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MECHANISM ANTAGONISM of *Trichoderma viride* AGAINST SEVERAL TYPES of PATHOGENS and PRODUCTION of SECONDARY METABOLITES

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ABSTRACT

Biological control agents using antagonistic fungi have the ability to inhibit the development of disease-causing pathogens by various mechanisms such as competition for space and nutrients, antibiosis by producing antibiotics in the form of chemical compounds, and parasitism by entangling pathogenic hyphae. Antibiotic mechanism is a condition in which an organism secretes one or more metabolites that have a negative effect on other organisms. One of the fungi that has the ability as an antibiosis is Trichoderma viride, where this fungus secretes secondary metabolites in the form of a viridiol phytotoxin compound. This study conducted to determine the antagonist mechanism of the fungus *T. viride* in suppressing the growth of *Alternaria solani, Fusarium oxysporum, Rhizoctonia solani,* and *Sclerotium rolfsii* which causes disease in some cultivated plants, as well as what compounds *T. viride* possesses in suppressing the growth of other pathogens. This research was carried out at the Plant Disease Laboratory, Department of Plant Pest and Disease, Faculty of Agriculture, Brawijaya University from November 2020 to August 2021. The research was conducted using *Trichoderma viride* as *antagonist fungus* and *Alternaria solani, Fusarium oxysporum, Rhizoctonia solani,* and *Sclerotium rolfsii* as pathogenic fungi. This research consisted of 3 stages, the first stage was rejuvenation and macroscopic and microscopic characterization of pathogenic fungi and antagonist fungi. The second stage is the in vitro antagonist test using the dual culture method using a completely randomized design with 6 replications. The third stage is the phytochemical test of secondary metabolites using 5 test, namely terpenoid and steroid test, the alkaloid test, the flavonoids test, the tannin test, and the saponin test. The results showed that *T. viride* had an inhibitory ability >50% against four types of pathogens. The mechanism of *T. viride* antagonist against four treatments, three treatments belonged to the competition mechanism and one treatment belonged to the microparasite mechanism. The content of secondary metabolites of *T. viride* are steroids and alkaloids.

Keywords: *Trichoderma viride,* antagonist, secondary metabolites.

INTRODUCTION

Biological control agents are living organism whose activities depend on the physiochemical environmental conditions encountered by fungus. Biological control using antagonistic fungi has advantages such as being easy to adapt because it naturally lives in the soil, usually functions as a decomposer of organic matter, and does not cause environmental pollution. Antagonistic fungi have the ability to inhibit the development of disease-causing pathogens with various mechanism such as, through competition for space and nutrients, antibiosis by producing antibiotics in the form of chemical compounds, and parasitism by entangling pathogenic hyphae.

Antibiosis mechanism is one of the mechanisms that occur between antagonistic fungi and pathogenic fungi, where this mechanism releases antibiotic compounds that can inhibit the growth of pathogenic fungi. Antibiosis is a condition which an organism secretes one or more metabolites that have a negative effect on other organisms. The antibiosis mechanism occurs when an empty zone is formed between the pathogenic fungus and the antagonist fungus, there is a change in the shape of the pathogenic hyphae, and produces pigment on the lower surface of the antagonist fungus colony.

There are several antagonistic fungi that have the ability to act as antibiotics against pathogenic fungi, including *Trichoderma viride*, where the antagonist fungi produce secondary metabolites in the form of antibiotic compounds to suppress the growth of microorganisms (Gusnawaty et al., 2014). *T. viride* as an antibiosis mechanism is a parasitic fungus that can attack and take nutrients from other fungi, and can kill or inhibit the growth of other fungi. The fungus *T. viride* secretes antibiotics from the viridiol phytotoxin compound that can inhibit the development of pathogens, parasitize pathogens by direct penetration and also more quickly use O_2, water (H_2O), and nutrients so that they can compete with pathogens (Wahyuni, 2018).

Antagonistic fungi *T. viride* can inhibit other microorganisms by producing secondary metabolites in the form of antibiotic compounds to suppress the growth of microorganisms. Therefore, this study was conducted with the aim of knowing the antibiotic mechanism of the antagonist fungus in suppressing the growth of the pathogens *Alternaria solani, Fusarium oxysporum, Rhizoctonia solani,* and *Sclerotium rolfsii* that cause disease in some cultivated plants, as well as what compounds *T. viride* contains in suppressing the growth of other pathogens.

