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EFFECTIVENESS TEST OF THE *TRICHODERMA* SP. AND *PSEUDOMONAS FLUORESCENS* SECONDARY METABOLITES AS BIOLOGICAL CONTROL AGENCIES OF THE *ONCOBASIDIUM THEOBROMA* PATHOGENS IN THE LABORATORY

Christina Oktora Matondang[1], Syamsafitri[2]*, Nurhayati[2] and Yenni Asbur[2]

[1]Master's Student in Agrotechnology, Faculty of Agriculture, Islamic University of North Sumatra, Medan, Indonesia.
[2]Agrotechnology Study Program, Faculty of Agriculture, Islamic University of North Sumatra, Medan, Indonesia.

***Corresponding Author: Syamsafitri**
Agrotechnology Study Program, Faculty of Agriculture, Islamic University of North Sumatra, Medan, Indonesia.

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ABSTRACT
Oncobasidium theobroma is a pathogen that causes Vascular Streak Dieback in cocoa plants. Vascular streak dieback or VSD is an important disease of cocoa and can makes plants become bare and decrease fruit production. Many attemps have been made to control VSD disease on cocoa but have not been succesfull yet. This study aimed to examine the potency of secondary metabolite produced by Trichoderma sp. and Pseudomonas fluorescens solely or in combination as biological control agencies of the pathogen O. theobroma in the laboratory at Balai Besar Perbenihan dan Proteksi Tanaman Perkebunan (BBPPTP) Medan. A completely randomized design was used yo assessed four treatments i.e Trichoderma sp., P. fluorescens, Trichoderma sp. + P. fluorescens secondary metabolites and control which was repeated six times. The observation parameter is the length of the colony of pathogen O. theobroma on the culture medium or PDA. The results showed that the secondary metabolites of Trichoderma sp., P. fluorescens, Trichoderma sp. + P. fluorescens secondary metabolites were able to suppress or inhibit the colony length of the pathogen O. theobroma which causes VSD disease in the laboratory. The percentage of inhibition of colony length by secondary metabolites of Trichoderma sp., P. fluorescens or a combination of both respectively was 92.55%; 72.05% and 63.6%.

KEYWORDS: *Vascular streak dieback (VSD), antagonistic microbes, bioactive compound, laboratory tests.*

INTRODUCTION

Cocoa (*Theobroma cacao* L.) is one of the plantation commodities that has potential to contribute the foreign exchange in Indonesia (Hartarto, 2019). Various processed cocoa bean products can contribute to the country's foreign exchange. However, currently this contribution is decreasing along with the decline in Indonesian cocoa production from year to year, since 2018 to 2020 (Ahmad, 2020). Based on data from the Central Statistics Agency (BPS, 2022), Indonesian cocoa production in 2018 was recorded at 767,400 tons. Furthermore, in 2019 it decreased to 734,700 tons and in 2020 it was 713,400 tons. This shows that Indonesia's cocoa production potential is still far from the expected achievement, about around 1 million tons with an area of 1.7 million ha (BPS, 2022).

One of the causes the production was declined, beside inferior seeds and old plants, is the lower productivity of cocoa. Low productivity is caused by several things, one of which is attacks by pest and disease plants or called OPT. OPT attacks can not only reduce production yields, but can also reduce the quality of a product. According to Defitri (2018), one of the important disease that attacks cocoa plants from seedlings to producing plants is *Oncobasidium theobromae*. This pathogen can cause *Vascular Streak Dieback* (VSD) (Semangun, 2000).

Yunasfi (2018) said that *O. theobroma* attacks are detrimental because they can cause chlorosis on the leaves, thereby disrupting the photosynthesis process and causing stunted growth. Even the growth of lateral shoots in the axils of fallen leaves will die too. Over time, the plant's leaves will run out from the tips and the last the plant will die. This is due to abnormal changes in chloroplast function, which can inhibit the development of young tissue. The cause of this abnormality is due to

the toxin released by the *O. theobroma* pathogen (Semangun, 2000).

Control of VSD disease is generally carried out by applying synthetic chemical fungicides. However, continuous application of fungicides can cause negative impacts not only on the environment, but also on the environment and human health (Ika, 2020). Beside it, it is difficult for fungicides to reach the disease because it is located in the vascular bundles of wood (xylem) (Hendra, et al., 2019). Matondang (2018) added that generally existing systemic fungicides are transported through sieve vascular bundles (phloem), so they will not affect the fungus. Infection occurs through young leaves that grow quickly, making it difficult to protect evenly with protectants.

