

# Potency of secondary metabolites of *Trichoderma asperellum* and *Pseudomonas fluorescens* in the growth of cocoa plants affected by vascular streak dieback

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**Abstract.** Simamora M, Basyuni M, Lisnawita. 2021. Potency of secondary metabolites of *Trichoderma asperellum* and *Pseudomonas fluorescens* in the growth of cocoa plants affected by vascular streak dieback. *Biodiversitas* 22: 2542-2547. Vascular streak dieback (VSD) is the main disease in cocoa caused by *Oncobasidium theobromae*. Using secondary metabolites of *Trichoderma asperellum* and *Pseudomonas fluorescens* is one of the methods to control disease in cocoa plants. The aim of this research is to evaluate the potency of secondary metabolites of *T. asperellum* and *P. fluorescens* to control VSD and study its effect on the growth of cocoa plants. The study used a non-factorial, completely randomized design (CRD) with eight treatments and four replications. The secondary metabolites were applied every week for ten times. The results showed that the secondary metabolites of *T. asperellum* and *P. fluorescens* were effective in controlling VSD. The combination of organic fertilizer and secondary metabolite of *P. fluorescens* and *T. asperellum* as found to be the best treatment to increase cocoa growth and the number of shoots increased by 43.58 shoots.

**Keywords:** *Oncobasidium theobromae*, *Pseudomonas fluorescens*, *Trichoderma asperellum*, vascular streak dieback

## INTRODUCTION

Vascular streak dieback (VSD) is an important wood vessel disease on cocoa caused by *Oncobasidium theobromae* (Samuels et al. 2012). This fungus (Basidiomycetes) penetrates through the young leaves and then enters the network of wood vessels (xylem). Within 6 to 16 weeks (depending on the age and variety of the cocoa plant), symptoms appeared on the second and third leaves of the shoot (Harni et al. 2017). The most characteristic symptoms of the disease are green-spotted leaf chlorosis or necrotic blotches and blackening of infected xylem in the vascular traces at the leaf scars resulting in the abscission of infected leaves (Samuels et al. 2012).

The disease is invariably fatal if the infection occurs in the main stem of seedlings or clonal plants, but if the infection reaches the main stem of mature plants, the disease is usually fatal only in susceptible genotypes (Guest and Keane 2018). In susceptible cocoa, the fungus grows through the xylem down into the main stem and can kill a mature cocoa tree (Samuels et al. 2012). The disease can cause a decrease in the quality and quantity of cocoa production and attack all stages of the plant, from the seedling to the productive stage (Ekowati et al. 2009). According to Harni et al. (2019), the development of the disease is due to the interaction of several factors, such as planted clones, a wetter climate, improper plant cultivation systems (spacing, shade trees, terracing, and drainage), and minimal plant maintenance (crop pruning and shade, fertilization, garden sanitation, and pest and disease control).

Attempts have been made to control chemically, but maximum results have not been found, as VSD disease occurs in the bundle of wood vessels (xylem), making it difficult to access fungicides. Meanwhile, according to Syahnen (2011), systemic fungicides are usually transported through phloem, thus they cannot hit the fungus. Therefore, other alternatives are needed to control this disease. One of them is using secondary metabolites of biological control agents.

Secondary metabolites, secreted by entomopathogenic fungi, are a rich source of bioactive chemicals, including polyketides, non-ribosomal peptides, polyketide-peptide hybrid metabolites, and terpenes. Many of these secondary metabolites have been reported to have antifeedant and insecticidal properties (Elbanhawey et al. 2019; Subbanna et al. 2019). Keswani et al. (2019) stated that microbial pesticides are considered as imperative alternatives to chemical pesticides as they have high host specificity, biodegradability, and environmental safety.

Many studies have been conducted to explore the biocontrol capacity of biological agents to produce antibiotics that make them a target for the biological control of plant diseases, such as *Trichoderma* sp. and *Pseudomonas fluorescens*. According to Vinale et al. (2014), *Trichoderma* sp. produces hydrolytic enzymes such as  $\beta$ -1,3 glucanase, chitinase, and cellulase, which can dissolve pathogenic cell walls. Secondary metabolites of *Trichoderma* sp. can reduce the intensity of the disease by up to 62%, caused by *O. theobromae* (Hutapea 2017). Furthermore, Soesanto et al. (2005) reported that *P. fluorescens* P19 and *P. fluorescens* P20 could suppress

fusarium wilt disease in tomato plants by 23.42% and 28.20%, respectively. Besides, Soesanto et al. (2010) stated that *P. fluorescens* P60 could be beneficial as it acted as plant-growth-promoting rhizobacteria and also has a tendency to increase plant height and root length. *P. fluorescens* P60 antagonist produces indole acetic acid which acts as growth support to increase the plant's dry weight. The objective of this study was to evaluate the effectiveness of secondary metabolites of *T. asperellum* and *P. fluorescens* for VSD and their effects on cocoa growth.