MATERIALS AND METHODES

Research Time and Place

This research was carried out at the Plant Disease Laboratory, Department of Plant Pest and Disease, Faculty of Agriculture, Brawijaya University from November 2020 to August 2021. This research was conducted using a completely randomized design using one antagonist fungus and four pathogenic fungi, with each treatment being repeated six times

Rejuvenation of Pathogenic Fungi

Plants showing symptoms of *A. solani, R. solani, F. oxysporum,* and *S. rolfsii* were cut as much as 1 cm with half healthy and half sick. The cut plant parts were then sterilized using sterile distilled water twice, alcohol 70%, NaOCl 2% each of which was soaked for 1 minutes, then dried on sterile tissue. The sterilized plant parts were then placed in a petri dish filled with PDA media, then incubated at room temperature to grow fungal mycelium for approximately 7 days.

Rejuvenation of *Trichoderma viride*

The rejuvenation was carried out by taking some of the fungal hyphae that had been grown in PDA media on a petri dish and then inoculation on a new sterile PDA medium in a petri dish. Fungal were incubated at room temperature (25-27^{o}C) for 7 days until the fungal mycelia became abundant.

Characterization of Pathogenic Fungi and Antagonist Fungi

Characterization of fungi *T. viride, A. solani, F. oxysporum, R. solani,* and *S.rolfsii* were characterized by macroscopic and microscopic observations. The macroscopic characterization included the shape, color, and growth of the colony, while the microscopic characterization observed included the structure of hyphae, conidia and conidiophores.

In vitro Antagonist Test with Dual Culture Method

The antagonist activity test using the dual culture method was carried out by placing pieces of the mycelium of the antagonist mold and the pathogenic mold with a diameter of 5 mm, aged 7 days, on PDA media in the same petri dish. Isolates of *A. solani, F. oxysporum, R. solani,* and *S. rolfsii* were planted 3 days earlier than *T. viride,* with each fungus positioned facing each other at a distance of 3 cm. Cultures were incubated at room temperature for 7 days accompanied by the observation of the radial growth of the colonies. The placement of the antagonism test can be seen in Figure 1.

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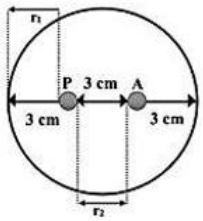

Figure 1. Schematic of placement of antagonistic fungi and pathogenic fungi

Percentage Growth Inhibition (PGI) is determined based on the equation according to Sinaga et al., (2009):

$$\mathbf{P}\ (\%) = \frac{(\mathbf{r_1} - \mathbf{r_2})}{\mathbf{r_1}} \times \mathbf{100}\%$$

Description:
P : Percentage of Inhibitory Power (%).
r1 : Radius of the pathogenic fungal colony away from the antagonist fungal colony.
r2 : Radius of the colony of the pathogenic fungus that is close to the colony of the antagonist fungus.

Antagonist Fungal Fermentation

Pure fungal colonies that have been incubated for 5-7 days are taken 3 pieces with a size of ± 1 × 1 cm. Inoculated into PDB medium as much as 50 mL in a 100 mL Erlenmeyer flask and fermented using a rotary shaker at 150 rpm for 7 days at a temperature of 27ºC (Hasiani et al., 2015).

Phytochemical Test of Secondary Metabolites of *Trichoderma viride*

Terpenoid and Steroid Test

The terpenoid and steroid tests were carried out by taking 4 mL of the sample dissolved in 20 drops of 30% ethanol which was then heated. After being heated, the filtrate formed was allowed to evaporate until the remaining part of the precipitate was left, which was then added with 2 mL of ether. Then 10 drops of the ether fraction were taken which was reacted with 6 drops of acetic anhydride and 2 drops of concentrated sulfuric acid. If the result is green, it indicates that the positive result contains steroids, while if it is red or purple, it indicates that the positive result contains terpenoids (Ergina et al., 2014).

