

## Raw Secondary Metabolites Application of Two *Trichoderma harzianum* Isolates towards Vascular Streak Dieback on Cocoa Seedlings

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### Abstrack

Vascular streak dieback is an important disease on cocoa that recently is still difficult to control. This research aimed to study the raw secondary metabolites application effectiveness of *Trichoderma harzianum* T10 and T213, or their combination to vascular streak dieback and its effect on growth and on phenolic compound of cocoa seedlings. Randomized block design was used with nine replicates. The treatments were control, secondary metabolites of *T. harzianum* T10 and T213, their combination and fungicide with active ingredient of phosphic acid. Variables observed were incubation period, disease intensity, plant height, number of leaves, wieght of fresh and dry plants, weight of fresh and dry roots, roots length, and phenolic compound. Result of the research showed that the raw secondary metabolites of *T. harzianum* T10 could suppress the disease intensity and delay the incubation period as 62.17 and 24.97%, respectively. The raw secondary metabolites of *T. harzianum* T213 and T10 combination could increase plant height 36.21%, number of leaves 19.07%, fresh weight of plant 20.6%, dry weight of plant 17.5%, fresh weight roots 27%, dry weight of root 34.36%, and roots length 21.30%. All raw secondary metabolites of *T. harzianum* were able to increase phenolic compounds (saponins, tannins, and glycosides) qualitatively of cocoa seedlings.

**Keywords:** Cacao seedlings, raw secondary metabolites, *Trichoderma harzianum*, vascular streak dieback

### INTRODUCTION

Cocoa is one of plantation crops with high economic value. In 2010, Indonesia is the third largest exporter of cocoa beans world after Ivory Coast and Ghana (Bowers *et al.*, 2001; Rubiyo & Siswanto, 2012). According to data from the BPS (2015), in 2014, cocoa production has decreased by 11.53 thousand tons.

The decrease in production due to the constraint in improving the quality and production yield. *Oncobasidium theobromae* Talbot & Keane cause xylem disease or

Vascular Streak Dieback (VSD) (Guest & Keane, 2007; Samuels *et al.*, 2012). Severe attacks can be deadly immature plants to 70% (Keane, 1981). According Mulyatni *et al.* (2011), in Indonesia VSD reduce productivity by 40%. If not promptly treated, the disease can be a major problem in the cultivation and production of cocoa.

Control is often done with fungicide. However, control with fungicides cannot be recommended, because the fungus is in the vessels (xylem), so it is difficult to obtain by fungicides (Yadeta & Thomma, 2013). In addition, excessive use of fungicides has

negative impacts for the environment and human health (Eskenazi *et al.*, 1999; Desneux *et al.*, 2006; Geiger *et al.*, 2011).

Accordingly, necessary to find other alternatives to control pathogen-causing VSD on cocoa. Secondary metabolites of biological agents, such as *Trichoderma* sp., were able to answer the challenges, which can reach the presence of the pathogen in plant tissue, and with diverse mechanisms (Ortuño *et al.*, 2016).

A secondary metabolite is a chemical compound produced by a limited number of fungal species in a genus, an order, or even phylum. A profile of secondary metabolites consists of all the different compounds a fungus can produce on a given substratum and includes toxins, antibiotics and other outward-directed compounds (Frisvad *et al.*, 2008). Raw secondary metabolites of *T. harzianum* are such as antibiotics, enzymes, hormones, and toxins (Reino *et al.*, 2008; Keswan *et al.*, 2014). Raw secondary metabolites of *Trichoderma* spp. may have a role in both plant growth regulation and activation of plant defence responses (Vinale *et al.*, 2008a; Ortuño *et al.*, 2016).

This study aimed to assess the effectiveness of raw secondary metabolites application of *T. harzianum* T10 and T213, or a combination thereof, to the vascular streak dieback and its impact on growth and the phenolic compounds in cocoa seedlings.

## MATERIALS AND METHODS

### Pathogen Antagonist and Isolates

Fungal pathogen isolate (*Oncobasidium theobromae*) was isolated from infected cacao stem (Prior, 1977), according to Baloiloi & Akanda (2013). The purified culture was transferred to PDA slants and stored in the refrigerator for later use.

