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Joint Action of *Trichoderma hamatum* and Difenoconazole on Growth of a Phytopathogen *Sclerotinia sclerotiorum* under Laboratory Conditions

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Authors' Contribution
ZH conceived and designed the study. DY , CL and YH performed the experiments and analyzed the data. ZH analyzed the data and wrote the article.

Key words
Phytopathogen, *Trichoderma hamatum*, Difenoconazole, Mycelial growth, Sclerotia, *Sclerotinia sclerotiorum*.

ABSTRACT

Sclerotinia sclerotiorum is a serious phytopathogen resulting in significant loss of crop yield. *Trichderma hamatum* has the capacity to inhibit *S. sclerotiorum* growth by production of toxic metabolites and mycoparasitism. In vitro bioassays were used to evaluate the efficacy of *T. hamatum* and difenoconazole alone or its combinations against *S. sclerotiorum*. The results showed that *T. hamatum* could infect and destroy the mycelia and sclerotia of *S. sclerotiorum by* mycoparasitism. The cell-free culture supernatant and ethylacetate extracts from *T. hamatum* inhibited mycelial growth of *S. sclerotiorum*, with EC_{50} value of 0.54 µl/cm^2 and 2.99 µg/ml, respectively. The sclerotia production of *S. sclerotiorum* decreased when treated with ethylacetate extracts. The level of additive inhibition on *S. sclerotiorum* using both fungal ethylacetate extracts and difenoconazole was directly related to the concentration of each component used in mixtures. Additive effects were observed in all treatments according to the mycelial growth inhibition, Me and Chi-square values. The use of *T. hamatum* offers a promising and effective co-formulation product or alternative to chemical fungicides in controlling popcorn disease of mulberry.

INTRODUCTION

Sclerotinia sclerotiorum (Libert) de Bary is a devastating and cosmopolitan plant pathogen. It can infect more than 400 species of plants in 75 families, including mulberry, oilseed rape, soybean, peanut, sunflower, and lettuce (Bolton *et al*., 2006; Boland and Hall, 2009; Hu *et al*., 2011). *S. sclerotiorum* infection of mulberry blossoms causes a fruits disease known as "popcorn disease", resulting in yield loss and reduced quality of mulberry fruits (Hu *et al*., 2011). The fungus can also attack oilseed rape and soybean crops causing "sclerotinia stem rot" in China and elsewhere (Ma *et al*., 2009; Wang *et al*., 2014). *S. sclerotiorum* produces structures known as sclerotia which permit it to overwinter or over summer in the absence of a host (Abawi and Grogan, 1979). During favorable conditions, the sclerotia can germinate and produce infectious hyphae that can directly infect host plants, or else the fungus can develop carpogenically to produce windborne ascospores that can infect crops perpetuating and expanding the disease cycle. Paraffin sections of diseased fruits have shown that the fungal mycelium infects mulberry drupelets and then disintegrates into small black particles, which form the outer wall of newly forming sclerotia. Sclerotia have two structures with a hard, dense black outer layer called the pseudoparenchyma and a loose inner layer called the prosenchyma (Lǔ *et al*., 2017). Traditionally, chemical fungicides have been used to control this disease, including difenoconazole, carbendazium, dimethachlon, iprodione, procymidone, vinclozolin and dicloraz (Beny-Yephet *et al*., 1986; Hubbard *et al*., 1997; Mueller *et al*., 2002; Matheron and Porchas, 2004; Jo *et al*., 2006; Zhu *et al*., 2016).

Difenoconazole having both protective and curative activity is a triazole chemical fungicide extensively used in many countries to control various fungal pathogens, including phytopathogens belonging to ascomycetes (deuteromycetes) and basidiomycetes in a wide range of crops (Allen *et al*., 2004; Gopinath *et al*., 2006) The mechanism of difenoconazole inhibition of fungal growth and spore germination is due to its activity as a systemic sterol demethylation inhibitor and as an inhibitor of the activity of various enzymes, including dehydrogenase, urease, phosphatase and protease (Filimon *et al*., 2015). However, repetitive and extensive applications of chemical fungicides in controlling plant diseases has resulted in the increase of resistant strains of *S. sclerotiorum* in the field and these resistant varieties pose significant risk for susceptible plants crops, can act as sources of

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environmental contamination, and may have risks for human health because of toxic residues in agricultural products (Gossen *et al*., 2001; Kuang *et al*., 2011; ID EI Mouden *et al*., 2012). Biological control of *S. sclerotiorum* is one of the important alternative measures to chemical control in phytopathogen management. Examples of biological control include application of fungal antagonists and vaccination to use of rhizosphere fungal antagonists and/or mycoparasites or mycoparasitic fungi such as *Trichoderma* and *Gliocladium* species (Mishike, 1998).

