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The In Vitro Antifungal Impact of Secondary Metabolites from *Trichoderma* sp. on *Fusarium* sp.

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ABSTRACT

Fusarium sp. is a significant pathogenic fungus responsible for causing wilt in various plants, including chilies, eggplants, and shallots. One approach to address this issue involves utilizing secondary metabolites from the fungus *Trichoderma* sp. These metabolites were applied at concentrations of 10%, 20%, and 30% alongside a fungicide called M-Dithane 45, following the recommended dosage specified on the packaging. This research aims to evaluate the impact of these secondary metabolites in suppressing the *Fusarium* sp. pathogen. The study occurred at the Plant Health Laboratory I within the Faculty of Agriculture at Universitas Pembangunan Nasional "Veteran" East Java from October to December 2022. It adopted a single-factor, Completely Randomized Design (CRD), and data analysis was carried out using R-studio software. In vitro analysis was conducted through the dual culture technique on a PDA medium, assessing inhibition percentages and observing the morphology of *Fusarium* sp. fungi. The results demonstrated that the highest inhibition percentage of *Fusarium* sp. occurred at a 30% concentration, reaching 26% compared to the control group. Morphological examinations of *Fusarium* sp. indicated that all treatments affected the growth of abnormal hyphae, including bending, curling, coiling, and lysis.

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1. Introduction

Plant wilt disease is one of the essential plant diseases caused by *Fusarium* sp. It is also a significant disease in several plants, such as eggplant, chili, tomato, and other Solanaceae (Djaenuddin, 2011). Losses caused by wilt disease are reported up to 50% and even threatened with crop failure. According to Semangun, (2004), the disease can kill the entire plant, especially during the rainy season, and the cultivation area is easily flooded with water.

Control of *Fusarium* wilt generally uses chemical pesticides because they are considered easy and effective. Using chemical pesticides in the long term can harm the ecosystem and the consumer. Thus, innovation is needed in utilizing biological control agents to reduce chemical pesticide usage. A safe control method is using bio-agents that do not pollute the environment (Agustina *et al*., 2019).

Trichoderma is a fungus with potential applications as a natural agent for controlling pathogens. *Trichoderma* sp. employs various mechanisms to hinder the proliferation of

harmful organisms, encompassing competition, parasitic actions, antibacterial and antifungal activities, and cell lysis (Purwantisari & Hastuti, 2009). As per the findings of Mukherjee *et al.*, 2012), *Trichoderma* sp. secretes enzymes, antibiotics, and toxic compounds, which not only suppress but also eliminate pathogens. The *Trichoderma* fungus generates secondary metabolites with antibacterial and antifungal properties, such as polyketides, pyrones, and terpenes (Laila *et al.*, 2012). They can be harnessed as biopesticides, leveraging compounds like antibiotics, toxins, hormones, and enzymes. Antibiotic compounds include cytosperone, viridins, mannitol, trichodermol, kiningins, and 2-hydroxymalonic acid (Vinale *et al.*, 2014). This investigation seeks to assess the impact of secondary metabolites on inhibiting the *Fusarium* sp. pathogen.

2. Methodology

The research occurred between October and December of 2022 at Plant Health Laboratory I, located within the Faculty of Agriculture at Universitas Pembangunan Nasional "Veteran" East Java. To facilitate the research, a range of equipment and instruments were utilized, including petri dishes with a diameter of 9 cm, loop needles, cork borers, shakers, centrifuge tubes, analytical scales, centrifugation apparatus, funnels, microscopes, and a Bunsen burner.

The materials used in this study were *Trichoderma* sp. from Pelaihari, South Kalimantan. *Fusarium* sp. isolate from Tarik, Sidoarjo. PDA media, PSE (*Potato Sugar Extract*) media, Aquades, cotton, tissue, 25mm PTFE Syringe filter 0.22μm (all pure), 70% alcohol, Whattman paper, M-Dithane 45 fungicide, label and wrap.

This research employed a Completely Randomized Design (CRD) featuring a solitary factor, incorporating 5 treatments of Control (K), M-Dithane-45 Fungicide, 10% Concentration, 20% Concentration, and 30% Concentration. Each experiment was repeated 4 times.