Preparation of *T. harzianum* T10 isolated from ginger (Soesanto *et al.*, 2005) and T213 isolated from shallot (Santoso *et al.*, 2007) rhizospheres was carried out on PDA, then, incubated for 7 days at room temperature. Both isolates of *T. harzianum* also be prepared in the corn broke medium, then incubated at room temperature about a week (Cavalcante *et al.*, 2008; Panahian *et al.*, 2012).

Raw secondary metabolites of two *T. harzianum* isolates was prepared in a liquid formula, which was composed of rice water as much as 8 L, 2 L coconut water and sugar 10 g per L, sterilized and put into a sterile jerry cans (Soesanto *et al.*, 2015). Furthermore, after cooling down, the medium is inoculated by *T. harzianum* derived from corn broke medium as many as 150 g with sterile water as much as 250 mL, stirred until homogeneous then filtered and put into jerry cans and shaken using a shaker for 7 days at room temperature with a speed of 150 rpm and then counted up the density of  $10^7$  conidia per mL of solution, and filtered (Whatman no. 42).

### Inoculation Treatments

*Oncobasidium theobromae* was inoculated on all seedlings leaves. The leaf was surface sterilized with 1% sodium hypochlorite for one minute and rinsed twice with distilled water. *O. theobromae* culture diameter of 5 mm cork borer was attached on the underside of the leaves, then covered the damp cotton as well as duct tape and wrapped in plastic (Baloiloi & Akanda, 2013). Inoculation was also done on the roots of cacao seeds as much as 2 points with 5 mm cork borer of the culture, then, covered with soils.

Application of raw secondary metabolites of *T. harzianum* was performed using the root infusion of 50 mL per seedling. Application of raw secondary metabolites

of *T. harzianum* do as much as 6 times at intervals of 6 days. Randomized block design with five treatments and six replications was used. The treatments tested included control, raw secondary metabolites of *T. harzianum* T10, *T. harzianum* T213, combination of *T. harzianum* T213 and T10, and fungicide with active ingredient of phosphite acid.

Patosistem component observed is the incubation period (dai) and the disease intensity (%). The intensity of the disease is calculated using the formula according to Kranz (1988):

$$I = \frac{\sum (U \times V)}{ZN} \times 100\%$$

where: S(u×v) = Sum of the symptomatic plant and their corresponding score scale, N = Total number of sampled plant, and Z = Highest score scale.

According Nur'aini (2014), VSD categories based on the value of scoring as follows: 0 = healthy; 1 = <5% leaves infected; 2 = 5–10% leaves infected, chlorosis/necrosis, no fallen leaves, lenticel swelling; 3 = 10–25% leaves infected, chlorosis/necrosis, lenticel swelling leaves falling down; 4 = 25–50% leaves infected, chlorosis/necrosis, lenticel swelling leaves falling down; 5 = 50–75% of infected leaves, chlorosis/necrosis, lenticel swelling leaves falling down; and 6 => 75% leaves infected, chlorosis/necrosis, lenticel swelling, leaves falling down, fruit body present, twigs are dead/dry.

Components of growth in terms of height, number of leaves, plant fresh weight, dry weight of plants, fresh root weight, dry root weight and root length. Analysis of phenolic compounds qualitatively carried out at the end research. Test of saponins and tannins conducted by Astuti (2011), and glycosides test conducted by Chairul (2003).

Data were analyzed by analysis of variance at the level of 5% error. If significantly different between treatments, was tested fur-

ther using Duncan Multiple Range Test with a level of 5%.

## RESULTS AND DISCUSSION

### Incubation and Disease Intensity

The application of raw secondary metabolites of *T. harzianum* T10 significantly affected the incubation period compared to control and fungicide (Table 1). The raw secondary metabolites of *T. harzianum* T10 did not differ with the other treatments. The raw secondary metabolites of *T. harzianum* T10 could prolong the incubation period as long as 15 and 18.89 dai or as 24.98% and 31.45% compared to control and fungicide, respectively. Although the other application did not differ significantly with *T. harzianum* T10, the raw secondary metabolites of *T. harzianum* T213 and the combination could lengthen the incubation period respectively as 13.05 dai or 22.46% and 6.72 dai or 12.98% compared to control and as 16.94 dai or 29.15% and 10.61 dai or 20.49%, respectively, compared to fungicide.