## MATERIALS AND METHODS

### Study area

The research was conducted in the cocoa plantation at Lau Mulgap Village, Selesai, Langkat District, North Sumatra, Indonesia, from July to September 2020. The plant samples were determined by random sampling of plants that showed symptoms of VSD attack, such as distinct leaf color toothless twigs, pimples, or rashes on twigs. Each sample plant was given a sign/label to facilitate observation.

### Experimental design

This study used a non-factorial completely randomized design (CRD) with eight treatments and four replications. The treatments were as follows: P0 (control/without treatment), P1 (organic fertilizer), P2 (secondary metabolites of *T. asperellum*), P3 (secondary metabolites of *P. fluorescens*), P4 (organic fertilizers + secondary metabolites of *T. asperellum*), P5 (organic fertilizers + secondary metabolites of *P. fluorescens*), P6 (secondary metabolites of *T. asperellum* + secondary metabolites of *P. fluorescens*), and P7 (organic fertilizers + secondary metabolites of *T. asperellum* + secondary metabolites of *P. fluorescens*).

### Production of secondary metabolites

*Trichoderma asperellum* and *P. fluorescens* isolates used in this study were identified and collected from the Field Laboratory of the Center for Seedlings and Plantation Protection, Medan. Preparation of secondary metabolites from *T. asperellum* and *P. fluorescens* was based on the method of Soesanto et al. (2005).

### Provision of organic fertilizer

The organic fertilizer used in this research was flour organic fertilizer (Nitrogen (0.69%), P<sub>2</sub>O<sub>5</sub> (3.18%), C-organic (17.02%), C/N (24.66%), pH (8.72%), and water content (18.87%). 5 kg per stem fertilizer was sprinkled on the stem area of the cocoa plant used as sample plants (based on dosage recommendations by the producer of Pamorganic Mas (CV. Utama Karya Tani).

### Treatment applications

All cocoa plants were first treated with dolomite to improve soil pH so that plants could easily absorb nutrients from the soil. Moreover, maintenance pruning was also performed for all sample trees. 400ml secondary metabolites of each biological control agent were applied by root infusion technique at four points at 7 days of interval. A root infusion was carried out by inserting the roots of cocoa plant into a plastic bag containing a suspension of secondary metabolites of biological control agents (Figure 1.A). Furthermore, the plastic bag was covered with dry leaves (Figure 1.B).

### Phytochemical analysis

A qualitative phytochemical analysis was performed to test the phenolic components such as saponin, tannin, and glycoside after the seventh week of application. Saponin analysis was carried out based on the modified method of Harbone (1984). Tannin analysis was carried out based on the modified method of Moelyono (1996). Glycoside analysis was performed based on the modified method of Kristianti (2008). The instrument used was the GCMS-QP2010 Shimadzu. The column used was DB-5MS (non-polar column). Its length was 30 m, diameter 0.25 mm, injector temperature 250 °C, detector temperature 280 °C, program temperature 40 °C/2 (increments of 10 °C per minute to 280 °C/3), pressure 68 Kpa, flow rate of 0.9 mL/min, and velocity 34.2 L.

### Observation parameters

The observation parameters included phytochemical analysis, level of efficacy, and increase in the number of shoots.



**Figure 1.** Application of secondary metabolites of biological control agents on cocoa roots. A. Root infusion using plastic bag containing secondary metabolites suspension, B. Covered with dry leaves

### Data analysis

The observational data were analysed with analysis of variance at an error level of 5%. If there was a significant difference between treatments, then it is continued with the Tukey's test at the 5% level (Sastrosupadi 2000).

## RESULTS AND DISCUSSIONS

### Phytochemical analysis

The results of the phytochemical analysis showed that all treatments contained phenolic compounds in the form of saponins, tannins, and glycosides. The content of saponins, tannins, and glycosides in the combined application treatment of *T. asperellum* and *P. fluorescens* secondary metabolites was higher than that in control (Table 1).