Alkaloid Test

The alkaloid test was carried out using 0.6 mL of sample added with 3 mL of chloroform and 6 drops of ammonia, the sample will be divided into two fractions where one of the fractions is chloroform. Then the chloroform fraction was added with 4 drops of sulfuric acid and after that it was divided into three parts which would be reacted with three types of reagents. Ten drops of Dragendroff's reagent will be added to the first test tube, ten drops of Meyer's reagent will be added to the second test tube, and ten drops of Wagner's reagent will be added to the third test tube. The test positive if Dragendrof's reagent will form a red to orange precipitate, on Meyer's reagent a yellowish white precipitate will be formed, and on Wagner's reagent a brown precipitate will be formed (Wahid dan Safwan, 2019).

Flavonoid Test

Flavonoid test using a diluted sample with a ratio of 1:2. Then 0.6 mL of the sample was taken which would be mixed with 3 mL of methanol and heated at 50ºC for five minutes. Then the heated sample is reacted with concentrated sulfuric acid in a ratio 1:1. If the compound is red, it indicates a positive result containing flavonoids (Ergina et al., 2014).

Tannin Test

The tannin test used a sample that was diluted in a ratio of 1:5 after which the sample was boiled for five minutes. Next, 6 drops of the boiled sample were taken and 6 drops of FeCl3 1%. If the resulting compound changes color to blue or blackish green, it indicates that the positive result contains tannin (Ergina et al., 2014).

Saponin Test

The saponin test was carried out by diluting the sample in a ratio of 1:10 which was then heated for five minutes. Then shaken for 10 seconds and if the results obtained are stable foam for 10 minutes, the results obtained are positive containing saponins (Wahid dan Safwan, 2019).

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RESULTS AND DISCUSSION

Inhibition of *Trichoderma viride* Against Fungal Pathogens

Observation of T. viride inhibitory test calculation on the growth of pathogenic fungi was calculated using the dual culture inhibition formula for 168 hours with observations made every 24 hours. Inhibition test was conducted to determine the potential of T. viridae in inhibiting the growth of pathogenic fungi. The data presented in Table 1 is the percentage data on the inhibition of the fungus T. viridae which was taken from observations for 96 hours to 168 hours.

Table 1. The results of the antagonist test of *T. viridae* on the growth of pathogenic fungi

Pathogenic Fungus	Inhibition (%)			
	96 hour	120 hour	144 hour	168 hour
Rhizocthonia solani	40.30 a	52.34 ab	65.80 ab	82.77 ab
Alternaria solani	42.34 ab	50.58 a	58.80 a	74.29 a
Fusarium oxysporum	60.71 c	71.09 c	80.56 c	95.71 bc
Sclerotium rolfsii	76.11 d	84.40 d	93.92 d	100 c

The numbers followed by the same letter notation are significantly different in the BNT follow-up test at the 5% level.

Antagonistic test of *T. viride* against the growth of pathogenic fungi showed varying results in the percentage of inhibition, the four isolates had an increase in the percentage of inhibition which indicated that the isolates of *T. viridae* were effective in suppressing the growth of pathogenic fungi. Seen in observation for 168 hours, the fungus *T. viridae* showed a positive inhibitory ability with an average of about 70% against four types of pathogens. This is in accordance with Suanda's statement (2019b), that *T. viridae* has fast growth and high ability to suppress the growth of pathogenic fungi. The threshold limit of antagonistic fungi is able to inhibit pathogenic fungi reaching 30% on the surface of a petri dish, then antagonist fungi only have a minimal inhibitory effect in suppressing the growth of pathogenic fungi, but if inhibition is >60%, then antagonist fungi can be said to be able to inhibit the growth of pathogenic fungi.

Based on the graph (Figure 7) the highest ability of *T. viride* in inhibiting the growth of pathogens was in inhibiting *S. rolfsii* which was observed for 168 hours the percentage of inhibition was 100% (Table 1). The results of this observation are in accordance with Suanda's statement (2016a), the requirement for organisms as biological agents is to have the ability to antagonism or to have the ability to inhibit the development of other organisms. The greater the inhibitory power produced, the higher the antagonistic power of the isolate. The difference in inhibition is the difference in the ability of each isolate to inhibit the growth of other organisms. Differences in inhibition are influenced by the type, amount, and quality of the antibiotic or other substances produced by *T. viride*.