The raw secondary metabolites of *T. harzianum* T10 significantly different with control and fungicides (Table 1). Control tended to show the higher disease intensity compared to the raw secondary metabolites of *T. harzianum*. The same thing happened in the fungicide that the fungicide has not been able to lower the disease intensity. In contrast, the raw secondary metabolites of *T. harzianum* T10 had a smaller intensity of the disease or was able to suppress the disease intensity by 62.17% and 69.76% when compared to the control and fungicide, respectively. Application of *T. harzianum* T213 raw secondary metabolites was not significantly different to controls, but significantly different with fungicide. The raw secondary metabolites of the combined *T. harzianum* isolates were not significantly

different either to the control or fungicide. Although it was not significantly different statistically (Table 1), the raw secondary metabolites of *T. harzianum* T213 and the combined isolates generally suppressed the development of *O. theobromae* as 44.68 and 22.44%, respectively, when compared to control.

Table 1. The effect of treatments on pathosystem component

Treatment	Incubation period (dai)	Disease intensity (%)
Control	45.06 bc	15.15 b
<i>T. harzianum</i> T10	60.06 a	5.73 c
<i>T. harzianum</i> T213	58.11 ab	8.38 bc
<i>T. harzianum</i> T10 + T213	51.78 abc	11.75 abc
Fungicide (phosphite acid)	41.17 c	18.95 a

Note: - dai = days after inoculation.  
- Figures in the same column followed by the same letter are not significantly different.

### Plant Growth

The application of raw secondary metabolites of *T. harzianum* was significantly different from the control treatment and fungicide (Table 2). The raw secondary metabolites of *T. harzianum* T10, T213, and the combination could promote the cacao seedling height respectively as high as 3.35, 2.29, and 2.89 cm or as 39.69, 31.03, and 36.22% compared to control and as 3.09, 2.03, and 2.63 cm or as 36.61, 27.51, and 32.96% compared to fungicide, respectively.

Application of *T. harzianum* raw secondary metabolites was significantly different to the control and fungicide. The raw secondary metabolites of *T. harzianum* T10, T213, and their combination significantly affected in increasing the number of leaves by 20.67, 18.50, and 19.08%, respectively, when compared

to control and as 30.31, 28.40, and 28.91%, respectively, when compared to fungicide.

The weight of fresh cacao seedlings showed no significant difference between treatments (Table 2). The raw secondary metabolites of *T. harzianum* combined isolates were significantly different to control and fungicide. Control and fungicide had lower fresh weight of the plant compared with the combination of *T. harzianum* isolates. All applications of *T. harzianum* raw secondary metabolites were not significantly different but the use of raw secondary metabolites of *T. harzianum* combined isolates showed an increase of 20.6% compared to all treatments.

The treatments were not significantly different on weight of dry cacao seedlings. Although no difference statistically (Table 2), it was presumably that the raw secondary metabolites of *T. harzianum* generally gave rise to dry weight of the plant each at 8.8, 14.7, and 17.5% when compared to controls.

The raw secondary metabolites of *T. harzianum* alone or in combination affected significantly in increasing fresh and dry root weight compared with the control and fungicide. Fungicide and controls show fresh and dry root weight is lower compared to the secondary metabolites of *T. harzianum*. The raw secondary metabolites of *T. harzianum* both T10 and T213 isolates and their combination was able to increase the weight of fresh root as 25.7, 18.2, and 34.36%, respectively, and increase the weight of the dried root as 34.76, 24.94, and 34.36%, respectively.

