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Potential of culture filtrate from *Trichoderma* spp. as biofungicide to *Colletotrichum gloeosporioides* causing anthracnose disease in chili

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Abstract. *Nurbailis, Djamaan A, Rahma H, Liswarni Y. 2019. Potential of culture filtrate from* Trichoderma *spp. as biofungicide to* Colletotrichum gloeosporioides *causing anthracnose disease in chili. Biodiversitas 20: 2915-2920. Trichoderma* spp. have the potential to be used for controlling the airborne pathogenic fungi such as *C. gloeosporioides.* The purpose of this study was to evaluate the antifungal activity of the culture filtrate of five isolates of *Trichoderma* spp. (*T. harzianum, T. viride, T. koningii, Trichoderma* PP2, *Trichoderma* PP3) against C. *gloeosporoides* causing anthracnose disease in chili. Culture filtrate of *Trichoderma* spp. was produced from single culture and dual culture techniques. The design was a Completely Randomized Design with six treatments and four replications. The treatments were culture filtrate from *T. harzianum*, *T. viride*, *T. koningii*, *Trichoderma* PP2, *Trichoderma* PP3, and negative control (without culture filtrate from *Trichoderma* spp.*).* Parameters observed were: the diameter of the colony, colony coverage, conidial germination, and conidial density. The results of this research showed all the culture filtrate from *Trichoderma* spp. produced by single and dual culture techniques can inhibit the growth of *C. gloeosporioides*. The culture filtrate from *Trichoderma* PP2 and *T. koningii* were the most potential in inhibiting the growth, conidial density, and conidial germination of *C. gloeosporioides*.

Keywords: Biofungicide, *Colletotrichum gloeosporioides*, culture filtrate, *Trichoderma*

INTRODUCTION

Colletotrichum gloeosporioides is one of the fungi that cause anthracnose disease in chili. This pathogen infects the mature and immature fruit of chili, causing high loss of production (Roberts et al. 2008; Robert et al. 2015; Diao et al. 2017). In general, the control of anthracnose disease is carried out by the extensive use of fungicides which have a negative impact on the environment and consumers (Sharma and Kulshrestha 2015) Therefore; it is necessary to find alternative controls which are environmentally friendly by utilizing the antagonistic fungi such as *Trichoderma* spp. as biological control agents.

Trichoderma spp. are free-living fungi, antagonistic, nonvirulent, plant symbionts, as well as hyperparasite against plant pathogenic fungi (Harman et al. 2004). They are commonly found in all climatic zones with habitats that dispersed in various types of soil and agricultural land. The most typical habitats of these fungi include soil and rotting wood (Samuel 2006; Druzhinina et al. 2006). *Trichoderma* has several mechanisms in inhibiting the growth of plant pathogens which are: competition of space and nutrients, parasitic to the plant pathogens and antibiosis by producing secondary metabolites as antimicrobial (Howell 2003; Vinale et al. 2008). *Trichoderma* spp. induces pathogen inhibition by secreting secondary metabolites. Different *Trichoderma* species secrete different substances, including isonitrile, diketopiperazines, sesquiterpenes, stemids, polyketides, alkylpyrones, and peptaibols (Wu et al. 2017).

Utilization of *Trichoderma* spp. to control pathogens that attack parts of the plant surface such as leaves and fruit have not been widely reported. *Trichoderma* has the potential to be developed for controlling this disease by utilizing secondary metabolites that are produced as antifungal and antibacterial compounds (Dubey et al. 2011; Leelavathy et al. 2014). Secondary metabolites produced by *Trichoderma* are chemically diverse, and their production varies between species and between isolates of the same species (Vinale et al. 2009). The vast structural and functional diversity of *Trichoderma* metabolites necessitates the continuous search for new metabolites. The generated knowledge may be necessary for the selection of new biocontrol agents, or the compounds themselves may be used as bioactive compounds in pesticide and antibiotic applications.(Mutawila et al. 2016).