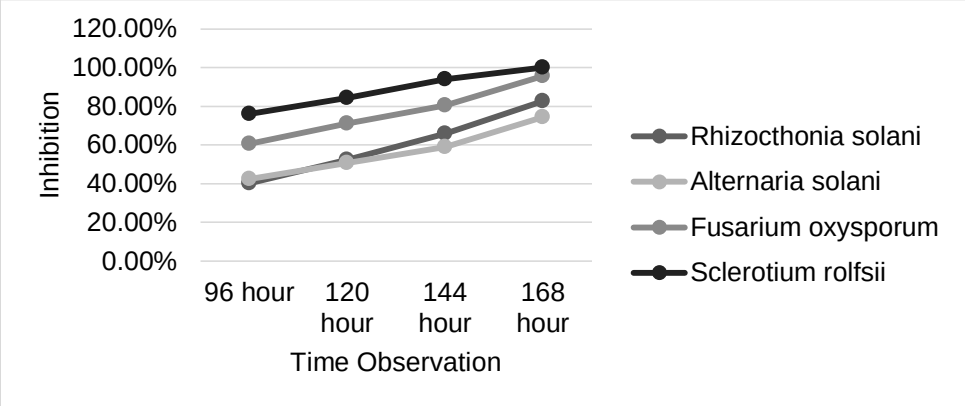

Figure 2. Inhibitory Percentage Curve of Trichoderma viride for 168 Hours

Mechanism of antagonist of *Trichoderma viride* **against Pathogens in vitro**

Mechanism of antagonist *Trichoderma viride* vs. *Alternaria solani*

Based on observations, it was seen that *T. viride* could inhibit the growth of *A. solani,* this inhibition was due to competition in the living space and also the nutrients of the two fungi. On microscopic observation the hyphae of *T. viride* grow side by side with the hyphae of the pathogen *A. solani*. The relatively faster growth of antagonistic fungi is an indicator of the mechanism of nutrient space competition with pathogenic fungi, the faster the growth of antagonistic fungi, it can be said that the antagonistic fungi are more effective in suppressing fungal growth. It can be said that *Trichoderma sp.* has many benefits in inhibiting pathogenic fungi that cause disease in several plants (Agustina et al., 2019).

This antibiosis mechanism is characterized by the discovery of an empty zone (clear zone) between the growth of *T. viride* and *A. solani* fungi. In addition, microscopically, it can be seen that the hyphae of the pathogen have an empty zone between the growths of the hyphae. *Trichoderma sp.* produce volatile and nonvolatile antibiotic compounds that can inhibit functional systems and make pathogens susceptible (Susandi et al., 2018). *Trichoderma sp.* form an antibiosis mechanism because it has an antibiotic substance, glitoxin, and toxic compounds that affect and inhibit the functional system of pathogenic fungi (Ruswandari et al., 2020).

Another mechanism that occurs between *T. viride* and the pathogen *A. solani* is the presence of a parasitism mechanism, this mechanism is characterized by the presence of colonies of T. viride that grow through the pathogen *A. solani.* According to Ruswandari et al. (2020), the mechanism of parasitism is characterized by the presence of antagonistic fungal hyphae that penetrate the hyphae of pathogenic fungi and lyse pathogenic fungi.

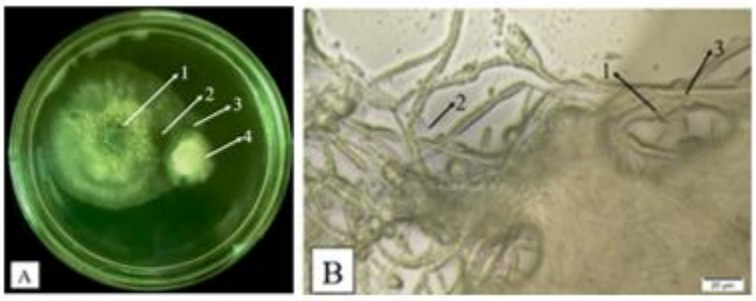

Figure 1. Mechanism of antagonist *Trichoderma viride* against *Alternaria solani*; A. *Trichoderma viride* grown side by side with *Alternaria solani*; (1) *Trichoderma viride*, (2) Empty zone, (3) *Trichoderma viride* that grows through the pathogen *Alternaria solani*, (4) *Alternaria solani*, B. Inhibitory interaction of *Trichoderma viride* with *Alternaria solani*; (1) Hyphae undergo lysis, (2) Hyphae undergo empty zone, (3) Competition between *Trichoderma viride* and *Alternaria solani*

Mechanism of antagonist *Trichoderma viride* vs. *Fusarium oxysporum*

The antagonistic process that occurs between *T. viride* and *F. oxysporum* is due to the competition that occurs between the two fungi grown side by side. Microscopically, it was seen that the hyphae of *T. viride* and the hyphae of the pathogen *F. oxysporum* coexisted and competed with each other. The competition that occurs between the two fungi is due to the need for a place to grow and the same media nutrients between the two fungi (Dwiastuti *et al.,* 2015). Susanti *et al.* (2021b), stated that the inhibition of pathogens in their growth was influenced by the high growth rate of antagonistic fungi in fighting for space and nutrients.