Table 2. The effect of the treatments on growth

Treatment	TT (cm)	JD	BTS (g)	BTK (g)	BAS (g)	BAK (g)	PA (cm)
Control	5.09 b	6.83 b	24.38 b	5.77	6.44 b	3.19 b	24.89 b
<i>T. harzianum</i> T10	8.44 a	8.61 a	25.72 ab	6.33	8.67 a	4.89 a	30.97 a
<i>T. harzianum</i> T213	7.38 a	8.38 a	26.16 ab	6.77	7.88 ab	4.25 ab	30.63 a
<i>T. harzianum</i> T10 + T213	7.98 a	8.44 a	30.72 a	7.00	8.83 a	4.86 a	31.63 a
Fungicide (phosphite acid)	5.35 b	6.00 b	20.55 b	5.61	6.56 b	3.56 b	26.65 ab

Note: Figures followed by the same letter in the same column showed no significant difference in DMRT 5%. TT = plant height, JD = number of leaves, BTS = Weight of fresh plants, BTK = Weight of dried plants, BAS = Weight of fresh roots, BAK = Weight of dry roots, and PA = length of roots.

The raw secondary metabolites of *T. harzianum* T10, T213, and the combined isolates were significantly different when compared to control (Table 2). The raw secondary metabolites of *T. harzianum* T10, T213, and the combined isolates were able to increase the length of roots as 19.63, 18.73, and 21.30%, respectively, when compared to control.

### Phenolic Compounds

Cacao seedlings contained phenolic compounds (Table 3). The content of saponins, tannins, and glycosides in raw secondary metabolite application of *T. harzianum* was more compared to the control and fungicide. The raw secondary metabolites *T. harzianum* T10, T213, and the combined isolates could increase the phenolic compounds content of cacao seedlings qualitatively.

Table 3. The cocoa seedlings phenolic compound qualitatively

Treatment	Saponin	Tannins	Glycosides
Control	+	+	+
<i>T. harzianum</i> T10	++	++	++
<i>T. harzianum</i> T213	++	++	+++
<i>T. harzianum</i> T10 + T213	++	++	++
Fungicide (phosphite acid)	+	+	+

Note: - = no phenol content. + = a little, ++ = sufficient, and +++ = lots.

The different data of incubation period is presumably because the plants in the control was not applied secondary metabolites of *T. harzianum*, so it does not have a mechanism of suppression against *O. theobromae* infection. This is in accordance with the opinion of Soesanto & Rahayuniati (2009), that the plants are not applied biological agents do not have a mechanism of suppression against pathogen infection. The same thing happened to fungicide which shows the faster incubation period compared to the treatment of *T. harzianum* raw secondary metabolites applications. The fungicide active

ingredient of phosphite acid have not been able to suppress the growth of pathogenic *O. theobromae*.

The delay was allegedly due to the application of *T. harzianum* raw secondary metabolites that was able to induce resistance against *O. theobromae* in cocoa seedlings. Inducement is evident with the increase of phenolic compounds in cocoa seedlings were treated with raw secondary metabolites of *T. harzianum* (Table 3). This is supported by Soesanto *et al.* (2015), that the secondary metabolite is able to induce plant resistance, so that the plant can withstand and avoid the attack of pathogen. Plants that are applied with the raw secondary metabolites of *T. harzianum* suspected to have the suppression infection mechanism against *O. theobromae*. This is in accordance with the opinion of Suganda (2000), that presses against pathogen infection can occur with performed award antagonistic microbes, chemicals, compost, and plant extracts instead of the host.

High disease intensity was found in the control and fungicide because of no raw secondary metabolites application of *T. harzianum* so that in the control and fungicide, young leaves faster attacked because young leaves are more susceptible to pathogens (Guest & Keane, 2007). *O. theobromae* is an airborne pathogens and faster infect young leaves. This statement is supported the fact that *O. theobromae* infect plants through young leaves, on the second or third flush behind the tip (Guest & Keane, 2007; Baloioli & Akanda, 2013).

In contrast, the raw secondary metabolites applications of *T. harzianum* T10 is able to suppress the disease intensity by 62.17% when compared to the control and fungicide. It is because the raw secondary metabolites of *T. harzianum* able to increase the phenolic compounds in plants (Table 3), which serves as a plant defense against

pathogen infection. According Schulz *et al.* (2002), the higher the content of phenolic compounds in plants, the higher the plant resistance to pathogen infection so that the disease intensity is low.