Biological control agents that are often studied are *Trichoderma* spp. and *Pseudomonas fluorescens*. *Trichoderma* spp. has been proven to be able to overcome various plant diseases (Munir et al., 2013; Kumar et al., 2017; Ghazanfar et al., 2018). Meanwhile, *P. fluorescens* is also used and is able to biologically control various plant diseases (Sivasakthi et al., 2014). Antagonistic microbes (Rashid et al., 2016) and chemicals (Muturi et al., 2017), will be directly degraded by sunlight when applied in the field, thereby limiting their capabilities. One strategy to avoid degradation of biological agents needs to be developed the use of the secondary metabolites they produce (Mutawila et al., 2015).

Trichoderma spp. produces secondary metabolites consisting of various bioactive compounds (Mukherjee et al., 2012b; Vinale et al., 2014). *P. fluorescens* produces secondary metabolites that contain antibiotics and are PGPR (Plant Gowth Promoting Rhizobacter), which inhibit the growth of pathogens and stimulate plant growth (Sahu et al., 2018; Alsohim, 2020). The use of these bacteria has reportedly provided positive results on growth and production in agricultural plants (Jain & Das, 2016). However, secondary metabolites from these two microbes have never been tested either singly or in combination to inhibit the colony length of the pathogen *O. theobroma* in the laboratory. The aim of this research is to examine the effectivity of secondary metabolites produced by *Trichoderma* sp. and *P. fluorescens* either singly or in combination as biological control agencies pathogens of *O. theobroma* in the laboratory.

MATERIAL AND METHODS

Preparation of antagonist isolates

Two antagonistic microbes used, *Trichoderma* sp. and *P. fluorescens*, collection of the Balai Besar Perbenihan dan Proteksi Tanaman Perkebunan (BBPPTP) Medan. *Trichoderma* was grown again on PDA medium and incubated at room temperature for 5 days (Yun et al., 2017), while *P. fluorescens* was grown on King's B medium and incubated at room temperature for 3 days (Lamichhane & Varvaro, 2013). Specifically for *Trichoderma*, the antagonist is then propagated in cracked corn by washing the cracked corn thoroughly first, then putting it in 50 g of plastic, sterilizing with an autoclave at 121°C, 15 psi pressure for 30 minutes, and cooling. *Trichoderma* as many as 3 cork drill plugs (diameter 6 mm) were inoculated into cold sterile cracked corn in plastic using a sterile spatula, then the plastic mouth was closed with staples and incubated for 6-7 days at room temperature (Soesanto et al., 2014).

Preparation of secondary metabolites

Two plastic wraps of cracked corn (@ 50 g) that had *Trichoderma* growing on them were dissolved in 1 L of sterile water. Next, stir until the spores are released from the corn and filtered. The filtered water is ready for use. The medium used to produce secondary metabolites was a mixture of rice washing water and coconut water (4:1, v/v) and 10 g L^{-1} of sugar, then cooked until boiling, filtered, and put into sterile jerry cans and cooled. After the medium had cooled, 100 mL L^{-1} conidium solution was added (Soesanto et al., 2020). Next, the jerry cans were closed tightly and incubated by shaking manually until homogeneous for 14 days (Hudson et al., 2021). After incubation, the conidia density was calculated using a haemocytometer to determine the level of dilution, and a conidia density of 106 conidia mL^{-1} was obtained. The solution was then filtered using Whatman filter paper no. 1 to separate the fungal propagules from the supernatant. Next, the conidia solution was centrifuged at a speed of 9,000 rpm for 2 minutes to separate the conidia from the crude extract solution of secondary metabolites (Shehata et al., 2019). *Trichoderma* secondary metabolites are ready for application.

The stages of making *P. fluorescens* secondary metabolites are as follows; The snails are collected, then the shells are broken and the meat is taken. The meat is cleaned and weighed in 400 g L^{-1} water plus 2 g L^{-1} shrimp paste and boiled until soft (Soesanto et al., 2014). Next, the broth is filtered, placed in sterile jerry cans and cooled. Then *P. fluorescens*, which was harvested from King's B by adding 10 mL of sterile water, was added to the broth medium, shaker for 3 days at a speed of 150 rpm (Soesanto et al., 2014). After incubation, the spore density was calculated using multilevel dilutions, using Nutrient Agar (NA) media and obtained a density of 109 upk mL^{-1}. The supernatant was prepared by centrifuging the antagonist suspension at 5,000 rpm for 30 minutes (Decoin et al., 2014). The supernatant obtained is ready for use.