Antibiosis is a mechanism of antagonistic fungi in inhibiting the growth of pathogens with antagonistic chemical products released into their environment, which include antibiotic components and extracellular enzyme systems that damage pathogens (Harman, et al. 2004; Woo, et al. 2006; Dubey et al. 2011; Leelavathy et al. 2014; Daniel et al. 2014). *T. harzianum* T22 and T39 strains produce secondary metabolites in the form of antibiotics that can suppress many plant pathogenic fungi such as *Leptosphaeria muculans*, *Phytophthora cinnamomi*, and *Botrytis cinerea* (Vinale et al. 2006).

Kumar et al. (2014) reported that secondary metabolites from *T. harzianum* (Th. Azad) and *T. viride* (01PP)

produced with dual culture with various soil-borne pathogenic fungi showed that the metabolites produced were more effective in suppressing the growth of *F. oxysporum* growth. f.sp. lenti, *Rhizoctonia solani,* and *Sclerotinia rolfsii*. The higher the concentration of secondary metabolites, the more depressed pathogen growth.

Nurbailis et al. (2006) reported that *T. viride* and *T. koningii,* indigenous isolates from banana rhizosphere were able to inhibit the growth of *F. oxysporu*m f.sp. *cubense* with an antibiosis, hyperparasite and competition mechanism. Nurbailis et al. (2014) also reported that *Trichoderma* PP2 and PP3 isolates originating from chili rhizosphere were effective in inhibiting the growth of *C. gloeosporioides* in vitro by the mechanism of competition, hyperparasites, and antibiosis.

There was an indication that *Trichoderma* spp. have an antibiosis mechanism in controlling plant diseases caused by *Colletotrichum* in chili. Therefore, it is necessary to conduct research on the utilization of secondary metabolites produced in culture medium of *Trichoderma* spp. to inhibit the growth of *C. gloeosporioides* that cause anthracnose in chili. The purpose of this study was to evaluate the antifungal activity of the culture filtrate of five isolates of *Trichoderma* spp. (*T. harzianum, T. viride, T. koningii, Trichoderma* PP2, *Trichoderma* PP3) against C. *gloeosporoides* causing anthracnose disease in chili

MATERIALS AND METHODS

The study was conducted in the Phytopathology Laboratory of the Agriculture Faculty, Andalas University, Padang from March to September 2018. The design was a Completely Randomized Design (CRD) consisting of six treatments and four replications. The treatments were culture filtrate of five *Trichoderma* isolates, namely: *T. viride*, *T. harzianum*, *T. koningii*, *Trichoderma* PP2, and *Trichoderma* PP3 isolates and negative control (without culture filtrate)). Each treatment has four replications. Data were statistically analyzed using Analysis of Variance and Duncan's Multiple Range Test (DMRT) at the 5% level.

Cultivation of *Trichoderma* spp.

Trichoderma spp.: *T. viride, T.harzianum, T. koningii, Trichoderma* PP2, and *Trichoderma* PP3 isolates with indication of having antibiosis activity were cultured on Potato Dextrose Agar (PDA) medium for seven days

Cultivation of *Trichoderma* spp in the liquid medium

Cultivation of *Trichoderma* in liquid medium was carried out in two techniques: 1) single culture technique of *Trichoderma: Trichoderma* was cultivated in a liquid medium of Potato Dextrose Broth, contains of 200 g potato, 20 g dextrose and 1000 mL distilled water and pH were adjusted 6.0. The medium was put as much as 100 ml into Erlenmeyer tube 250 ml and sterilized in an autoclave for 30 minutes at 15 psi and 121º C. The sterilized medium was placed in laminar airflow for the inoculation of *Trichoderma* spp. Inoculation was carried out by adding 10 ml conidial suspension of *Trichoderma* (10^6 conidia/mL) in to an Erlenmeyer tube containing 100 ml of PDB medium (10% of the total medium). Subsequently incubated for five days at room temperature and then incubation was continued in a rotary shaker at a speed of 180 rpm for seven days. 2) dual culture technique of *Trichoderma* and *C. gloeosporoides* simultaneously. Cultivation was carried out in the Potato Dextrose Broth (PDB) as mention above. Inoculation was carried out by adding 10 ml conidial suspension each of *Trichoderma* and *C. gloeosporoides* (10^6 conidia/mL) into an Erlenmeyer tube containing 100 ml of PDB medium. Next incubated for five days at room temperature, incubation was continued on a rotary shaker at a speed of 180 rpm for seven days. (Kumar et al. 2014).