Trichoderma species are important biocontrol agents against several plant pathogens and their diseases via direct action (mycelial coiling), *e.g.* by producing cell-wall degrading enzymes and a wide spectrum of (toxic) secondary metabolites that have antifungal activity including terpenes, polyketides and peptaibols (Cardoza *et al*., 2005; Alfano *et al*., 2007; Pandya and Saraf, 2010; Ye *et al*., 2014a, b). They may also act as competitors to potentially harmful microbes that can cause plant disease by adhering to root surfaces thus competing for space and nutrients and/or promoting local and systemic defense responses, as has been observed in many plant species including cucumber, cotton, and bell pepper (Mishra, 1996; Yedidia *et al*., 2003; Harman *et al*., 2004). Studies have shown that *T. hamatum* can reduce the foliar diseases incidence in several vegetable crops and induces systemic defense response by altering genes involved in stress and protein metabolism (Khan *et al*., 2004; Aldahmani *et al*., 2005; Horst *et al*., 2005). The aim of the present study was to evaluate if *T. hamatum* and its metabolite extracts alone or together with difenoconazole is efficient to inhibit the mycelial growth and reduce sclerotia production of *S. sclerotiorum.*

MATERIALS AND METHODS

Fungal strains

The *Trichoderma hamatum* strain Th-N5 (CCTCC accession # M 2016251) was isolates from soil in mulberry orchard in Guangzhou city, China. *Sclerotinia sclerotiorum* isolate (Ss01), isolated from infected mulberry fruit in Guangzhou city, deposited at Laboratory of Entomopathogenic Fungus, College of Agriculture, South China Agricultural University, identified as the method described by Hu *et al*. (2011), was used in this study. To produce the inoculum for each assay, *S. sclerotiorum* and *T. hamatum* were routinely cultured on potato dextrose agar (PDA) medium (Potato infusion 200 g/L; Dextrose 20 g/L and Agar 20 g/L dissolved in deionized water 1000 ml and sterilized at 121°C at 15 psi for 25 min) and incubated at 20±1°C and 26±1°C for 10 days, respectively. Conidia of *T. hamatum* were harvested from PDA plates into sterile distilled H_2O+0.1% Tween-80 (Liu and Robat, 1993). Conidia were counted using a Fuchs-Rosenthal hemocytometer and a compound microscope. Conidial suspensions were adjusted to indicate concentrations as needed. Sclerotia of *S. sclerotiorum* was harvested and deposited under 4°C condition for use.

Preparation of fungal cell-free culture supernatant and ethyl acetate extracts of T. hamatum

Fungal conidia (10 ml, 1×10^6 conidia ml^{-1}) of *T. hamatum* was inoculated into shake cultures in a 1 liter flask containing 300 ml of Czapek-Dox broth + 1% peptone (CZP, composed of peptone 0.5%, $NaNO_3$ 0.2%, K_2HPO_4 0.1%, $MgSO_4\cdot7H_2O$ 0.05%, KCl 0.05%, $FeSO_4\cdot7H_2O$ 0.001%, sucrose 3% (w/v)) and incubated with aeration (180 rpm) at 26±1°C for 3 days for the production of the seed inoculum. The seed inoculum were then added to fresh CZP at 1:9 ratios (v/v, 3000 ml total volume) and the mixture was incubated with aeration (240 rpm) at 26±1°C for 4, 6 and 8 days. After 4, 6 and 8 days, fungal cells were removed by centrifugation (12000 x g, 15 min) and the cell-free culture supernatant stored at 4°C for use.

Proteins from the cell-free culture supernatant were extracted using trichloroacetic acid (TCA) and acetone mixture. Three volumes (6 ml) of pre-cooled 15% (w/v) TCA-acetone solution was added to the cell-free culture supernatant and after mixing, the suspension was centrifuged (4°C, 3000 x g, 10 min) to separate out the precipitated proteins. The resulting protein precipitate pellets were washed three times with pre-chilled acetone (1 ml) and stored at 4°C until use. Protein extracts were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% gels. After electrophoresis the gels were stained using a Coomassie blue staining procedure.

Metabolites were extracted from the cell-free culture supernatant using ethyl acetate (EthOAc). Aliquots (6000 ml) of the cell-free supernatant were mixed with an equal volume of ethyl acetate (1:1) and mixed vigorously for 30 min. The organic phase from above extraction mixture was collected and concentrated (rotary evaporator RE -52A, Shanghai Ya Rong Biochemical Instrument Factory, Shanghai, China) under reduced pressure to obtain EthOAc-fraction and stored at -20°C for use. All chemicals used in present experiment were purchased at Qianghui Bio-chemical Company.

T. hamatum *infection of mycelia and sclerotia of* S. sclerotiorum

A freshly prepared fungal suspension of *T. hamatum* (100 µl of 1×10^6 conidia ml^{-1}) was inoculated in the centre of plates containing PDA medium using a micro applicator

to spread and cover the whole plate with the fungal cells. The plates were incubated for mycelial growth at 20±2°C, 80±5% R.H., and L14:D10 h for 2-3 days. A piece of sclerotia of *S. sclerotiorum* was put in the center of plates containing PDA medium for mycelial growth. Mycelial discs of each strain together with medium (Ø1 cm) were removed and cultured together on one new plate with PDA medium, and incubated at 20±1°C. In the new plate, *S. sclerotiorum* mycelial disc was put in the centre of plate and *T. hamatum* disc was placed about 2.5 cm away from *S. sclerotiorum* disc (Fig. 1). Medium containing only one mycelial disc of each strain were used, respectively, as a controls. The mycelial growth and sclerotia production of *S. sclerotiorum* on different treatments were observed daily. At the same time the mycelia growth and conidia production of *T. hamatum* was also detected daily. All experiments were performed in triplicate, and experiments were repeated with at least one independent batch of *S. sclerotiorum* or *T. hamatum* mycelial disc.