2.1 *Fusarium* sp. Isolation

Fusarium sp. was obtained from the root base of eggplant, showing *Fusarium* wilt symptoms. The stem's bottom is divided in the center, then sliced into small sections, sterilized with 70% alcohol sterile distilled water, and dried using sterile tissue. The plant components were subsequently placed into petri dishes filled with solid PDA medium and allowed to culture for around one week. The fungi were then purified on PDA media incubated for 7 days and observed macroscopically and microscopically (Rabha *et al.*, 2014).

Rejuvenation of *Trichoderma* sp.

Trichoderma sp. fungi rejuvenated on PDA media by inoculating *Trichoderma* sp. using a cork drill and an ose needle. Rejuvenation is carried out to obtain isolates at a young age and still active in metabolism.

2.2 Extraction of Secondary Metabolites *of Trichoderma* sp.

A strain of *Trichoderma* sp. fungi was grown using PSE medium, resulting in a spore density of 108 spores, which were subsequently inoculated in quantities of five into a 250 ml media volume. The mixture underwent agitation for seven days at 120 revolutions per minute at room temperature, as documented by Soesanto *et al.* in 2014. Afterward, centrifugation was carried out at 10,000 revolutions per minute for 30 minutes to separate the supernatant from the pellets. The supernatant was further clarified through filtration using a 0.22 μm syringe

filter to obtain a transparent filtrate. The process of separating suspensions during the secondary metabolites production followed the steps described by Sukapiring *et al.* in 2016 Antagonist Test on Potato Dextrose Agar (PDA) Media In the laboratory setting, PDA media was utilized for testing through the antagonist test method, which was carried out by placing filter paper face to face and *Fusarium* sp. 5mm in size. The secondary metabolites were dissolved using sterile distilled water and soaking the filter paper at each concentration for 30 minutes. In the fungicide treatment, use the dosage according to the recommendations listed on the packaging label, which has previously been diluted using 10 ml of sterile distilled water. Filter paper (A) was placed on the PDA media at a distance of 3 cm from the edge of the petri dish. *Fusarium* sp. cut using a cork borer and placed opposite the filter paper. Placing pieces of *Fusarium* sp. (P) was carried out at a distance of 3 cm from the edge of the petri dish (Ningsih *et al.*, 2016)

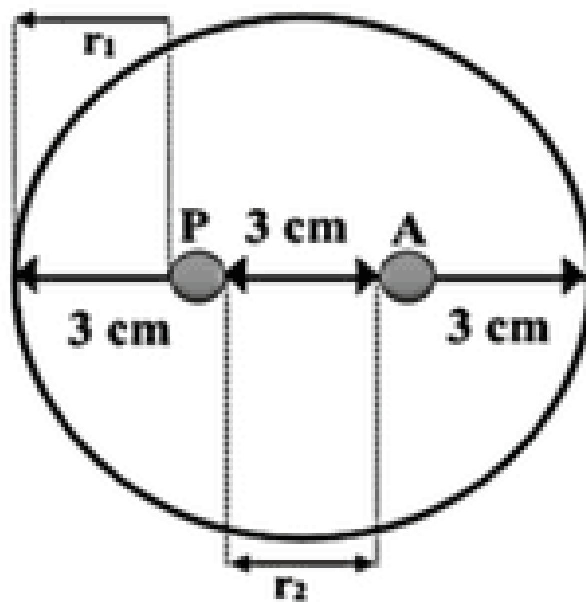

Figure 1. *Trichoderma* sp. Metabolite Antagonist Test against *Fusarium* sp. invitro
Note: A (filter paper) and P (Patogen *Fusarium* sp.)

2.3 Observation Parameters

The parameters in the antagonist test are the antagonistic mechanism and the percentage of inhibition. Observation of antagonistic mechanisms was carried out macroscopically and microscopically. Macroscopic observation was carried out by observing the inhibition zone between cultures. Microscopic examinations involved extracting segments of mycelium from the interaction zone between the two fungi and subsequently positioning them on a glass surface for observation through a microscope.