Culture filtrate of *Trichoderma* spp

Liquid growth media as mention above was separated from biomass of *Trichoderma* spp for single culture and biomass of *Trichoderma* spp and *C. gloeosporoides* for dual culture techniques using Whatman filter paper. The filtrate was centrifuged at 4000 rpm for 30 minutes, then filtered again using Whatman paper into another Erlenmeyer. The obtained filtrate was prepared by passing through Millipore filter membrane 0.2 µm

Culture of *Colletotrichum gloeosporioides*

The pathogenic fungus *C. gloeosporioides* was isolated from infected chili fruit with anthracnose symptoms characterized by dry brownish-black rot with sunken lesions in fruit skin. The isolation was carried out by the moist chamber method. First the surface sterilization is carried out on symptomatic fruit by washing with sterile aquadest, then the fruit is soaked in a plastic box containing Natrium hypoclorit 1% (NaOCl 1%) for tree minutes and then rinsed with sterile aquadest. The fruit is cut into 0.5 cm size by inserting symptomatic and healthy parts. Five pieces of fruit were placed in a plastic petri dish covered with moistened filter paper and incubated for 48 hours. The fungus that grows on the fruit was isolated in a petri dish containing a PDA medium, the fungal colony that has the characteristic of *C. gloeosporoides* is isolated again until its pure culture is obtained. Furthermore pure cultures are identified macroscopically and microscopically by referring to the literature (Alexopoulos et al. 1995; Agrios 2005).

Inhibition test of culture filtrate against *C. gloeosporoides*

One mL of *Trichoderma* culture filtrate from single or dual culture techniques was mixed evenly with 9 ml of PDA medium at 40^0C and poured onto Petri dishes. Cultures of *C. gloeosporioides* on PDA was cut using a core borer with a diameter of 0.5 cm and placed in the center of a Petri dish. Petri dish was incubated at room temperature until the culture in control covered the entire PDA surface.

Parameters observed

Growth inhibition of C. gloeosporioides *colonies*

Diameter and colony coverage of *C. gloeosporoides* colonies were observed by measuring the diameter of the fungal colonies using a ruler. Colony coverage was

measured using millimeter plastic paper. observations were done every day until up to 12 days after inoculation of *C. gloeosporoides*.

Evaluation of conidial germination and conidial density of C. gloeosporioides

Conidial germination of *C. gloeosporoides* was determined by the method of Junianto and Sukamto (1995). PDA in the form of a plate with an area of about 1 cm^2 and 1-2 mm thickness was placed on a sterile object-glass, then 10 µl of conidial suspension containing 10^6 conidia /mL was transferred on PDA medium. The object-glass was placed in sterile Petri dishes containing a moistened filter paper and incubated at room temperature for 24 hours. Observation was using a light microscope with a magnification of 400 times. Germination was observed by counting the number of conidia that germinated divided by the total number of conidia observed.

Conidial density of *C. gloeosporoides* was determined by preparing; *C. gloeosporoides* culture on PDA medium that has been added with filtrate of *Trichoderma* spp. that incubated for 12 days (the control dish was full colony coverage). Furthermore, the conidia were crushed using a small brush and stirred evenly with distilled water. The suspension was inserted into the test tube, and a dilution of 10^{-1} was made. The number of *C. gloeosporioides* conidia which cultivated on PDA incorporated with culture filtrate of *Trichoderma* spp. was calculated using a hemocytometer. The observation was carried out using a light microscope with a magnification of 400x.