Effects of the cell-free culture supernatant and ethylacetate extracts on mycelial growth and sclerotia production of S. sclerotiorum

Mycelial discs of *S. sclerotiorum* was prepared as described 2.3, mycelial discs were removed and cultured on PDA medium having different concentrations of cell-free culture supernatant or ethylacetate extracts of *T. hamatum*, respectively, and incubated at 20±1°C. The same mediums without the cell-free culture supernatant or ethylacetate extracts were served as a control. There were 5 Petri dishes for different concentrations of cell-free culture supernatant or ethylacetate extracts treatments. Colony diameters were measured at 12, 24, 36, 48 and 60 h post treatment. Sclerotia production of *S. sclerotiorum* was recorded from 8 to 15 days post-treatment. All experiments were performed in triplicate, and experiments were repeated with at least one independent batch of *S. sclerotiorum* mycelial disc, cell-free culture supernatant and ethylacetate extracts.

Inhibitory effect of difenoconazole on mycelial growth of S. sclerotiorum

Mycelial discs of *S. sclerotiorum* was prepared as described above, mycelial discs were removed and cultured on PDA medium having fungicide difenoconazole (Novartis, China, Shanghai) and incubated at 20±1°C. The same media without difenoconazole were used as controls. Replicates (5 Petri dishes) for each different concentrations of difenoconazole treatments were used.

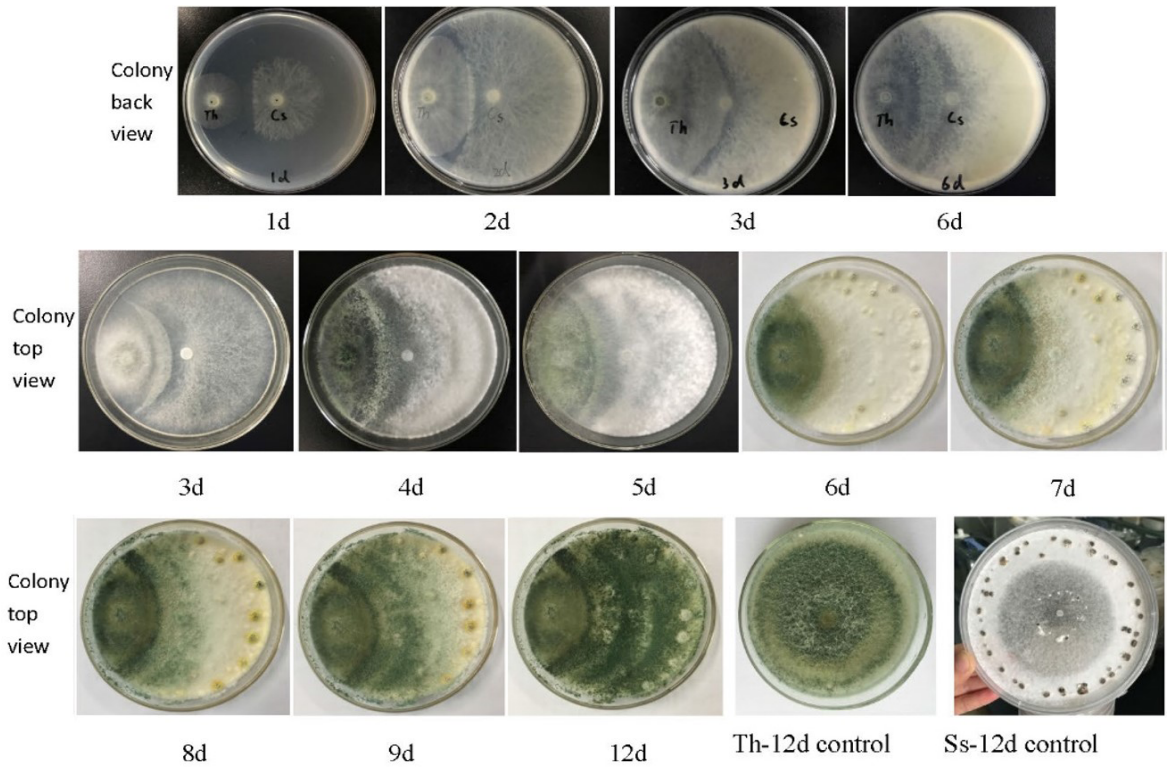

Fig. 1. Inhibition of *Trichderma hamatum* on mycelial growth and Sclerotia formation of *Sclerotinia sclerotiorum*.

Colony diameters were measured at 12, 24, 36, 48 and 60 h post treatment. All treatments were replicated three times, and experiments were repeated with at least one independent batch of difenoconazole and mycelia disc.