Preparation of culture media

In the laboratory testing, the culture medium used is Potato Dextrose Agar (PDA) media which is already in the form of melted flour. Take 39 grams of PDA flour, put it in 1 liter of water, cook it while stirring until it boils. Next, the PDA solution was put into 10 erlenmeyer tubes, 100 ml each and sterilized in an autoclave at 121°C, 15 psi pressure for 30 minutes.

Laboratory test

PDA solution in an erlenmeyer tube that has been sterilized in the autoclave, then put into laminar air flow. When the PDA solution begins to cool, then drop 1 mL of the secondary metabolite solution of *Trichoderma* sp, *P. fluorescens* or a combination of both into each erlenmeyer tube and stir until evenly mixed. After mixing thoroughly, 10 mL of the PDA solution was poured into a petri dish and left to harden. Then the PDA media is left for 1x24 hours to ensure it is not contaminated with other pathogens. Next, the 7 day old pure culture of *O. theobromae* was planted in a petri dish containing PDA media + secondary metabolite solution.

Research design

This study used a completely randomized design with secondary metabolite treatment of *Trichoderma* sp. (P1), *P. fluorescens* (P2), *Trichoderma* sp. + *P. fluorescens* (P3), and control (P0). The number of repetitions was six; Each replication consisted of two petri dishes, so the total was 48 petri dishes.

Observation variables

Observations were made by counting the length of colonies on PDA culture media every day, 7 observations. Measurements were carried out from the time the *O. theobroma* pathogen was inoculated until the colony growth in the control treatment filled the petri dish. Next, the percentage of colony length inhibition was calculated using formula (1).

$$Percentage\ of\ inhibitory\ power\ (\%) = \frac{colonies\ diameter\ before\ (cm) - colonies\ diameter\ after\ (cm)}{colonies\ diameter\ before\ (cm)}\ x\ 100\% \quad .. (1)$$

Data analysis

Data were analyzed using analysis of variance at a confidence level of 95%. If the differences were significant between treatments, it was continued with BNJ at the 95% level.

RESULTS AND DISCUSSION

Effect of secondary metabolites of *Trichoderma* sp. and *P. fluorescens* on the length of *O. theobromae* colonies in the laboratory.

Data on the growth of the colony length of the pathogen *O. theobromae* on PDA media that has been treated with secondary metabolites of *Trichoderma* sp. and *P. fluorescens* were obtained from observations 7 times. Colony length data is presented in Table 1.

Table 1: Data on the Growth of Colony Length (cm) of the O. theobroma Pathogen on PDA Media at the BBPPTP Medan Laboratory (dai).

Treatment	*The colony length of the O. theobroma pathogen on the … observation (dai)*						
	I	II	III	IV	V	VI	VII
P0	0,000	2,475	4,550	5,042	5,425 [a*)]	6,167 [a]	6,708 [a]
P1	0,000	0,000	0,083	0,192	0,333 [b]	0,400 [b]	0,500 [b]
P2	0,000	1,233	1,375	1,442	1,517 [b]	1,608 [b]	1,875 [b]
P3	0,000	1,342	1,592	1,683	1,775 [b]	2,067 [b]	2,442 [b]

*) Numbers in the same column followed by different letters indicate significantly different at the 5% level based on the LSD test

Table 1, 7 days of observation shows that the length of the *O. theobroma* pathogenic colonies on media treated with antagonistic microbial secondary metabolites, either alone or in combination, was significantly different when compared to the control. The lowest colony length for the pathogen *O. theobroma* was located on PDA media treated with secondary metabolites of *Trichoderma* sp. and *P. fluorescens* compared to controls. These two biological control agencies are types of fungi and bacteria which the contain ingredients can be prevent and suppress the growth of useless pathogens such as *O. theobroma*. Secondary metabolite solution of *Trichoderma* sp. able to release antibiotic compounds such as gliotoxin, glioviridine, trichodermol and 1,3-β-glucanase (Arya and Perello, 2010). This statement was confirmed by Vey et al., (2001), who stated that the mechanism of the antibiotic compounds gliotoxin and glioviridine actions can affects and inhibits protein synthesis and disrupts the integrity of cell membranes.