RESULTS AND DISCUSSION

The effect of culture filtrate of *Trichoderma* spp. to the growth of *C. gloeosporioides:* Single culture technique

Culture filtrate of *Trichoderma* spp. incorporated with PDA affected the growth of *C. gloeosporioides* significantly over control (Table 1). Culture filtrates of *Trichoderma* spp. contain secondary metabolites derived from *Trichoderma* spp were able to suppress the growth of *C. gloeosporioides* in various degree. The highest growth suppression was recorded in *Trichoderma* PP2 with the effectivity 67.80%, and *T. koningi* had the effectivity 25.89 % in suppressing the colony coverage r. The highest colony growth was recorded in negative control without culture filtrate of *Trichoderma* spp.

The high ability of culture filtrates of *T. koningii* and *Trichoderma* PP2 in inhibiting the growth of *C. gloeosporioides* was due to the secondary metabolites contain in culture filtrate that has antifungal activity. Vinale et al. (2006) reported that the secondary metabolites of *T. harzianum* strain T22 and T39 have antifungal activity so that they can inhibit the growth of pathogenic fungi like *Leptosphaera muculans*, *Phytophthora cinnamomi*, and *Botrytis cinerea*. In this study, *T. koningii* and *Trichoderma* PP2 have better ability in inhibiting the growth of *C. gloeosporioides* than *T. harzianum*. It is due to different species or isolates in the same species of *Trichoderma* produce different types of secondary metabolites (Wu et al. 2017) so that they have different abilities in inhibiting the growth of pathogenic fungi.

Conidial germination and conidial density of C. gloeosporioides

Culture filtrate of *Trichoderma* spp. showed a significantly different effect on the conidial density and germination of *C. gloeosporioides* conidia (Table 2). *Trichoderma* spp. produce secondary metabolites that able to suppress the formation of *C. gloeosporioides* conidia with the conidial density of 1.3-1.65 x 10^8 /mL and the germination of conidia was in the range 21.33-32.37% which were much lower than the control treatment (4.60 x10^8 /mL) .

In general, culture filtrate of *Trichoderma* spp. reduces the germination of *C. gloeosporoides* conidia. The lowest germination (21.33%) was in the treatment of culture filtrate from *T. harzianum* with the effectiveness of 72.21%. The results of this study showed that reduction of germination caused by culture filtrate of *Trichoderma* spp. was varied. Vinale et al. (2014) reported that viridin, a secondary metabolite produced by *T. koningii* and *T. virens* was able to inhibit conidial germination of the fungi *Botrytis alii*, *C. line*, and *F. cauruleum*. Differences in ability to reduce germination rate by different *Trichoderma* isolates may be caused by different secondary metabolites that were produced so that the ability to inhibit the conidial germination of pathogenic fungi was also different.

Table 1. Effect of culture filtrates of *Trichoderma* spp. on the growth *C. gloeosporioides* colonies in single culture

Treatment	Colony coverage (mm^2)	Effecti-veness (%)	Diameter of colony (cm)	Effecti-veness (%)
Control	4792.30[a]	0	8.87[a]	0
T. viride	4514.50[ab]	5.79	8.00[b]	9.8
Trichoderma PP3	4492.30[ab]	6.26	7.37[c]	16.91
T. harzianum	4230.30[b]	11.72	7.42[c]	16.34
T. koningi	3551.30[c]	25.89	6.60[d]	25.59
Trichoderma PP2	1543.00[d]	67.80	7.85[b]	11.49
	CV = 8.30%		CV = 9.42%	

Note: Mean values having the same uppercase letter in the column do not significantly different at the 5% level by DMRT.