Joint action of ethylacetate extracts and difenoconazole on mycelial growth of S. sclerotiorum *under laboratory conditions*

The activities of ethylacetate extracts and difenoconazole mixtures were tested against mycelial growth of *S. sclerotiorum*. On basis of the above results, three concentrations of difenoconazole (0, 0.275, 0.55 µg/mL) and ethylacetate extracts (0, 1.5, 3.0 µg/mL) were used, respectively. The different mixtures of ethylacetate extracts and difenoconazole were prepared by serial dilutions with sterilized water, and bioassays were carried out using the methods previously described. The different mixtures of ethylacetate extracts (µg/mL) and difenoconazole (µg/mL) were designed as follows: T1=1.5+0.0, T2=3.0+0.0, T3=1.5+0.275, T4=1.5+0.55, T5=3.0+0.275, T6=3.0+0.55, T7=0.0+0.275, T8=0.0+0.55 and T9=0.0+0.0.

Statistical analysis

The average mycelial growth inhibition of colony was calculated as (long diameter + short diameter)/2. Inhibitory percentage on mycelial growth or sclerotia production was calculated as: % inhibition = [(Gc-Gt)/Gc]*100, where Gc is radial growth diameter or sclerotia production in control and Gt is radial growth diameter or sclerotia production in treatment. Inhibitory percentage on mycelial growth or sclerotia production data were analyzed by Analysis of variance (ANOVA) and treatment means were compared by using Tukey's HSD test for mean comparisons at 5% level of significance. The curves of (log concentration – probit line (LC-p)) were calculated and tested by chi-square test, median effective concentrations (EC_{50}) and their confidence intervals were calculated by probit analysis using SPSS (Statistical Package for Social Science) 8.0 for windows (SPSS, 2008). The data were control-corrected (Abbott, 1925) first, and converted into proportion (*i.e.*, 0-100% → 0-1). The inhibitory percentage expected for no interaction (additive effect) was calculated as follows:

$$Me=Ma+Mb*(1-Ma)$$

$$X^2= [(Mab-Me)*100]*[(Mab-Me)*100]/(Me*100)$$

Where, Me is the expected inhibitory percentage for additive mortality; Ma, Mb and Mab are the observed inhibitory percentage for agents ethylacetate extracts, difenoconazole and their combinations, respectively. Then P-value were looked up in a chi-square table for df=1. If Mab significantly <Me, it meant antagonism; if Mab significantly >me, it meant synergism. Otherwise the inhibition rate was additive.

RESULTS

T. hamatum *infection of mycelia and sclerotia of* S. sclerotiorum

Mycelial discs of *T. hamatum* and *S. sclerotiorum* with media (Ø1 cm) were cultured together on one plate with PDA medium to evaluate mycelial growth inhibition each other. The mycelium of *S. sclerotiorum* grew faster and the area covered of *S. sclerotiorum* colony was bigger than that of *T. hamatum* after 1, 2 days cultured (Fig. 1). The mycelium of *S. sclerotiorum* stopped spreading forward when it prior to contact with the *T. hamatum* colony, however, the mycelium of *T. hamatum* grew and spread directly into the *S. sclerotiorum* colony after 2 d culture on PDA (Fig. 1, colony back 2d-6d). A "interaction belt" or zone was clearly apparent between the *T. hamatum* and *S. sclerotiorum* colonies after 3 d co-culture. Conidia appeared in the *T. hamatum* colony at 4 d post inoculation on PDA (colony top view 3d), and subsequently, it conidiation was observed in the *S. sclerotiorum* colony with *T. hamatum* mycelium growing forward over the time course (colony top view 3d-12d). *S. sclerotiorum* sclerotia appeared at 6 d to 9 d at the place with no *T. hamatum* mycelium infection. Finally, the mycelium and conidia of *T. hamatum* covered the whole *S. sclerotiorum* colony, and the color of sclerotia turned to grey white (colony top view 12 d) as compared to the sclerotia of *S. sclerotiorum* grown alone on PDA medium (colony top view Ss-12d). No sclerotia were seen in co-cultured PDA medium. The results of microscopic observation showed that *T. hamatum* mycelium grew, twined round and stretched into the mycelia and sclerotia of *S. sclerotiorum*, and the infected sclerotia of *S. sclerotiorum* were empty of conidia.

Effects of the cell-free culture supernatant and ethylacetate extracts on mycelial growth and sclerotia production

Cell-free culture supernatants of *T. hamatum* derived from cells grown for 4, 6 and 8 d incubation were used to evaluate the optimum culture time for production of metabolites toxic to *S. sclerotiorum*. The final concentrations of cell-free culture supernatants covered on PDA assay plates were 0.1, 0.2, 0.4, 0.8 and 1.6 µl/cm^2. The EC_{50} value of *T. hamatum* cell-free culture supernatant on mycelial growth inhibition of *S. sclerotiorum* increased over time, and the EC_{50} values of supernatants from 4 d incubation at 36, 48 and 60 h were higher than that of the supernatants from 6 and 8 d incubation, respectively (Fig. 2). The best time for harvest cell-free culture supernatant was at 6 d post incubation, with no difference between EC_{50}