The secondary metabolites of *T. harzianum* suspected of having the suppression infection mechanism against *O. theobromae*. The pathogen can not spread to the entire network of plants or the location of the attack is limited, so that the disease intensity is not high. This statement is supported by Guest & Keane (2007).

According to Agrios (2005), a pathogen that generally colonize dead tissue will be inhibited by biological agents, so as to reduce the progression of disease in the plant tissue. The disease intensity in the raw secondary metabolites of *T. harzianum* T213 and their combined treatment in harmony with the incubation period. In addition, the raw secondary metabolites contains more phenolic compounds than in controls (Table 3). According Schulz *et al.* (2002), phenolic compound serves as a plant defense against pathogen infection. Further explained Agrios (2005), that an increase in phenolic compounds in plants due to the addition of fungal metabolites antagonist is absorbed by plants and translocated systemically to all parts of the plant, causing the substance responsible in induced resistance, among which is a phenol compound.

The application of *T. harzianum* raw secondary metabolites affected significantly seedlings growth components (Table 2). The low height of plants is found in the control and fungicide allegedly because the intensity of the attack pathogens and lack of *O. theobromae* attack control by secondary metabolites of biological agents so the plants become stunted growth. According Guest & Keane (2007), the attack of pathogens that cause chlorosis on the leaves can disrupt the process of photosynthesis so the growth becomes stunted.

The increase in plant height alleged activity of raw secondary metabolites of *T. harzianum*. Besides being able to suppress the pathogen attack, secondary metabolites can trigger the growth of plants. This is according to Kliebenstein *et al.* (2005), that the secondary metabolite of biological agents was capable of double plays both in reducing the pest attack plantations as well as the growth and production of the host plant.

The low amount of leaves in the control and fungicide due to the control and fungicide are not applied the secondary metabolites, thus leading to high intensity resulting disease infected leaves become deciduous. This is consistent with Guest & Keane (2007) that the disease can cause twig toothless VSD for leaf experiencing hair loss. Increasing the number of leaves presumably because the secondary metabolites of *T. harzianum* can affect plant growth. This statement is supported by Vinale *et al.* (2008b).

Control and fungicides have a fresh weight of plants is low compared with the treatment of *T. harzianum* isolates combination. This is presumably because the high pathogenic attack resulted in the growth of the plants become stunted. This is in accordance with the intensity of the disease in fungicide and control for the percentage intensity is higher than other treatments. In addition, the process of photosynthesis in symptom leaves will be disrupted because of damage to the chloroplast. The result would not be optimum photosynthesis process so that the plant growth will be impaired. According to McMahon & Purwantara (2016), that pathogen infected young leaves of cacao seedlings and mature trees, symptoms of leaf chlorosis, leaf fall, and branch dieback or seedling mortality leading to inhibition of plant growth.

Increased weight of the fresh plant allegedly because the secondary metabolites is used to have a mechanism of plant growth

promoting fungi so that the secondary metabolites of *T. harzianum* were able to stimulate plant growth. This is consistent with an increase in the number of leaves and plant height at application treatment of secondary metabolites of *T. harzianum* combined isolates. This statement is supported by Vinale *et al.* (2008a) that one of the biological agents secondary metabolites functions was to stimulate and generate growth regulators.

Application of *T. harzianum* raw secondary metabolites was able to increase saponins, tannins, and glycosides in plants that can induce plant resistance (Table 3), so that the development of the pathogens in the plants become inhibited. The symptoms are not widespread and the process of photosynthesis in the leaves still occur. This causes good plant growth so it affected plant fresh weight (Anggraeni & Wibowo, 2007). Increased weight of the fresh plant in line with the increase in the value of the plant dry weight, root wet weight, dry root weight, and root length that have better value compared to growth component of other treatments.

Increased weight of the dried plants in secondary metabolites applications of *T. harzianum* in line with the increased weight of the fresh plant. The increased weight of the dried plant suspected because of the application of *T. harzianum* secondary metabolites. The provision of secondary metabolites of *T. harzianum* allegedly able to suppress the growth of pathogenic *O. theobromae* so the optimum cocoa seedlings can grow and be able to increase plant growth. This statement is supported by the results of research Aziz *et al.* (2015) that the application of secondary metabolites of *T. harzianum* T213, T10, and their combination can suppress the intensity of bacterial wilt by 51.72% and to increase the potato crop dry weight by 34.9%.