In addition to the mechanism of competition, in testing the mechanism of *T. viride* antagonist in suppressing the growth of the pathogen *F. oxysporum*, a mechanism of parasitism occurred. It was seen that in the petri dish the mycelium of *T. viride* grew penetrated into the mycelium of *F. oxysporum*, and microscopically there was a twisting that occurred in the hyphae of *F. oxysporum* caused by the hyphae of *T. viride*. According to Arsih *et al.* (2015), the fungus *Trichoderma* sp. has the ability to parasitize the mycelium of pathogenic fungi by penetrating the cell wall and entering the cell to take nutrients, this causes the pathogenic fungus to die. *Trichoderma* sp. secrete enzymes and toxins that are toxic to *Fusarium* sp. In addition, *Trichoderma* sp. produce viridian antibiotics, glotoxin, and paraceltin which have

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the ability to destroy fungal cells and the enzymes β (1, 3) glucanase and chitinase which can thin the cell walls of pathogenic fungi.

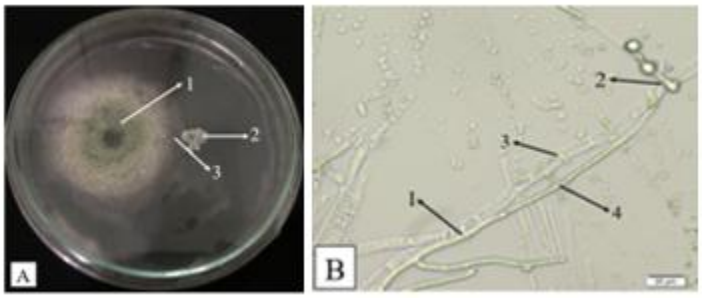

Figure 2. Mechanism of antagonist of *Trichoderma viride* against *Fusarium oxysporum*; *A. Trichoderma viride* grown side by side with *Fusarium oxysporum; (1) Trichoderma viride, (2) Fusarium oxysporum* pathogen, *(3) Trichoderma viride* that grows through the *Fusarium oxysporum* pathogen, *B.* Inhibitory interaction between *Trichoderma viride* and *Fusarium oxysporum; (1)* Competition between *Trichoderma viride* hyphae and *Fusarium oxysporum, (2) Trichoderma viride* hyphae entangling *Fusarium oxysporum* hyphae and *Fusarium oxysporum* hyphae were damaged*, (3) Fusarium oxysporum* hyphae, *(4) Trichoderma viride* hyphae

Mechanism of antagonist *Trichoderma viride* vs*. Sclerotium rolfsii*

The results of the antagonist mechanism test showed that the T. viride isolate tested with *S. rolfsii* had a microparasite antagonist mechanism. The mycoparasite mechanism that occurs is seen that in the petri dish the hyphae of *T. viride* grow through the culture of *S. rolfsii.* According to Sriwati *et al*. (2014), between *Trichoderma* sp. can control pathogens by mycoparasites, namely by parasitizing the mycelium of the pathogen, producing antibiotics, competing for growth space and nutrients, and intervening with pathogenic hyphae. *Trichoderma* sp. have antagonistic mechanisms such as competition for life, parasitism, antibiosis, and lysis.

S. rolfsii in petri dishes did not grow from the beginning of the observation, this is because *T. viride* has the ability to grow faster so that it can suppress the growth of other pathogens grown side by side. This is in accordance with the statement of Amaria *et al.* (2013a), *T. viride* can control soil-borne pathogenic fungi such as Sclerotium rolfsii and Phythium spp. with a microparasitic mechanism and can also induce plant resistance. In addition, T. viride can inhibit the growth of pathogens because this fungus can produce secondary metabolites that function as antifungals, namely trichodermin and 3-4 dihydroxycarotane.