of supernatants from 6 d and 8 d incubations, and high inhibition of *S. sclerotiorum* at relatively short incubation times. The mycelial growth inhibition of cell-free culture supernatant from 6 d incubation, treated in 70°C for 10 min, on *S. sclerotiorum* was consistently up to 50% during the test period at the treatments with the concentration of 1.6 and 3.2 μl/cm^2, respectively (Fig. 3). Inhibition was up to 50% even when the supernatant was treated in 70°C for 20 min (Fig. 4) and the inhibitions were different among the samples from 0, 20 and 30 min treatments. SDS-PAGE of proteins from cell-free culture supernatant treated in 70°C for 0, 20 and 30 min, respectively, showed a range of protein bands (Fig. 5). Some bands were clearly present in gel when samples treated in 70°C for 20 and 30 min as compared to control. The results showed that some proteins in the *T. hamatum* derived supernatant kept activity, *i.e.* high mycelial growth inhibition, towards *S. sclerotiorum* even when treated at 70°C for 20 or 30 min.

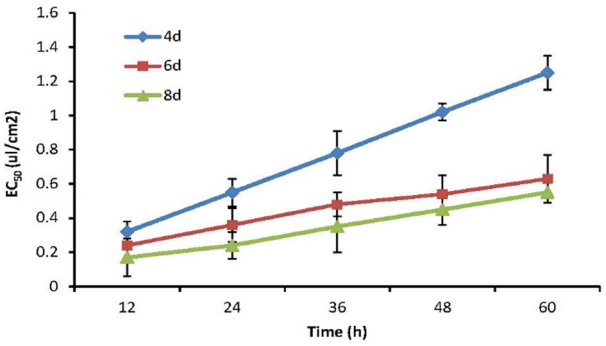

Fig. 2. The inhibition of EC_{50} of *T. hamatum* cell-free culture supernatant on mycelial growth of *S. sclerotiorum*.

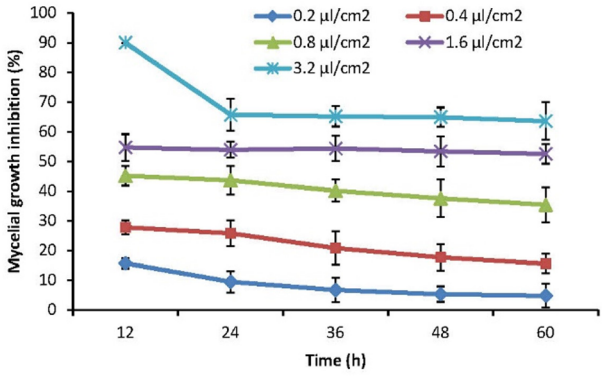

Fig. 3. The inhibition of *T. hamatum* cell-free culture supernatant on mycelial growth inhibition of *S. sclerotiorum* after treated in 70°C for 10 min. Data on mean (±SE) inhibitory percentage were subjected to arcsine transformation prior to computation

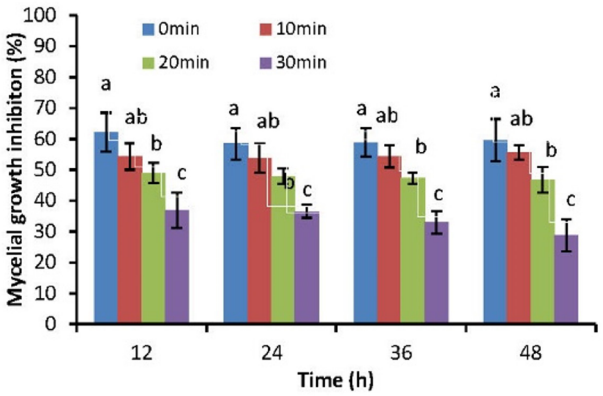

Fig. 4. The inhibition of *T. hamatum* cell-free culture supernatant on mycelial growth inhibition of *S. sclerotiorum* after treated in 70°C for 0, 10, 20 and 30 min. Data on mean (±SE) inhibitory percentage were subjected to arcsine transformation prior to computation. Means±SE in time dots (the column) marked with different letters are significantly different (Tukey's HSD, a=0.05).

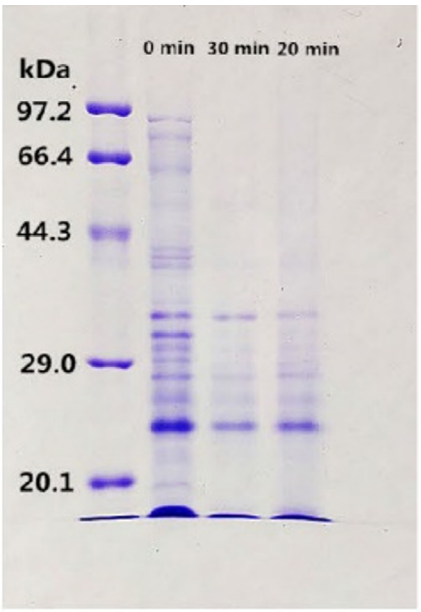

Fig. 5. SDS-PAGE of protein from *T. hamatum* cell-free culture supernatant after treated in 70°C for 0, 20 and 30 min.