Calculation of the percentage of inhibition of growth of the fingers by the formula (Putro *et al.*, 2014).

$$I\ (\%) = \frac{(R1 - R2)}{R2} \times 100\%$$

Information:
I = Percentage of inhibition
R1 = The pathogenic fungal colony's radius that is directed away from the metabolites
R2 = The pathogenic fungal colony's radius, which direction is close to metabolites
Data analysis

Analysis of Varience (ANOVA) then analyzed the data obtained to determine whether each treatment had an effect. If significantly different, proceed with the BNJ Test using the *R-Studio Application*

3. Results and Discussion

3.1 Isolation of *Fusarium* sp.

The isolation results of *Fusarium* sp. on PDA media macroscopically show that it has the characteristics of a white colony with spreading margins and has a fibrous mycelium-like cotton accompanied by a yellow color in the center of the colony (Figure 2. a). By Syaifudin, (2020), one of the *Fusarium* was found to have white colonies on the top, the bottom surface of the colony was cream colored, and the margins spread. According to Sholihah *et al.*, (2019), *Fusarium* sp. isolate has sporodochium, which, when in large quantities, will cause the mushroom colony to change from white to yellow-orange.

Microscopic observation showed the cultures had round, single-celled microconidia and crescent-shaped macroconidia with 3-5 partitions (Figure 2. b). Sastrahidayat, (2013) states that many microconidia are produced by fungi in all conditions, single or double-celled, not insulated and round. Macroconidia are curved and long, with narrow ends having three or five partitions.

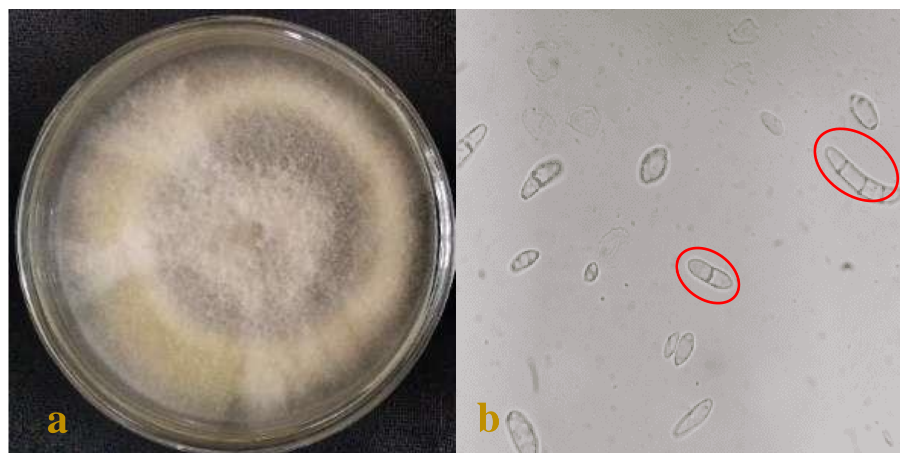

Figure 2. Morphology of *Fusarium oxysporum* a) 7 days old colony on PDA media b) *Fusarium* sp. 400x magnification

3.2 Isolation of *Trichoderma* sp.

Trichoderma sp. fungi isolate grows with white hyphae at the beginning of its age, then after 4 days, it changes color to dark green and has a concentric shape (Figure 3. a). This aligns with the viewpoint expressed by Wijaya *et al.*, (2012) who characterize the *Trichoderma* sp. fungus colony has a white color at the initial age of isolation, then changing color to yellow to dark green at a later incubation age.

Microscopic observation was carried out by taking hyphae or fungal spores using an ose needle, placing them on the preparation that had been dripped with sterile aquadest, covering them using an *object glass,* and observing them using a microscope. The microscopic feature is that the conidiophore branches form a pyramid-like shape, each branch having a phialid (Figure 3. b). Round green conidia are attached to the ends of the conidiophores, and conidia are also formed in clusters with short fialid stalks (Ajayasree & Borkar, 2018).