Table 2. Conidial density and germination of *C.gloeosporoides* treated with culture filtrate of *Trichoderma* spp. in single culture

Treatment	Conidial density (x 10^8/mL suspension)	Effecti-veness (%)	Germi-nation of conidia (%)	Effecti-veness (%)
Control	4.60a	0	76.76a	0
T. viride	1.65b	64.13	32.37b	57.82
T. harzianum	1.60b	65.21	21.33c	72.21
T. koningi	1.50bc	67.39	21.94c	71.41
*Trichoderma.*PP2	1.50bc	67.39	31.27b	59.26
*Trichoderma.*PP3	1.30c	71.73	23.74c	69.07
	CV =8.59		CV= 12.32	

Note: The numbers followed by the same uppercase letter on the same column are not significantly different at the 5% level by DMRT

Growth rate (colony diameter) of *C. gloeosporioides* treated with culture filtrate of *Trichoderma* spp. was lower than control. The lowest growth rate was in the treatment of culture filtrate produced by *T. koningii* (Figure 1).

Colony growth of *C. gloeosporioides* on Potato Dextrose Agar medium incorporated with culture filtrate of *Trichoderma* spp. was dominated by mycelium formation while conidia formation was inhibited compared to the control (Figure 2).

The effect of culture filtrate of *Trichoderma* spp. to the growth of *C. gloeosporioides:* Dual Culture Technique

Growth inhibition of C. gloeosporioides

The treatment of culture filtrate incorporation of *Trichoderma* spp. produced by dual culture technique showed a significantly different effect among treatments to the colony coverage and diameter of *C. gloeosporioides* colonies (Table 3). *Trichoderma* spp. produced secondary metabolites that could suppress the growth of *C. gloeosporioides*. Culture filtrate of *Trichoderma* PP2 results in complete growth inhibition of *C. gloeosporioides*, while culture filtrate of *T. harzianum* caused growth inhibition of *C. gloeosporioides* with colony coverage was 1568.80 mm^2 (effectiveness 39.45%), and diameter of colony was 5.37 cm. Colony coverage and diameter of colony in negative control were 4792.30 mm^2 and 8.87 cm respectively.

Dual culture technique results in higher growth suppression of pathogenic fungi because they grew simultaneously on the same media at the same time so that *Trichoderma* during its growth and propagation will produce secondary metabolite compounds maximally for competing against *C. gloeosporoides*. Kumar et al. (2014) reported that secondary metabolites of *T. harzianum* (Th Ahad) and *T. viridae* (O1PP) produced in liquid medium simultaneously with pathogenic fungi were more effective to suppress the growth of *F. oxysporum* f.sp. lenti, *Rhizoctonia solani*, and *Sclerotinia rolfsii*. The higher of the secondary metabolite concentration, the more inhibition of pathogenic growth.

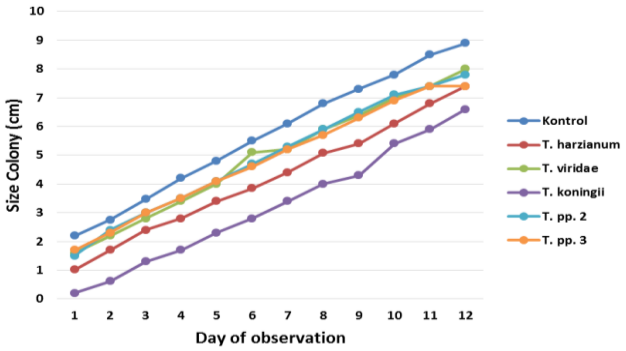

Figure 1. Growth rate (colony diameter) of *C. gloeosporioides* treated with culture filtrate of *Trichoderma* spp produced by Single Culture Technique