The mycelial growth inhibition of *T. hamatum* ethylacetate extracts, used at different concentrations (0.5, 1, 2, 4 and 8 μg/ml), against *S. sclerotiorum* was determined (Fig. 6). The mycelial growth inhibition increased with increased concentration of *T. hamatum* ethylacetate extracts, reaching 50% at 8 μg/ml ethylacetate extract. The LC_{50} values of *T. hamatum* ethyl acetate extracts on

mycelial growth inhibition of *S. sclerotiorum* were 1.49, 2.25, 2.77, 2.99 and 2.96 µg/ml at 12, 24, 36, 48 and 60 h post treatment. Sclerotia production of *S. sclerotiorum* decreased significantly when treated by *T. hamatum* ethyl acetate extract, with the sclerotia production inhibition of ethyl acetate extract up to 50% at 4 and 8 µg/ml extract at 8, 9 and 10 d post treatment (Fig. 7). These results indicate that ethyl acetate extracts inhibited mycelial growth and reduced sclerotia production.

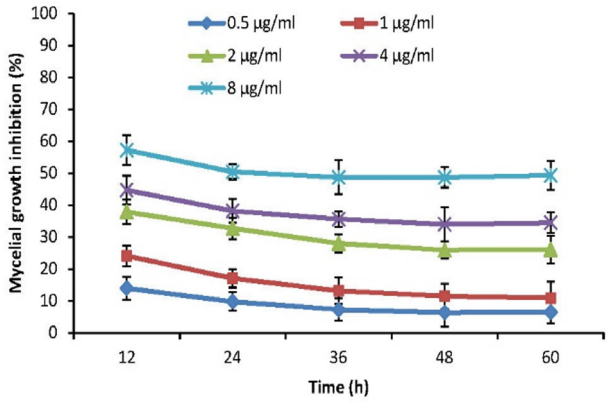

Fig. 6. The inhibition of *T. hamatum* ethylacetate on mycelial growth of *S. sclerotiorum.* Data on mean (±SE) inhibitory percentage were subjected to arcsine transformation prior to computation.

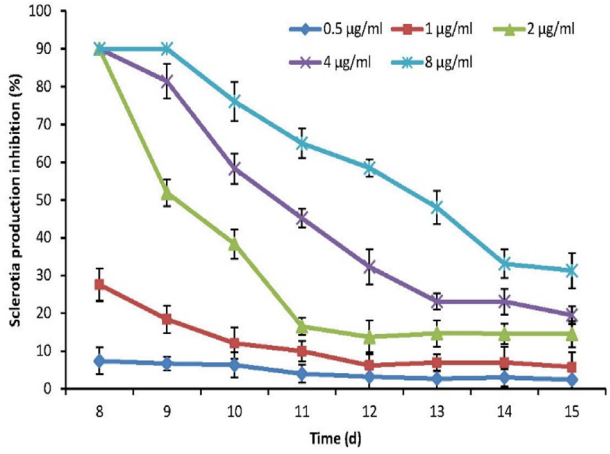

Fig. 7. The inhibition of *T. hamatum* ethylacetate extracts on sclerotia production of *S. sclerotiorum.* Data on mean (±SE) inhibitory percentage were subjected to arcsine transformation prior to computation.

Inhibitory effect of difenoconazole on mycelial growth

Difenoconazole was capable of inhiniting *S. sclerotiorum* mycelial growth (Fig. 8). The highest inhibition rate of difenoconazole on the mycelia growth of *S. sclerotiorum* (~50%) was seen using 1.6 µg/ml of fungicide. For inhibition of mycelial growth, the EC_{50} values of difenoconazole towards *S. sclerotiorum* were 0.32, 0.41, 0.50, 0.55, 0.53 and 0.60 µg/ml at 12, 24, 36, 48, 60 and 72 h post treatment.

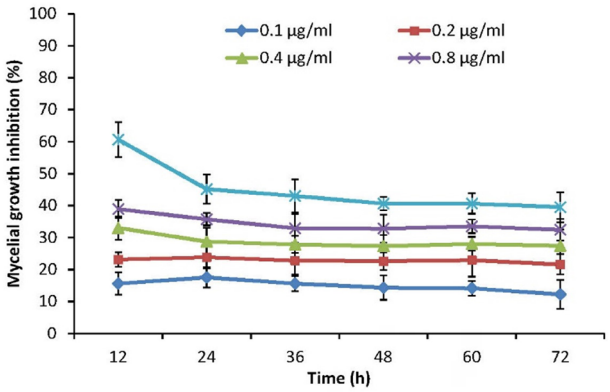

Fig. 8. The inhibition of difenoconazole on mycelial growth of *S. sclerotiorum.* Data on mean (±SE) inhibitory percentage were subjected to arcsine transformation prior to computation.

