppressed sporulation of *B. cinerea* B05.10 and *S. sclerotiorum* A367 on leaves of rapeseed. Recent evidence suggested that *C. rosea* simultaneously suppresses the *B. cinerea* sporulation via mycoparasitism (Moraga-Suazo et al. 2011). Throughout this study, these two approaches can appear in the inhibition of *B. cinerea* B05.10, and *S. sclerotiorum* A367 sporulation by *Trichoderma* HbGT6-07 isolates, as it can actively penetrate the *B. cinerea* B05.10 and *S. sclerotiorum* A367 colonies in dual culture methods. *Trichoderma* isolates HbGT6-07 substantially improved the rapeseed vigour index as linked to the control group. Also, they demonstrated both growth-enhancing and immunity-inducing impact on rape seedlings. The experimental data of this study revealed that the targeted *Trichoderma* isolate HbGT6-07 can enhance the production of adjacent plants by releasing several plant VOCs which boost the resistance response (Blande et al. 2014). However, according to our results, we are suggesting in details research on natural biocontrol agent like *Trichoderma* to turn on plant defense system to avoid the action of pathogens.

Conclusion

Trichoderma was identified as a globally recognized biocontrol fungus due to its effective and broad-spectrum of antimicrobial actions. This study provides empirical evidence that VOCs released by *Trichoderma* isolates HbGT6-07 have an inhibitory effect and may be used as an effective alternative option of synthetic fungicides to prevent various fungal pathogens growth. Besides, *Trichoderma* isolate HbGT6-07 cultural filtrates are known to become the most useful and efficient agents for regulating a broad range of microorganisms like *B. cinerea* and *S. Sclerotiorum*. Finally, the upsurge in VOCs from *Trichoderma* isolate HbGT6-07 may be attributed to changes in gene expression so that the VOCs effect assessment would be the focus of further study *in vivo* assays and genetic analysis.

Declarations

Supplementary Materials The Supplementary Material for this article can be found online.

Author contributions Md. Kamaruzzaman conceived and designed the experiments. Md. Kamaruzzaman and Md. Samiul Islam performed the experiments. Md. Kamaruzzaman and Md. Samiul Islam analyzed the data. Md. Kamaruzzaman and Md. Samiul Islam, Shakil Ahmed Polash and Razia Sultana contributed formal analysis. Md. Kamaruzzaman and Md. Samiul Islam, and Shakil Ahmed Polash wrote the manuscript with input from all co-authors. All authors read and approved the final manuscript.

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Table

Table 1. HS-SPME/gas chromatography-mass spectrometry (GC-MS) profile of volatile organic compounds (VOCs) produced by *T. asperellum* HbGT6-07

SL	RT	Compound Name and molecular formula (MF)	Molecular weight (MW)	Peak Area (%)	CAS No.
1	6.67	Ethanol (C ₂ H ₆ O)	46.07	1.3	64-17-5
2	7.53	Propan-2-one (C ₃ H ₈ O)	60.10	1.5	67-64-1
3	8.59	Butane-2,3-dione (C ₄ H ₈ O)	86.08	1.1	431-03-8
4	9.23	Acetic acid (C ₂ H ₄ O ₂)	60.05	1.6	64-19-7
5	10.26	3-Methylbutanol (C ₅ H ₁₀ O)	86.13	1.8	590-86-3
6	11.17	2-Methylbutanol (C ₅ H ₁₀ O)	86.13	2.1	96-17-3
7	12.30	3-Methylbutan-1-ol (C ₅ H ₁₂ O)	88.14	0.9	123-51-3
8	12.72	2-Methylpropanoic acid (C ₄ H ₈ O ₂)	88.10	1.6	79-31-2
9	13.33	Butanoic acid (C ₄ H ₈ O ₂)	88.10	2.4	107-92-6
10	13.99	2-Ethylhexanal (C ₈ H ₁₆ O)	128.21	18.8	123-05-7
11	14.63	3-Methylbutanoic acid (C ₅ H ₁₀ O ₂)	102.13	4.2	503-74-2
12	15.26	Ethylbenzene (C ₈ H ₁₀)	106.16	1.5	100-41-4
13	15.44	Xylene (C ₈ H ₁₀)	106.16	2.4	1330-20-7
14	16.08	Heptan-2-one (C ₇ H ₁₄ O)	114.18	1.6	110-43-0
15	16.52	Styrene (C ₈ H ₈)	104.14	1.5	100-42-5
16	16.72	Heptanal (C ₇ H ₁₄ O)	114.18	1.9	111-71-7
17	17.62	Octan-2-one (C ₈ H ₁₆ O)	128.21	5.6	111-13-7
18	18.14	Octan-3-one (C ₈ H ₁₆ O)	128.21	11.2	106-68-3
19	18.72	Octanal (C ₈ H ₁₆ O)	128.21	1.1	124-13-0
20	19.00	2-Ethylhexan-1-ol (C ₈ H ₁₈ O)	130.22	2.8	104-76-7
21	19.28	Limonene (C ₁₀ H ₁₆)	136.23	0.9	138-86-3
22	20.36	Undecane (C ₁₁ H ₂₄)	156.30	1.3	1120-21-4
23	22.14	Camphor (C ₁₀ H ₁₆ O)	152.23	0.9	21368-68-3
24	22.35	Decanal (C ₁₀ H ₂₀ O)	156.26	1.7	112-31-2
25	23.34	Undecan-2-one (C ₁₁ H ₂₂ O)	170.29	5.8	112-12-9
26	23.80	Undecanal (C ₁₁ H ₂₂ O)	170.29	1.1	112-44-7
27	24.48	β-Cedrene (C ₁₅ H ₂₄)	204.35	1.0	546-28-1
28	25.40	Octadecane (C ₁₈ H ₃₈)	254.49	1.9	593-45-3
29	25.98	α-Bergamotene (C ₁₅ H ₂₄)	204.35	1.4	17699-05-7
30	26.84	2,6-Ditert-butyl-4-methylphenol (C ₁₅ H ₂₄ O)	220.35	1.7	128-37-0
31	27.85	Heptadecane (C ₁₇ H ₃₆)	240.46	0.9	629-78-7
32	28.74	6-Pentylpyran-2-one (C ₁₀ H ₁₄ O ₂)	166.22	2.3	27593-23-3