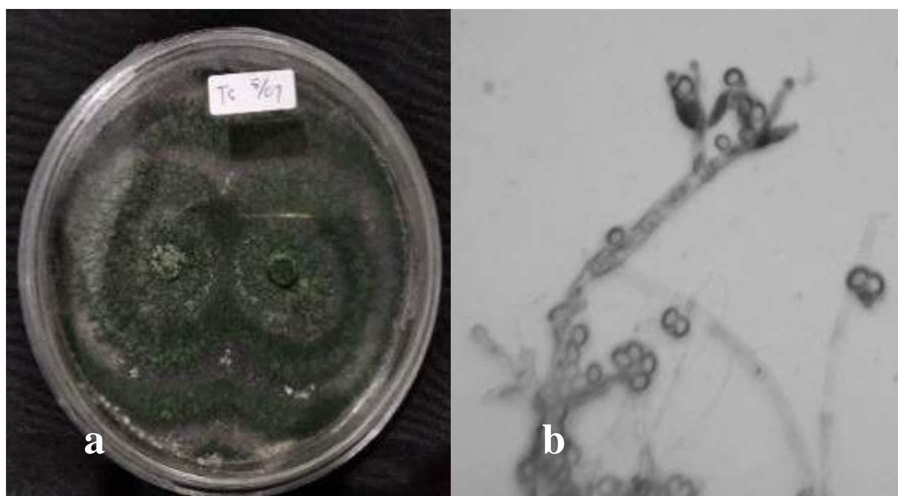

Figure 3. (a) *Trichoderma* sp. isolates 7 days old on PDA media (b) microscopic observation *of Trichoderma* sp. 400x magnification

3.3 Result of *Trichoderma* sp. Secondary Metabolite Antagonism Test against *Fusarium* sp. on PDA Media

The antagonist test was observed for 7 days after inoculation (DAI). The outcomes of the antagonist assessment using PDA media indicated that various levels of secondary compounds from *Trichoderma* sp. and fungicides can impede the growth of *Fusarium* sp. At the same time, the control did not experience inhibition, and it was seen that the colonies filled the petri dishes (Figure 4).

The inhibition test results of the secondary metabolites of *Trichoderma* sp. against pathogenic fungi *Fusarium* sp. had an effect on the control and between treatments (Figure 4). The findings from in vitro experiments indicated that the secondary metabolites under investigation effectively hindered the proliferation of the pathogenic fungus *Fusarium* sp.