The low weight of fresh and dry roots on the control and fungicide allegedly was not applied the secondary metabolites of

*T. harzianum* so that the intensity of the disease is high which results in the process of photosynthesis becomes blocked and the impact on root growth to be disturbed. According Wasis *et al.* (2011), root growth is dependent on the active area of leaf photosynthesis. because the roots will receive energy only when there is excess energy that is not used for the growth of the plant canopy.

Fungicides used allegedly does not contain growth hormones. According Mkdgroup (2011), fungicide active ingredient folirfos with phosphorous acid can only control the development of pathogens in plants.

The increase in root weight allegedly due to the application of secondary metabolites of *T. harzianum* can enhance root growth. Secondary metabolites of *T. harzianum* contains growth hormones that can stimulate the growth of plant roots. This statement is supported by Druzhinina *et al.* (2011) that *Trichoderma* sp. can stimulate the formation of lateral roots, for issuing a kind of hormone auxin active substance that stimulates lateral root formation.

This is presumably because the control was not applied to the secondary metabolites of *Trichoderma* sp. so that the roots of cocoa seedlings colonized by pathogens *O. theobromae*. This statement is supported by Harlina (2009) that the inhibition of root growth occurs in the area because of constraints on protein synthesis in the root elongation. Barriers protein synthesis is caused by the limited components of macromolecules as well as the existence of barriers synthesis and activity of enzymes in the process of protein synthesis. The inhibitory effect was caused by pathogenic fungi.

Increased root elongation allegedly was because the raw secondary metabolites capability of *T. harzianum* trigger the growth of plant roots. According to Moeinzadeh *et al.* (2010), microbial activity antagonist as

PGPF because it can produce growth hormone to stimulate the extension of the roots. This statement is supported by Nurahmi *et al.* (2012) that the application of *T. harzianum* can affect root extension cocoa seeds. More Cleland (1972) explains that the species *T. harzianum* can produce indolasetat acid (IAA) and other materials related to auxin.

The increase in phenolic compounds in plant tissue is suspected because the application of *T. harzianum* raw secondary metabolites on cocoa seedlings were able to increase the content of saponins, tannins and glycosides in the plant. This statement is reinforced by Agrios (2005) that the increase in phenol content in plants for fungal antagonist used supernatant is absorbed by plants and translocated systemically to all parts of the plant, causing the substance responsible for the resilience of the affected including phenolic compounds.

This proves that the raw secondary metabolite of *T. harzianum* can enhance phenolic compounds in cacao seedlings so the impact on plant resistance. The plants become susceptible to pathogens so the value of the intensity of the disease in the raw secondary metabolites of *T. harzianum* lower and a long incubation period compared with the control and fungicide. According to Wachjadi *et al.* (2013), phenolic compounds responsible for plant resistance to pathogen attack. The high intensity of the disease in fungicides and control due to low saponins, tannins and glycosides in plant. Phenolic compounds in plants plays an important role on the mechanisms of plant resistance to pathogen attack. If the content of phenolic compounds is low then the plant has a low resistance of infection pathogens. This is in accordance with Wachjadi *et al.* (2013), a plant that has a low content of phenolic compounds. The plant resistance to infectious diseases will decline. so that the intensity of the disease is high.

## CONCLUSION

Treatment using raw secondary metabolites of *T. harzianum* T10 could suppress disease intensity by 62.17%. and were able to delay the incubation period amounted to 24.97%. The raw secondary metabolites of the combined *T. harzianum* T213 and T10 could improve plant height, number of leaves, plant fresh weight, plant dry weight, root fresh weight, root dry weight, and root length in the range of 17.5–36.2%. Treatment of raw secondary metabolites of *T. harzianum* was capable of increasing the phenolic compounds (saponins, tannins, and glycosides) content in the plants tissues that play a role in building the plant resistance to VSD.

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