Phenolic compounds are found naturally in plants. Their function is to defend plants from pathogenic infections. Plant phenolics and their extracts can be excellent inhibitors of many foodborne pathogens (Gyawali and Ibrahim 2014; Zambrano et al. 2019). Inhibition of this signaling process can contribute to the biological control of pathogenic organisms and bacterial toxins causing food deterioration and/or poisoning (Nazzaro et al. 2013). Mandal et al. (2010) stated that phenolic compounds act as agents in plant defense. Phenolic will be synthesized by plants when plant receptors recognize the presence of potential pathogens (Newman et al. 2007) by conserved pathogen-associated molecular patterns (PAMPs), leading to PAMP-triggered immunity (Zipfel 2008). As a result, the progress of the infection is restricted long before the pathogen gains complete hold of the plant (Nicaise et al. 2009).

Plant-derived phenolics, such as phenolic acids, flavonoids, stilbenes, and tannins, can inhibit the growth and activity of many microorganisms, including food-related pathogens as well as clinically important bacteria, fungi, and protozoa (Daglia 2012; Schmidt et al. 2012; Li et al. 2014). Since the different molecules vary in their structure and chemical composition, they can display various antimicrobial effects, such as permeabilization and destabilization of the plasma membrane or inhibition of extracellular enzymes. Moreover, these mechanisms of action differ from traditional antibiotics, which could make

plant phenolics effective against drug-resistant pathogens (Górniak et al. 2019).

In this study, saponins, tannins, and glycosides were high in the combined application treatment of *T. asperellum* and *P. fluorescens*. This is presumably because the secondary metabolites of *T. asperellum* and *P. fluorescens* in the tested cocoa plants were able to increase the saponins, tannins, and glycosides in the plants. This statement is in accordance with the results of research by Soesanto et al. (2013) that the application of antagonistic microbes can increase the content of phenolic compounds in plants.

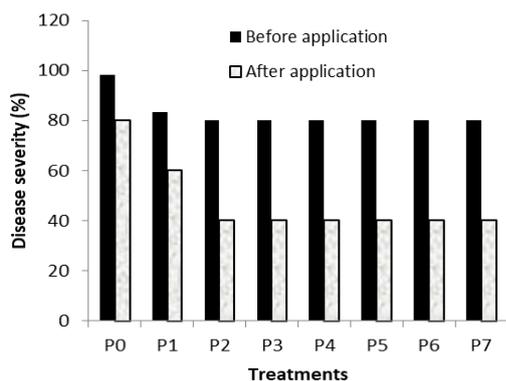
The results showed that secondary metabolites of *T. asperellum* and *P. fluorescens* were quickly absorbed by cocoa plants as they were treated with the root infusion method. This condition increases the phenol content in plants. Agrios (2005) stated that the increase in phenols in the plants because the secondary metabolites of antagonistic fungi are absorbed by plants and translocated systemically to all parts of the plant, giving rise to substances that are responsible for affected resistance, including phenol compounds. In addition to antagonistic fungi, Soesanto and Termoshuizen (2001), and Jankiewicz and Koltonowicz (2012) stated that *P. fluorescens* colonize roots to stimulate plants, increasing the production of secondary metabolite compounds so that they play a role in plant resistance. Hanada et al. (2010) reported that *Trichoderma* spp. is a plant growth promoter and biocontrol agent.

The combined treatment of *T. asperellum* and *P. fluorescens* secondary metabolites increases the amount of phenol in plants, proving that combination treatment can increase phenol compounds in tested cocoa plants, and also affects resistance to plant pathogens. The disease severity was found to be lower in the combined treatment of *T. asperellum* and *P. fluorescens* secondary metabolites compared to control (Figure 2). Soesanto et al. (2013) stated that phenol compounds are responsible for plant resistance to pathogen attack. Phenolic compounds have gained attention in food research as possible growth inhibitors of foodborne pathogens; many individual phenolics have promising anti-quorum sensing potential and can suppress biofilm formation and toxin production of food-related pathogens (Takó et al. 2020).