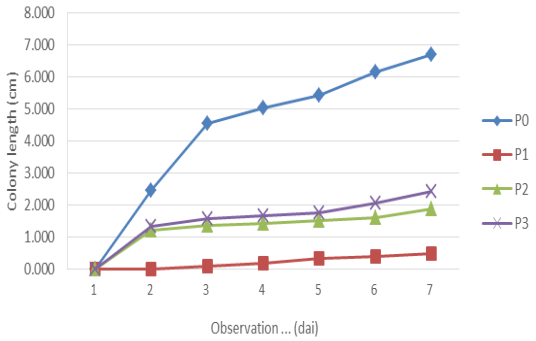

Figure 1: Development of the *O. theobroma* Pathogen Colony Lengths in each Treatment.

Note: P1 = secondary metabolite of *Trichoderma* sp., P2 = secondary metabolite of *P. fluorescens*, P3 = combination secondary metabolite of *Trichoderma* sp. + *P. fluorescens*, dan P0 = kontrol

Figure 1 shows that the development of colony length was slower in media treated with secondary metabolites of *Trichoderma* and *P. fluorescens*. Meanwhile, on the control media, the growth of colony length was faster. The delay in increasing colony length on treated media according to Soesanto (2008) is because these two metabolites contain contents that can suppress pathogen growth, where the contents have several mechanisms. Like *Trichoderma* antagonistic fungi against pathogens, namely parasitism, lysis, antibiosis and space competition. This parasitic characteristic makes the *Trichoderma* fungus able to effectively suppress the extensive growth of *O. theobroma* fungal colonies on PDA media.

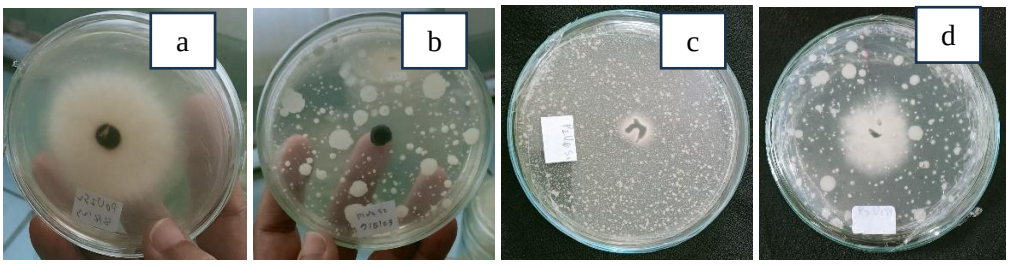

Figure 2: Growth of *O. theobroma* Pathogen Colonies in each Treatment on PDA.

Figure 2 (b, c, d) shows that the growth of *O. theobroma* colonies on treated PDA was not significant. This is different from control (a), which continues to increase. This is because *Trichoderma* secondary metabolites can be used directly to control pathogens. Soesanto (2020) added that *Trichoderma* filtrate is able to control plant pathogens on PDA media. Penetration of pathogen hyphae through the formation of an appressorium, production of cell wall degrading enzymes, and parasitization of the contents of the pathogen cell wall (Mukherjee et al., 2012). Degradation of pathogen cell walls during mycoparasitism is mediated by a suite of hydrolytic enzymes including proteases, chitinase, and β-(1,6)-glucanase.

Effect of secondary metabolites of *Trichoderma* sp. and *P. fluorescens* to inhibit the length of *O. theobromae* colonies in the laboratory.

With this increase in colony length, the inhibition of secondary metabolites of *Trichoderma* sp. and *P. fluorescens* on the growth of colonies of the pathogen *O. theobroma* on PDA media can be calculated. The results of the statistical analysis of inhibition of colony length growth of pathogens that cause VSD can be seen in Table 2.

Table 2: Data on the Percentage of Inhibition the Colony Length of the Pathogen *o. Theobroma* in the Laboratory.

Treatment	The percentage (%) of inhibition the colony length of *O. theobroma* on the … (dai)						
	I	II	III	IV	V	VI	VII
P0	0 a	0 a	0 a	0 a	0 a	0 a	0 a
P1	0 a	100 d	98,168 d	96,198 d	93,856 d	93,514 d	92,547 d
P2	0 a	50,168 c	69,780 c	71,405 c	72,043 c	73,919 c	72,050 c
P3	0 a	45,791 b	65,018 b	66,612 b	67,281 b	66,486 b	63,602 b

*) Numbers in the same column followed by different letters indicate significantly different at the 5% level based on the LSD test