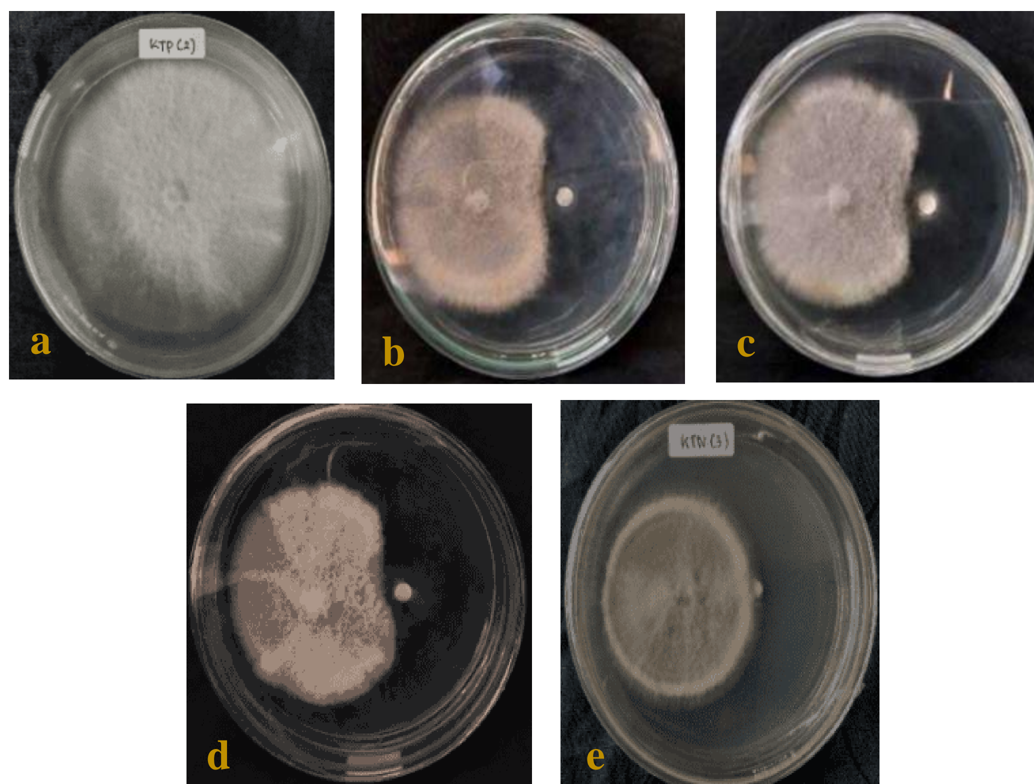

Figure 4. Inhibition of secondary metabolites of *Trichoderma* sp. against *Fusarium*. sp. invitro a). control b). concentration of 10% c). concentration of 20% d). concentration of 30% e) M-Dithane 45 fungicide.

The secondary metabolite of *Trichoderma* sp., which had more significant inhibition on the 7th day of observation, was a concentration of 30%, which was equal to 26% which was then followed by a concentration of 20%, equal to 24% and 20% in the fungicide treatment whereas, at a concentration of 10% the value was 17%. In the Fungicide (KTN) treatment, it had an inhibitory percentage of 20%, whereas in the KTP treatment (without treatment), it had an inhibitory percentage of 0%. The inhibition test results of the secondary metabolites of *Trichoderma* sp. against pathogenic fungi *Fusarium* sp. are presented in (Figure 5).

Growth inhibition of *Fusarium* sp. is thought to occur due to the antibiosis mechanism of secondary metabolites of the fungus *Trichoderma* sp. contained in the culture medium. Antibiosis is a common mechanism in antagonistic fungi due to the antibiotic compounds they produce so that they can block the pathogenic fungi's growth zone (Sriyanti *et al*., 2015). Saxena *et al*., (2015) stated that *Trichoderma sp.* can generate toxins (antibiotics) capable of eliminating other microorganisms even when present in minimal amounts. This statement is confirmed by Vinale *et al*., (2014), who state that the *Trichoderma* sp. secondary metabolite can produce enzymes, hormones, antibiotic compounds, and toxins. The antibiotic compounds contain 2-hydroxy malonate acid, cytosperone, kiningins, mannitol, trichodermol, and viridins. Factors that affect the ability of antagonistic fungi to produce antibiotics are the number of microorganisms, temperature, pH, and substrate type (Contreras-Cornejo *et al*., 2016)

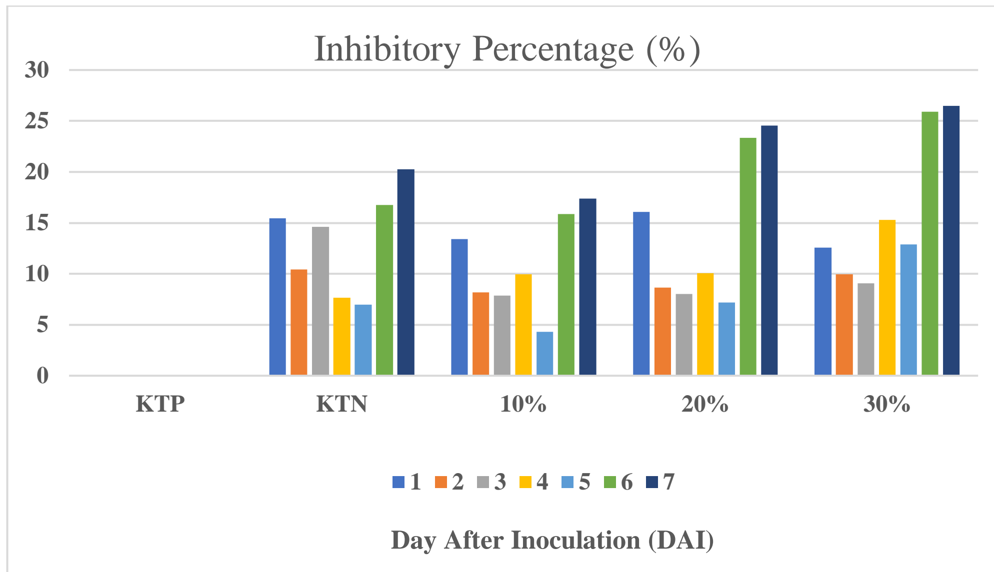

Figure 5. Test Results for The Inhibition Of Secondary Metabolites of *Trichoderma* sp. Against Pathogenic Fungi *Fusarium* Sp.