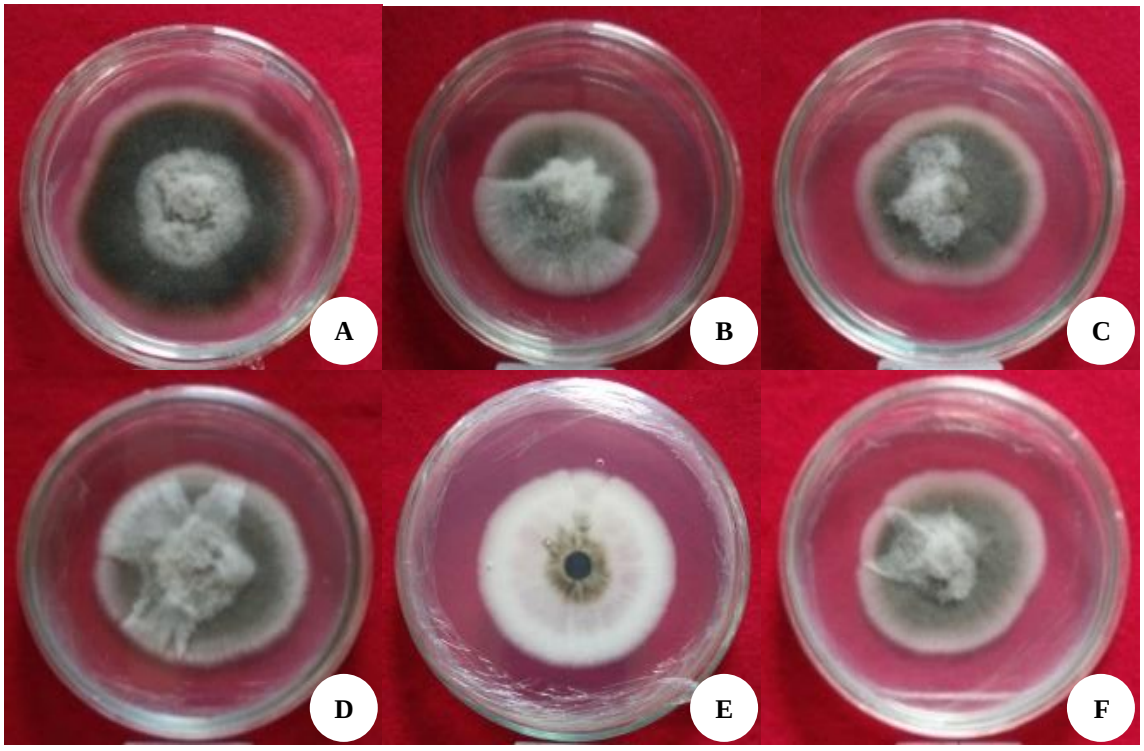

Figure 2. Growth of *C. gloeosporioides* treated with culture filtrate of *Trichoderma* spp at 10 days after inoculation. A. Control, B. *Trichoderma* PP3, C. *Trichoderma harzianum*, D. *Trichoderma koningii*, E. *Trichoderma* PP2, F. *Trichoderma viride*

The culture medium of *Trichoderma* PP2 was able to inhibit the growth of *C. gloeosporioides* completely. It indicated that *Trichoderma* PP2 was superior to other isolates in producing secondary metabolites as antifungal. According to Harman et al. (2004) *Trichoderma* spp. can produce hydrolytic enzymes that can destroy pathogenic cell walls such as chitinase, β 1.3 glucanases, and protease.

The culture medium of *T. koningii* and PP3 result in the growth of *C. gloeosporioides* colonies was slower than culture medium of *T. harzianum* and *T. koningii* treatment (Figure 3).

Table 3. Effect of culture filtrates of *Trichoderma* spp. produced by dual culture method on the growth *C. gloeosporioides* colonies

Treatment	Colony coverage (mm^2)	Effecti-veness (%)	Diameter of colony (cm)	Effecti-veness (%)
Control	4792.30a	0	8.87a	0
T.viride + Cg	4470.50a	6.71	7.85b	11.49
Trichoderma PP3 + Cg	2673.80b	44.20	5.40c	39.12
T.koningii + Cg	2173.80b	54.63	5.95c	32.91
T.harzianum + Cg	1568.80c	67.26	5.37c	39.45
Trichoderma PP2+ Cg.	0.00d	100	0.00d	100
	CV = 9.56		CV = 8.23	