Joint action of ethylacetate extracts and difenoconazole on mycelial growth under laboratory conditions

The mean inhibition rates of ethylacetate extracts from *T. hamatum*, difenoconazole and their combinations, together with Me (expected inhibition rate for additive inhibition rate) and Chi-square values are given in Table I. All combinations of extracts and difenoconazole tested caused high inhibition of *S. sclerotiorum* mycelial growth, revealing substantial levels of additive inhibition. The mycelial growth at 12, 36, 48 and 60 h in control were 1.63, 4.24, 6.96, 9.69 and 12.6 cm, respectively. The level of additive inhibition between the extracts and difenoconazole was affected by the concentrations of each component in the mixtures, *i.e.*, the inhibition rates of mycelial growth increased with an increase in the concentration of the extracts and difenoconazole. The inhibition rate in the treatments containing the extracts or difenoconazole alone differed significantly from the relevant mixtures of the extracts and difenoconazole. The best additive effect was observed in treatment T5 (3.0+0.275) against *S. sclerotiorum* having inhibitory percentage values of 45 to 60% on mycelial growth during the period of 12 to 60 h treatment according to their inhibition rate, Me and Chi-square values (Table I).

Table I.- Joint action of ethylacetate extracts and difenoconazole on the mycelial growth of *S. sclerotiorum.*

Treatment	12 h	24 h	36 h	48 h	60 h
T1	34.1 ± 2.3 d	28.1 ±3.5 d	24.3 ±2.2 d	22.1 ±3.1 d	21.1 ± 4.1 c
T2	41.0 ± 3.8 c	34.7 ±2.9 c	31.4 ±2.5 c	29.1 ±3.7 c	28.3 ± 4.3 c
T3	55.4 ± 4.2 b (54.4, 1.612)	45.9±3.6 b (46.7, 1.446)	43.6 ±4.1 b (42.7, 1.918)	39.2 ±3.4 b (40.4, 3.603)	38.4 ± 3.8 b (39.3, 2.18)
T4	56.1 ± 4.7 b (56.7, 6.427)	50.2 ±4.1 ab (49.5, 1.124)	47.2 ±4.7 ab (45.3, 7.592)	43.8 ±4.1 ab (42.8, 2.236)	42.9 ± 5.7 ab (41.8, 3.047)
T5	60.4 ± 4.9 a (59.2, 2.307)	52.5 ±4.3 a (51.6, 1.525)	49.2 ±3.9 a (48.1, 2.657)	47.5 ±4.4 a (45.8, 6.605)	45.3 ± 4.9 a (44.9, 0.426)
T6	62.2 ± 5.6 a (61.2, 1.514)	55.6 ±4.7 a (54.1, 4.192)	51.7 ±3.8 a (50.5, 2.994)	48.3 ±5.2 a (48.0, 0.242)	47.6 ± 6.2 a (47.1, 0.562)
T7	30.9 ± 3.4 d	25.9 ±2.6 d	24.3 ±2.4 d	23.5 ±3.5 d	23.1 ± 2.2 c
T8	34.3 ± 3.9 d	29.7 ±3.3 d	27.8 ±3.2 cd	26.6 ±2.4 cd	26.2 ± 2.8 c
F, df, P	109.2, 7, 0.0001	93.5, 7, 0.0001	67.4, 7, 0.0001	50.2, 7, 0.0001	82.7, 7, 0.0001

Means (M ± SE) in the same column followed by different letters are significantly different (Tukey's HSD Test, α=0.05). Data on mean (±SE) inhibition rate were subjected to arcsine transformation prior to computation. Data in bracket shows Me (the expected inhibition rate for additive inhibition rate) subjected to arcsine transformation and the chisquare value, respectively. T1=1.5 (µg/ml, metabolite extract) + 0.0 (µg/ml, difenoconazole), T2 = 3.0 + 0.0, T3 = 1.5 + 0.275, T4 = 1.5 + 0.55, T5 = 3.0 + 0.275, T6 = 3.0 + 0.55, T7 = 0.0 + 0.275, and T8 = 0.0 + 0.55.