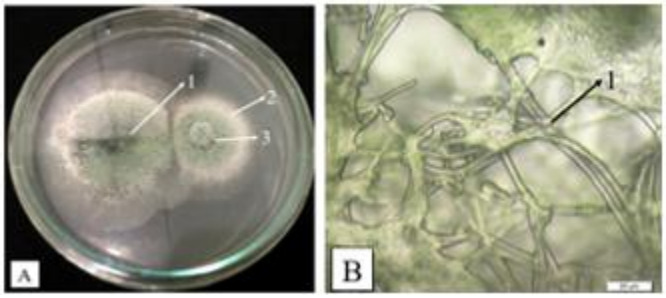

Figure 3. Mechanism of antagonist *Trichoderma viride* against *Sclerotium rolfsii*; A. *Trichoderma viride* grown side by side with *Sclerotium rolfsii*; (1) *Trichoderma viride* mycelium, (2) *Sclerotium rolfsii* mycelium*, Trichoderma viride* mycelium growing on the mycelium of *Sclerotium rolfsii*, B. Inhibitory interaction between *Trichoderma viride* and *Sclerotium rolfsii*; (1) *Trichoderma viride* hyphae wrapped around the hyphae of *Sclerotium rolfsii*

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Mechanism of antagonist *Trichoderma viride* vs. *Rhizoctonia solani*

The growth of *T. viride* showed a faster growth pattern, while the growth of *R. solani* tended to be slower than that of *T. viride.* From the visible characteristics, the mechanism that occurs between *T. viride* and *R. solani* is classified as a competition mechanism. This is in line with the opinion of Chamzurni *et al.* (2011), several species of *Trichoderma* sp. can produce gliotoxin and viridian metabolites which are used as antibiotics. *T. viride* produces an antibiotic compound viridiol phytotoxin which is a compound that is able to inhibit the growth of pathogens, parasitize pathogens by direct penetration and can use O2, water and nutrients faster than pathogens.

The results of observations obtained on the mechanism that occurs between *T. viride* in inhibiting the growth of *R. solani* in addition to the competition mechanism, there is also a mechanism of parasitism. This mechanism is characterized by the presence of mycelium from *T. viride* that grows through the mycelium of *R. solani*. The microparasite mechanism occurs when the mycelium of the fungus *Trichoderma* sp. penetrate the cell wall and enter the cells which then take nutrients from the cell which causes the pathogenic fungi to die (Tarigan *et al.,* 2017).

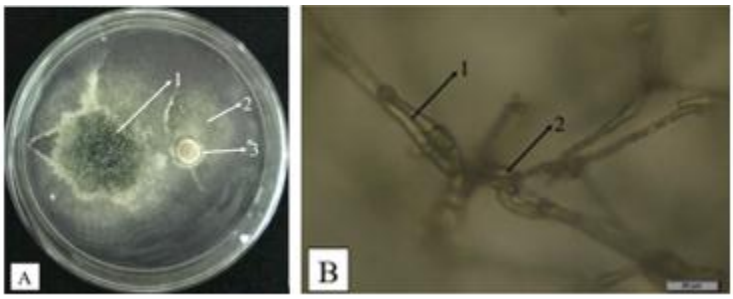

Figure 4. Mechanism of antagonist of *Trichoderma viride* against *Rhizoctonia solani*; A. *Trichoderma viride* grown side by side with *Rhizoctonia solani*; (1) *Trichoderma viride* mycelium, (2) *Trichoderma viride* that grows through the pathogen *Rhizoctonia solani*, (3) Rhizoctonia solani mycelium,
B. Inhibitory interaction between *Trichoderma viride* and *Rhizoctonia solani* (1) Competition between *Trichoderma viride* hyphae and *Rhizoctonia solani* hyphae, (2) *Trichoderma viride* hyphae wrapped around *Rhizoctonia solani* hyphae

Content of Secondary Metabolites *Trichoderma viride*

The results of the qualitative phytochemical test (terpenoid and steroid test, alkaloid test, flavonoid test, tannin test, and saponin test) against the fungus T. viride used as antifungal.