**Table 1.** Phenolic components in cocoa plants

Treatments	Phenolic compounds in cocoa plants		
	Saponin	Tannin	Glycoside
P0: Control (no treatment)	+	+	+
P1: Organic fertilizer	+	+	+
P2: Secondary metabolite of <i>T. asperellum</i>	++	++	++
P3: Secondary metabolite of <i>P. fluorescens</i>	++	+++	+++
P4: Organic fertilizer + secondary metabolite of <i>T. asperellum</i>	++	++	++
P5: Organic fertilizer + secondary metabolite of <i>P. fluorescens</i>	++	+++	+++
P6: Secondary metabolite of <i>T. asperellum</i> + <i>P. fluorescens</i>	+++	+++	+++
P7: Organic fertilizer + secondary metabolite of <i>T. asperellum</i> + <i>P. fluorescens</i>	+++	+++	+++

Note: (+) sign indicated the intensity level of the colour. + : Low, ++ : Medium, +++ : High



**Figure 2.** The effectiveness of secondary metabolites in reducing the disease severity of VSD in cocoa plants

*Trichoderma* sp. can stimulate plant-growth and biocontrol potential because disease severity was higher in control than other treatments that were directly proportional to the saponins, tannins, and glycosides in plants. This proves that the presence of phenolic compounds is very influential on plants attacked by pathogens. Lattanzio et al. (2006) reported that phenolic compounds in plants are secondary metabolites played a major role in the mechanism of plant resistance to fungal pathogens and insect herbivores attack.

#### Level of efficacy

The results of phytochemical analysis revealed that the treatment of secondary metabolites is quite effective in controlling VSD disease with an effective value of 40%. The application of secondary metabolites of *T. asperellum* and *P. fluorescens* either alone or in combination gives the same effectiveness (P2, P3, P4, P5, P6, and P7; Figure 2). The effectiveness of secondary metabolites is good enough if the level of efficacy is greater than or equal to 30%, provided that the level of damage in plants treated with secondary metabolites tested is lower than the level of damage in the control treatment (Aini 2014).

#### Growth of shoot (The number of shoots)

The growth parameters recorded in cocoa shoots were increase in number of shoots. It was measured by compare

number of shoots before and after treatments. The results of the analysis of shoot growth variance are shown in Table 2.

Based on Table 2, the number of shoots that appeared between treatment P0 (control) and P1 (organic fertilizer) was significantly different; P1 treatment and secondary metabolites (P2, P3, P4, P5, P6, and P7) did not significantly different, and P0 treatment was significantly different with P1, P2, P3, P4, P5, P6, and P7.

Shoot growth (increase in number of shoots) after treatment between P2, P3, P4, and P6 was not significantly different but highly significantly different for P0 and P1. Treatments between P2, P3, P4, and P6 were not significantly different from treatment P7 but significantly different from P5.

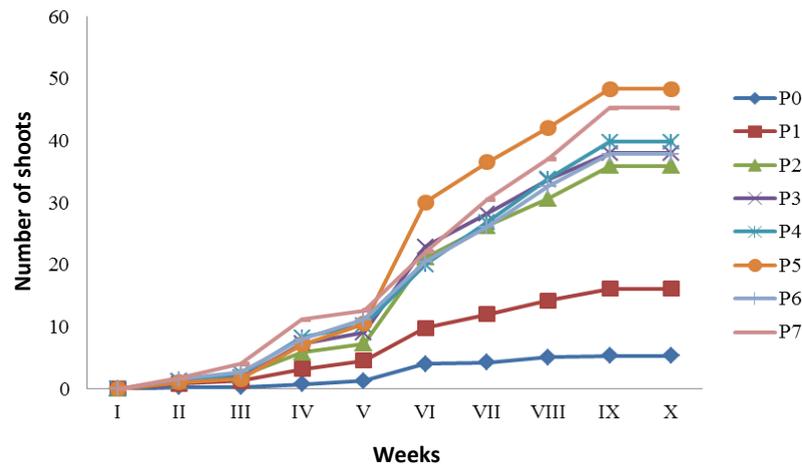
The application of secondary metabolites can increase the number of shoots. The highest increase in number of shoots was recorded in treatment P5 (47.25 shoots) followed by treatment P7 (43.58 shoots), treatment P4 (38.58 shoots), treatment P3 (36.83 shoots), treatment P6 (36.25 shoots) and P2 treatment (34.75 shoots). While the lowest number of shoots i.e. (5.08 shoots) and (15.33 shoots) were recorded in P0 treatment and P1 treatment, respectively (Figure 3).