Note: Mean values having the same uppercase letter in the column do not significantly different at the 5% level by DMRT. Cg: *C. gloeosporioides*

Table 4. Conidial density and germination of *C. gloeosporoides* treated with culture filtrate of *Trichoderma* spp. in Dual Culture Technique

Treatment	Conidial density (x 10^8/mL supension)	Effecti-veness (%)	Germi-nation of conidia (%)	Effecti-veness (%)
Control	40.60a	0	76.76a	0
T. harzinum + Cg	19.00b	53.20	34.47b	55.09
T. koningii + Cg	16.50b	59.35	33.70b	56.09
T.viride + *Cg*	16.00b	60.59	36.19b	52.85
*Trichoderma.*PP3 + Cg	8.50c	79.06	32.40b	57.79
*Trichoderma.*PP2 + Cg	0c	100	0c	100
	CV = 11.52		CV= 16.32	

Note: The numbers followed by the same uppercase letter on the same column are not significantly different at the 5% level by DMRT. Cg: *C. gloeosporioides*

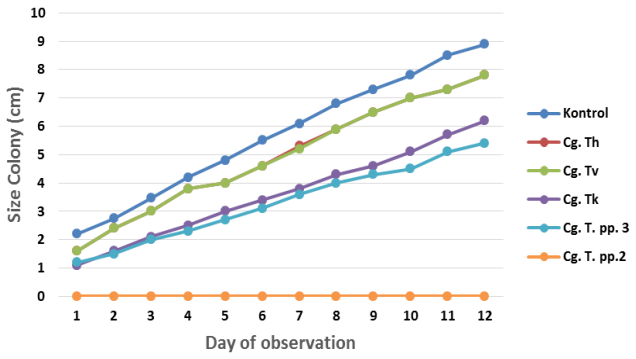

Figure 3. Growth rate (colony diameter) of *C. gloeosporioides* treated with culture filtrate s of *Trichoderma* spp. by dual culture

Conidial germination and conidial density of C. gloeosporioides

The treatment of *Trichoderma* spp in dual culture showed different effects significantly to the conidial germination and conidial density of *C. gloeosporioides* (Table 4). In general, culture medium of *Trichoderma* spp. was able to suppress the growth of *C. gloeosporioides* in various degree. Conidial density in *Trichoderma* spp. treatment was varied from 8.50 to 19.00 x10^8/mL, and conidial germination varied from 32.4 to 34.47%. Treatment with culture filtrate of *Trichoderma* PP2 causes complete inhibition of conidial germination and conidial density, while conidial density and conidial germination in negative control was 40.60 x10^8/mL and 76.76% respectively (Table 4).

Secondary metabolites produced in culture medium by *Trichoderma* PP2, *T. koningii* and *Trichoderma* PP3. were superior to suppress the formation of *C. gloeosporioides* conidia. It is related to the ability to suppress the growth of *C. gloeosporioides* (Figure 3) so that the conidial density and conidial germination were also inhibited. Meena et al. (2017) reported that *T. harzianum* and *T. viride* produce volatile and non-volatile compounds of secondary metabolites that effectively inhibit the growth and spore production of *Fusarium* spp. and *Alternaria alternata*.

Secondary metabolites of *Trichoderma* PP2 from dual culture inhibit the growth of *C. gloeosporioides* completely, so neither mycelium nor conidia were not formed. It indicated that the secondary metabolites of *Trichoderma* PP2 were very potential as antifungal. According to Daniel et al. (2014), secondary metabolites of *T. asperellum* T2-31 extract inhibit sporulation and germination of *F. oxysporum* conidia. The result of this study can be concluded that culture medium or culture filtrate from *Trichoderma* spp produced by single and dual culture used in this study can inhibit the growth of *C. gloeosporoides*. Secondary metabolites of *Trichoderma* PP2 and *T. koningii* were the best in inhibiting the growth, formation, and germination of *C. gloeosporoides* conidia.

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