RT^1 = Retention Time; MF^2 = Molecular Formula; MW^3 = Molecular Weight (Da); RPA^4 = Relative Peak Area; $CAS\ Nr^6$ = Chemical Abstracts Service number. The VOCs of *T. asperellum* HbGT6-07 with the RPAs less than or equal 0.85% were not included.

Supplemental Legends

Fig. S1 a *In-vitro* efficacy of *T. asperellum* HbGT6-07 isolates against *B. cinerea* (B05.10) and **b** *S. sclerotiorum* (A367) after 3 and 6 days-after-inoculation (DAI)

Fig. S2 PCR amplification of *T. asperellum* HbGT6-07 isolates were amplified the fragments with ITS1/ITS4 primer pairs in -600 bp region

Fig. S3 Diagram of detection of antifungal activity through the production of volatile organic compounds using dish within dish method (DwD)

Fig. S4 Hyphal fragments preparation outlines at different treatment ratios (33.33:66.67, 50:50 and 66.67:33.33)

Fig. S5 The reduction of lesion diameter by the isolates of *T. asperellum* HbGT6-07 against B05.10 and A367 fungal infection. **a** The formation of necrotic lesions (< 0.5 mm in size) by B05.10 isolates was reduced by the isolates HbGT6-07 after 3 dpi while **b** A367 causing lesion diameter was suppressed significantly at 3 dpi.

Table S1 *Trichoderma* strains screened for superior antifungal VOCs production.

Table S2 The list of species and GenBank accession numbers of DNA sequences for constructing phylogenetic tree.

Table S3 Primary screening of *Trichoderma* species against *B. cinerea* and *S. sclerotiorum* by dual culture.

Figures

Figure 1

Phylogenetic tree depending on the ITS region of the genomic rDNA gene of 2 isolates and 11 representative strains of *Trichoderma*. The Neighbor-Joining (NJ) method was done through MEGA7

where bootstrap values (n = 1000) higher than 50% are visible at the internodes in the tree. As the outer group, *Protocrea pallida* CBS299.78 strain was used

Figure 2

(a, b) Inhibition percentages of HbGT6-07 CF to *B. cinerea* and (a, c) HbGT6-07 CF to *S. sclerotiorum*

Figure 3

Effect of volatile organic compounds of *T. asperellum* HbGT6-07 isolate on *B. cinerea* B05.10. a The initial efficacy of VOCs of isolate *T. asperellum* HbGT6-07, *B. cinerea* B05.10 and AWG designated as control (CK). b Indicates colony diameter of AWG+HbGT6-07 and AWG+B05.10 isolates compare to treated and non-treated dishes CK (AWG). c Culture growth rate after 5 days. d The limited number of sclerotia were developed with an average colony size on the dish of isolates AWG+HbGT6-07

(A)

Ck (AWG)

AWG+HbGT6-07

AWG+A367

(B)

(C)

(D)

Figure 4

Effect of volatile organic compounds of *T. asperellum* HbGT6-07 isolate on *S. sclerotiorum* A367. a The initial efficacy of VOCs of isolate *T. asperellum* HbGT6-07, *S. sclerotiorum* A367. b Indicates colony diameter of AWG+HbGT6-07 and AWG+A367 isolates compare to treated and non-treated dishes CK (AWG). c Culture growth rate after 5 days. d The limited number of sclerotia were developed with an average colony size on the dish of isolates AWG+HbGT6-07

Figure 5

Biocontrol assay of disease suppression by isolate HbGT6-07 on rapeseed leaves. The hyphal fragments (HbGT6-07:B05.10) with the treatment level at ratios of 33.33:66.67, 50:50 and 66.67:33.33 was reduced the leaf lesion diameter of 43, 65 and 97% where HbGT6-07:A367 fragments moderately reduced lesion diameter of 16, 9 and 55%, respectively

Figure 6

Suppression of sclerotia formation with two different culture conditions a (HbGT6-07+B05.10 and HbGT6-07+A367). b Mixed culture showed significant reduction of sclerotia as compare to for B05.10 and A367. c The dry weight data of sclerotia production revealed HbGT6-07 isolates completely inhibit the sclerotia production

Figure 7

GC-MS profiling of volatile organic compounds emitted by the isolates *T. asperellum* HbGT6-07

Figure 8

Structure of thirty-two different volatile organic compounds emitted by the isolates *T. asperellum* HbGT6-07

Supplementary Files

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