From Figure 3, it is evident that the number of shoots increased every week in all treatments. An increase in the number of shoots was not significant in the treatment without secondary metabolites (P0 and P1) but was found to be highly significant in the treatment with secondary metabolites (P2, P3, P4, P5, P6, and P7). The number of shoots did not increase in P0 treatment, as disease severity of VSD was very high, so the shoot growth could not achieve optimum results. As a result, leaf growth could not run properly, and if left unchecked, the plant would have died. According to Hutapea (2017), the attack of *O. theobromae* that causes chlorosis in leaves can interfere in the photosynthesis process, inhibiting growth. Furthermore, New lateral shoots will grow in the axillary of fallen leaves, but the lateral shoots will not grow normally and eventually dry out and die. Over time, the plant leaves will run out from the tip, giving the plant the appearance of a broom, and eventually leading to death. This is caused by an abnormal change in chloroplast function, which can inhibit the development of young tissue. The cause of chloroplast abnormalities is considered to be due to the presence of toxins released by pathogens.

**Table 2.** Effect of treatments on cocoa shoots growth

Treatments	The number of early shoots (before treatments)	The number of final shoots (after treatments)	Increase in number of shoots
P0: Control (no treatment)	0.25 a	5.33 a	5.08 a
P1: Organic fertilizer	0.83 b	16.17 b	15.33 b
P2: Secondary metabolite <i>T. asperellum</i>	1.17 bc	35.92 bc	34.75 bc
P3: Secondary metabolite <i>P. fluorescens</i>	1.17 bc	38.00 bc	36.83 bc
P4: Organic fertilizer + secondary metabolite <i>T. asperellum</i>	1.25 bc	39.83 bc	38.58 bc
P5: Organic fertilizer + secondary metabolite <i>P. fluorescens</i>	1.08 bc	48.33 d	47.25 d
P6: Secondary metabolite <i>T. asperellum</i> + <i>P. fluorescens</i>	1.58 cd	37.83 bc	36.25 bc
P7: Organic fertilizer + secondary metabolite <i>T. asperellum</i> + secondary metabolite <i>P. fluorescens</i>	1.75 d	45.33 cd	43.58 cd

Note: Those numbers followed by the same letters indicated not significantly different by Tukey's test at  $\alpha = 5\%$



**Figure 3.** Increase in the number of shoots in each treatment

The increase in the number of shoots after treatment was due to the administration of secondary metabolites singly or to a combination of *T. asperellum* and *P. fluorescens* that could affect plant growth. This is in accordance with Soesanto et al. (2010), who reported that secondary metabolites of biological control agents play a dual role, both additively and synergistically, where these metabolites can not only overcome or control pests but are also able to trigger the growth and production of host plants. As per a previous study (Rosmana et al. 2019), the combination of *T. asperellum* and composted plant residues resulted in the highest decrease of VSD incidence that indicated a synergistic effect arising from a mixture of different mechanisms of the two treatments.

*Trichoderma* establishes symbiotic interactions with plants, and like other root-colonizing microorganisms such as rhizobia and mycorrhizae, these interactions can induce systemic resistance to plant diseases (Harman et al. 2008). According to Hyakumachi and Kubota (2003), the function of *Trichoderma* apart from being a biological control agent was also a plant growth-promoting fungus (PGPF). It has been reported that the mechanism of action of PGPF was known as a compound that functions as an antibiosis because it can produce antibiotics and growth hormones that can stimulate root extension so that plant growth can be better, increasing quality of yield, and improve plant health. Besides, Harni (2016) reported that as a biological agent, *P. fluorescens* is an endophytic bacterium that can increase plant growth because it produces growth hormones such as auxin and cytokinins. This indicates that the interaction between *T. asperellum* and *P. fluorescens* secondary metabolites affects the growth of cocoa plants.

It was observed that *P. fluorescens* had a potential to suppress the development of VSD disease and to produce secondary metabolites as high growth regulators, which enhance the growth of cocoa plants. According to Nasrun and Nurmansyah (2016), the formula with the active ingredient of *P. fluorescens* Pf19 can induce the resistance of patchouli against *Ralstonia Solanacearum* which causes bacterial wilt disease, and increased patchouli plant growth. Hariprasad et al. (2013) showed that a tomato-rhizosphere-

associated *Pseudomonas* sp. bacteria was able to produce the antimicrobial substance phenazine, which enhances intrinsic resistance in tomato root and attack of pathogens. It stimulated the intracellular accumulation of organic compounds (phenolics, lipoxygenase, and jasmonic acid) in the treated plant and provided protection against a wide range of pathogenic microbes (fungi, bacteria, and/or viruses).

In conclusion, secondary metabolites of *T. asperellum* and *P. fluorescens* were quite effective in controlling VSD and increasing cocoa growth as they reduced the severity of VSD by 40% and increased shoot growth by 43.58%.

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