DISCUSSION

S. sclerotiorum is a cosmopolitan phytopathogenic fungus that kills host plant cells and feeds on dead tissue. Ascospores from overwinter "sclerotia" spread by wind and are important sources of primary infection capable for example infecting mulberry including dead or senescent flower petals (Hegedus and Rimmer, 2005) resulting in named as "popcorn disease" in mulberry trees (Siegler and Jenkins, 1923; Blain, 1931; Bolton *et al*., 2006). The fungus produces a black structure called "sclerotium" with a thick black substance in diseased fruit (Li and Rollins, 2009). Sclerotia are highly stress resistant and can withstand adverse environmental impacts, solubilization and degradation by concentrated acids and hot alkali solutions, and bleaching by oxidizing agents (Butler and Day, 1998; Wu *et al*., 2008). *S. sclerotiorum* also secretes oxalic acid as a nonspecific phytotoxin, that may act as a signaling molecule or elicitor to subvert and redirect host pathways toward cell death, thus interfering with the functioning of plant guard cells (Cessna *et al*., 2000; Guimarães and Stotz, 2004; Kim *et al*., 2008; Williams *et al*., 2011). The fungus also produces cell-wall-degrading enzymes including pectinases, proteases, cellulases, polygalacturonases, and glucoamylases to weaken the plant cell wall during infection host plant (Martel *et al*., 1996; Poussereau *et al*., 2001; Yajima *et al*., 2009). Once *S. sclerotiorum* has infected and entered into plant structures (*e.g.* inflorescences), it is difficult to control mycelium growth in blossoms and fruit with chemical fungicide in particular due to potential human health consequences of systemic fungicide residue on edible parts of the plant. Thus, targeting the sclerotia, the source of apothecia and ascospores, is a good control tactics for decreasing primary infection resource of plant diseases. It is reported that *Trichoderma* spp. and *Gliocladium* species, *Coniothyrium minitans* have the capacity to degrade sclerotia of *S. sclerotiorum* reducing their viability and apothecial production (Mishike, 1998; Jones *et al*., 2014). Mycoparasitism is a process in which fungi (*T. hamatum*) attacked and kill other fungi. In this study, via microscope observation of infected sclerotia, we found that *T. hamatum* mycelium not only infect and parasitize the sclerotia of *S. sclerotiorum*, but that infected sclerotia can be destroyed and are reduced/empty of viable cells. Similarly, the result of Jones *et al*. (2014) showed that *T. hamatum* has the ability to degrade *S. sclerotiorum* sclerotia production. Apotheical production of *S. sclerotiorum* was reduced up to 70% when sclerotia infected by *T. hamatum* mycelium (Rabeendran *et al*., 2005). At the same time, the sclerotia production of *S. sclerotiorum* decreased significantly when treated by *T. hamatum* ethyl acetate extracts, with the sclerotia production inhibition rate up to 50% at concentrations of 4 and 8 µg/ml at 8, 9 and 10 d post treatment.

Our results show that *T. hamatum* mycelia can grow and spread directly into *S. sclerotiorum* colonis when they are co-cultured together on PDA medium, where

ultimately the mycelia and spores of *T. hamatum* cover the *S. sclerotiorum* colony. However, *S. sclerotiorum* mycelia stop spreading forward prior to contact with *T. hamatum* colonies. The reasons for this include metabolites secreted by *T. hamatum* and mycoparasitism. In order to survive and compete for nutrients, Trichoderma spp. secrete cell-wall degrading enzymes to hydrolyze the mycelium of competing fungi and produce a series of toxic and antibiotic metabolites that inhibit mycelial growth of other fungi (Thrane *et al*., 2000; Eziashi *et al*., 2006; Vinale *et al*., 2008; Andrabi *et al*., 2011). The enzyme, endo-1,3-β-glucanase and cellulose, from *T. hamatum* culture supernatant has been shown to inhibit the germination and growth of encysted *Pythium* zoospores (Thrane *et al*., 1997). In the present study, *T. hamatum* fermentation broth was tested and showed a strong capacity to inhibit *S. sclerotiorum* mycelial growth, even when the cell-free culture supernatant was treated at 70°C for 20 or 30 min. Possibly, a small molecular protease in the *T. hamatum* supernatant was capable of sustaining activity even at high temperature or some other heat stable factor(s) exist. These heat stable factor(s) are likely secondary metabolites secreted by *T. hamatum* that exhibit antifungal activity, and can include terpenes, polyketides and peptaibols (Cardoza *et al*., 2005; Alfano *et al*., 2007; Pandya and Saraf, 2010) that can readily diffuse into the PDA medium to inhibit *S. sclerotiorum* mycelium growth and hyphal elongation. Polyketides from *T. hamatum* have been demonstrated to inhibit the mycelia growth of *Gaeumannomyces graminis*, the causative agent of wheat disease (Cardoza *et al*., 2005). Our results show that *T. hamatum* ethylacetate extracts strongly inhibit the mycelial growth of *S. sclerotiorum* with the LC_{50} values of 1.49, 2.25, 2.77, 2.99 and 2.96 µg/ml at 12, 24, 36, 48 and 60 h post treatment under laboratory conditions.

Joint application of ethylacetate extracts from *T. hamatum* fermental broth and difenoconazole resulted in additive activities. The level of the additive effect was most evident under laboratory conditions when 1.5 or 3.0 mg/L of the ethylacetate extracts was mixed with 0.275 or 0.55 mg/L difenoconazole. The possible reason behind the additive action of both chemicals can be related to their different mode and mechanisms of action. The mechanism of difenoconazole in inhibiting fungal growth is a systemic sterol demethylation inhibitor of plant pathogen and as an inhibitor of the activity of various enzymes (Filimon *et al*., 2015). In our pre-experiment, difenoconazole was used as a systemic fungicide with both protective and curative activity up to 7 d post-treatment, with the mycelial growth of *S. sclerotiorum* inhibited by the ethylacetate extracts for 5 d. Our results indicate that *T. hamatum* ethylacetate extracts used as an alternative agent, or in combination with difenoconazole against *S. sclerotiorum* can allows for a reduction of the dose of fungicide applied in the field and delay the increase of emergence of fungicide resistant pathogens. In conclusion, our data show that *T. hamatum* has strong potential biocontrol activity against popcorn disease of mulberry caused by *S. sclerotiorum*, and can be used to format safer and more effective alternatives/ combnations to fungicides in plant disease control.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Statement of conflict of interest

No potential conflict of interest was reported by the authors.

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