Table 2. Phytochemical Test Results (Secondary Metabolites) of *Trichoderma viride*

Phytochemical Test	Results	Information
Terpenoid/Steroid	+	Green solution
Alkaloid		
Dragendrof	+	Orange precipitate
Meyer	+	White precipitate
Wagner	+	Brown precipitate
Tanin	-	Yellow solution
Flavonoid	-	Faded brown solution
Saponin	-	There is no foam

Based on the results of Table 2, it can be seen that the secondary metabolite compounds possessed by the fungus *T. viride* are steroids and alkaloids. The results obtained are in accordance with the statement of Hasanah *et al.,* (2015a), endophytic pathogens can secrete secondary metabolites such as steroids, terpenes, flavonoids, alkaloids, quinones, and phenols. This compound has the potential as a bioactive compound. *T. viride* contains alkaloid compounds, which these compounds have antimicrobial activity that can inhibit the growth of pathogenic fungi. The results of this observation are in accordance with the statement of Wulandari (2011), alkaloids are compounds that can inhibit fungal nucleic acid

biosynthesis which can cause fungi to not grow and eventually die. Alkaloids are cyclic organic compounds that contain nitrogen with a negative oxidation number with a limited level of distribution in living things. According to Illing *et al.* (2017), alkaloids can be classified into 3 groups, namely true alkaloids (having a basic heterocyclic nitrogen ring derived from amino acids), combined alkaloids (the derivatives of amino acids and nitrogen do not form heterocyclic rings, have basic properties), and pseudo alkaloids (plant bases containing heterocyclic nitrogen and having no biosynthetic relationship with amino acids, pseudo-alkaloids derived from terpenoid compounds derived from acetic acid and polyketonic acid). Phytochemical screening of *T. viride* using 30% ethanol, ether, acetic anhydride, and concentrated sulfuric acid containing steroid compounds found in plants can act as a protector. The results of this observation are in accordance with the statement of Ikalinus *et al.* (2015), the color change that occurred in the steroid test was due to an oxidation reaction in the terpenoid/steroid group through the formation of conjugate double bonds (pentaenyl compounds). In addition, steroid compounds can work to repel some pathogens and also attract some other pathogens.

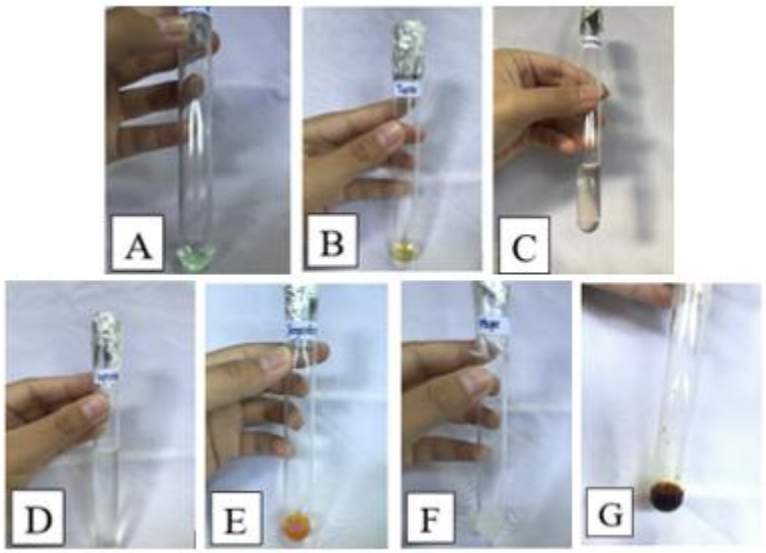

Figure 5. Phytochemical test results of secondary metabolites of *Trichoderma viride*; A. Steroid, B. Tanin, C. Flavonoid, D. Saponin, E. Dragendroff, F. Meyer, G. Wagner

CONCLUSION

1. The average percentage of inhibition of *T. viride* in suppressing the growth of pathogens A*. solani, F. oxysporum, R. solani,* and *S. rolfsii* has a yield of 70%. Based on the tests conducted, it can be seen that the fungus *T. viride* has a good ability to suppress the growth of disease-causing pathogens in plants.
2. In testing the antagonist mechanism of *T. viride* against four types of disease-causing pathogens, it was explained that the mechanisms possessed by *T. viride* include the competition mechanism, the antibiosis mechanism, and the parasitism mechanism.
3. The content of compounds owned by *T. viride* in suppressing the growth of disease-causing pathogens are steroids and alkaloids.

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