The percentage value of inhibition *of Fusarium* sp. was found at a concentration of 10%, showing the highest yield of 26%. However, the size of the inhibition zone is at most 30%, so it is said that the inhibition power still needs to be stronger. This follows the statement of Ratnasari & Isnawati, (2014) The inhibition of a biological agent with an inhibition percentage of less than 30% has a weak inhibitory effect. If more than 60% of the biological agents have a strong inhibitory category.

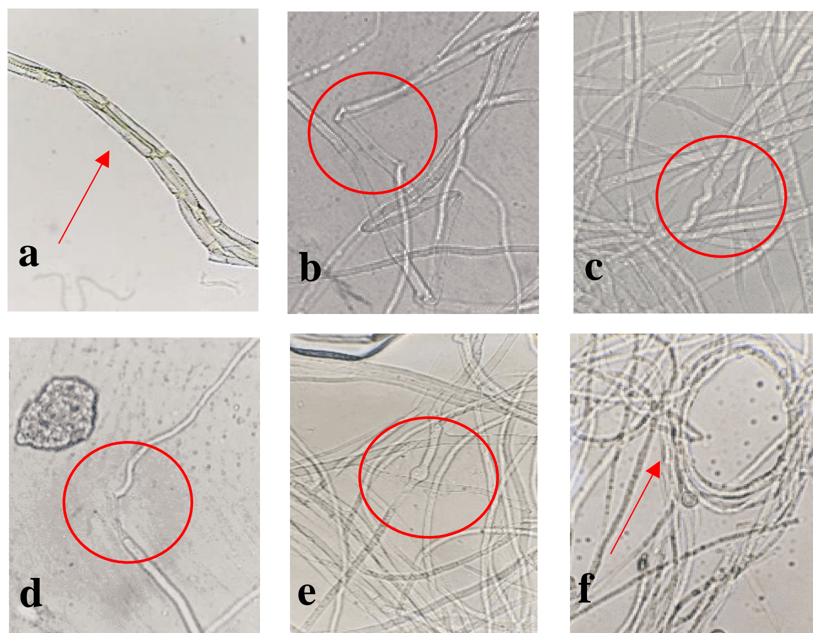

Figure 5. Hyphae *of Fusarium* sp. as a result of secondary metabolite treatment *of Trichoderma* sp. a) normal hypae b) hyphae bent c) hyphae curled d) hyphae lysis e) hyphae swollen f) hyphae coiled (Magnification 1000x)

Hyphae morphology of *Fusarium* sp. treated with secondary metabolites had different interactions by experiencing abnormal development, namely bending, curling, coiling, and lysis. Whereas in the fungicide treatment, there were also circular abnormal hyphae. This is following research by Fadiji & Babalola (2020) that Endophytic fungi employ a strategy involving the rotation and penetration of their hyphae, facilitated by the secretion of lyase enzymes capable of breaking down the cell walls of pathogens, enabling them to capture these harmful microorganisms. For example, *Trichoderma* sp. can capture and penetrate the hyphae of a known plant pathogen, such as Rhizoctonia solani; observations are associated with biocontrol activity. (Asril, 2011), added that abnormal reactions could occur due to interactions between external structures or direct contact between biological agents and pathogens and between substances or compounds produced by biological agents in the form of secondary metabolites with pathogens.

The antagonism is thought to be a reaction of antibiotic compounds produced by *Trichoderma* sp. secondary metabolites. In addition, the abnormality is an attempt by the pathogen to defend itself. This is following the statement of Sunarwati and (Sunarwati & Yoza, 2010) Malformations that occur in hyphae are a form of reaction with antibiotic compounds and as an effort to defend themselves. According to Lestari, (2019), this abnormality is caused by fungi-producing compounds that can inhibit or damage the structure of the cell wall of fungal hyphae. It will affect the growth of pathogenic fungi as a whole.

4. Conclusion

The secondary metabolites of *Trichoderma* sp. affect inhibiting the fungus *Fusarium* sp. antagonist in vitro testing on PDA media showed the best treatment at a concentration of 30% with an inhibition percentage of 26% compared to the control treatment.

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