Table 2, observations from 2-7 days show that the inhibition of the colony length of the *O. theobroma* pathogen on the treated media was significantly different when compared to the control. Application of secondary metabolites of *Trichoderma* sp. and *P. fluorescens*, both alone and in combination, were effective in inhibiting the growth of *O. theobroma* pathogen colonies between 63.60-92.54%. This is because secondary metabolites of *Trichoderma* sp. is a biological control agency that has ability to inhibit the growth of pathogens (Radder, 2019). Inhibition is carried out by dominating living places and food sources and destroying the cell walls of the antagonist fungy. Beside that, this fungus can also produce several antibiotic compounds such as alametichin, paracelsin, trihotoxin which can destroy antagonist fungus cells by destroying the permeability of cell membranes, as well as chitinase and laminarinase enzymes which can cause lysis of the cell walls of antagonist fungi.

Degradation of pathogen cell walls during microparasitism is mediated by a suite of hydrolysis enzymes including β-(1,6)-glucanase, chitinase and protease (Mutawilla et al., 2015). *Trichoderma* sp. produces secondary metabolites consisting of cellulolysis, xylanolysis, chitinolysis and β-1,3-glucanolysis enzymes. Apart from that, it also produces non-ribosomal peptides such as peptaibiotics, siderophores, gliotoxin and gliovirin as well as several lysis enzymes. The ability to produce large amounts of secondary metabolites makes *Trichoderma* sp. successful as a biocontrol agent. Mycoparasite *Trichoderma* sp. as an antagonist and kills pathogenic fungi with the combined action of bioactive compounds and hydrolysis enzymes that lyse the cell walls of pathogenic fungi (Ghazanfar et al., 2018).

As well as the secondary metabolites of *P. fluorescens* can produce siderophores, pterins, pyrroles, phenazines and various other antibiotic compounds which can kill and inhibit the growth of pathogens (Soesanto, 2015). Siderophores are able to bind and take up iron from the environment, thereby reducing its availability to the pathogen and preventing its growth. Pterins have the ability to suppress pathogen growth in various ways, including interfering with nucleic acid synthesis, inhibiting important enzymes, and damaging pathogen cell membranes. *P. fluorescens* is able to synthesize pterin and use it as an antimicrobial compound to fight pathogens. Pyrrole compounds have strong antimicrobial properties. The mechanism of pyrrole compounds is by disrupting the function of microbial cell membranes, inhibiting protein synthesis, and causing DNA damage, all of which lead to microbial death.

P. fluorescens produces phenazine compounds such as phenazine-1-carbinol, phenazine-1,6-dicarbonyl, and phenazine-1,7-dicarbonyl (Sivasakthi et al. 2014). These compounds have strong antimicrobial activity against various plant pathogens, including fungi and bacteria. Phenazin works by various mechanisms, including damaging microbial cell membranes, producing free radicals that damage DNA, and inhibiting vital enzymes.

Alsohim, A.S. (2020) stated in their research that *P. fluorescens* also produces various other antibiotics such as phytohormones, lipopeptides, and complex compounds such as pyoluteorin and 2,4-diacetylphloroglucinol (DAPG). These antibiotics work by interfering with various vital functions in pathogenic microbial cells, such as cell membrane synthesis, protein synthesis, and nucleic acid synthesis, which ultimately causes the death of the microbe. By utilizing a combination of these compounds, *P. fluorescens* is able to effectively fight pathogens and protect plants. Both secondary metabolites of *Trichoderma* and *P. fluorescens* contain bioactive compounds that function as antifungals, PGPR and induce plant resistance to attack by disease-causing pathogens (Ghazanfar et al., 2018; Sivasakthi et al., 2014).

Combined secondary metabolite treatment of *Trichoderma* sp. and *P. fluorescens* showed significant differences although all experienced an increase (Table 2). This condition is thought to indicate that the number or quantity of bioactive compounds in the combined secondary metabolites is not much due to synergism or the mutual neutralizing effect of the bioactive compounds in the combined secondary metabolites. In accordance with the opinion of (Ghazanfar et al., 2018) that there are interactions between bioactive compounds because the combination of several of these compounds is synergistic, additive or antagonistic.

CONCLUSION

Secondary metabolites produced by *Trichoderma* sp. and *P. fluorescens* had a significant effect in inhibiting the long growth of the pathogenic *O. theobroma* colony in the laboratory. The percentage of inhibition of the colony length of the pathogen *O. theobroma* by secondary metabolites of *Trichoderma* sp, *P. fluorescens* alone or in combination was 92.55%, 72.05% and 63.6%, respectively.

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