



Field Application of Trichoderma Suspension to Control Cacao Pod Rot (*Phytophthora palmivora*)

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ABSTRACT

Cacao pod rot caused by *Phytophthora palmivora*, is an important disease and contributes significant disease losses to global cocoa production. This research objective was to determine the effect of *Trichoderma harzianum* and *T. virens* suspensions to cacao pod rot disease on the field. This research was carried out in Pulo Hagu village, Pidie Regency, Aceh, Indonesia from March to July 2017. The single pattern randomized block design was adopted to evaluate three treatments, i.e. without suspension (control), suspensions of *T. Harzianum*, and *T. virens* for eight replications. Each replication consisted of three of experimental units. The result showed that both of *Trichoderma* species contained only Alkaloid metabolite based on Phytochemical test. On the field, the application of *T. harzianum* suspension reduced the percentage of fruit infection and disease intensity for 48.57 %, 46.04 % at 12 weeks after application (WAA) respectively. Based on the percentage reduction in the area of the spot between the metabolites *T. harzianum* suspension and control and *T. virens* and control are 47.24 % and 27.46 % at 87 WAA respectively. In addition, *T. virens* suppressed the percentage of infected fruit and the intensity of infected fruit for 40.61 % and 38.02 % at 12 WAA.

INTRODUCTION

On cocoa, black pod rot disease is caused by a plant pathogen in the genus of *Phytophthora* (Guest, 2007; Vanegtern, Rogers, & Nelson, 2015). In *Phytophthora* genus, more than 80 species caused plant diseases, including *P. palmivora*, *P. megakarya*, *P. citrophora*, and *P. capsici*, are responsible for black pod rot on cacao (Vanegtern, Rogers, & Nelson, 2015). Cacao pod rot caused by fungal pathogen, *Phytophthora palmivora*, is an important disease in cacao plantations and contributes significant losses to global cocoa production (Guest, 2007; Vanegtern, Rogers, & Nelson, 2015). *Phytophthora palmivora* infects fruits from various stages of fruit development, starting from young fruit or fruit nipples to almost mature fruit

(Muzuni, Indradewi, & Baharudin, 2015). From 20 to 30 % pod losses through black pod rot and ca. 10 % of trees annually through stem cankers are caused by *P. palmivora* (Guest, 2007).

Biological control agents (*Trichoderma*) available in the nature are able to find their own targets, relatively cheap, safe for the environment and health and can be reproduced simply. Many *Trichoderma* species, commonly are able to produce spores and secondary metabolites like 6-pentyl-alpha-pyrone (6-PP), a lactone with antibiotic properties and coconut aroma (Hamrouni, Molinet, Dupuy, Masmoudi, & Roussos, 2017). Plants wield an arsenal of structurally diverse chemical compounds called secondary metabolites, the active secondary metabolite compounds were classified into several

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groups, namely: alkaloid, terpen, steroid, flavonoid and saponin (Ahmed et al., 2017). Fungi can be grown in liquid culture Broth Agar for various purposes such as for releasing metabolite extract, enzyme and antibiotics (Nevalainen, Kautto, & Te'o, 2014).

Generally, the bio-control mechanism may be divided into five ways such as antibiosis, competition, mycoparasitism, cell wall degrading enzymes, and induced resistance (Lo, 1998). Antibiosis mechanism includes antimicrobial metabolites and antibiotics (Lo, 1998). Antimicrobial activities are produced by several *Trichoderma* species such as *T. koningii* (Song et al., 2006) and *T. virens* (Lo, 1998). *Trichoderma virens* can produce gliotoxins as antimicrobial compound (Vargas et al., 2014). In case of antibiotic, gliovirin is produced by *T. virens* to inhibit Oomycota (Lo, 1998).

Related to the important role of *Trichoderma* to control plant disease, it is necessary to evaluate the use of *Trichoderma* suspension from *T. harzianum* and *T. virens* species to control cacao pod rot disease on cacao plantation.

MATERIALS AND METHODS

This research was carried out in the cacao farm in Pulo Hagu Village, Padang Tiji Subdistrict, Pidie District. Laboratory activities were conducted at the Plant Disease Laboratory, Faculty of Agriculture, Syiah Kuala University and the Organic Chemical Laboratory, Faculty of Math and Science, Syiah Kuala University. All activities were carried out from March to July 2017.

The culture isolates of *T. harzianum* and *T. virens* were isolates of the Laboratory of Plant Diseases, Faculty of Agriculture, Syiah Kuala University. Phytochemical extraction has been conducted in The Organic Chemical Laboratory, Faculty of Math and Science, Syiah Kuala University.

The field experiment design used a single pattern Randomized Block Design (RBD) consisting of three treatments, namely: without suspension (as control), suspensions of *T. Harzianum* and *T. virens*. Each treatment was replicated eight times and each replication consisting of three units of cacao plants. It was obtained 72 test units.

Trichoderma Suspension

Trichoderma harzianum and *T. virens* isolates that used in this study, as collection of the Plant Disease Laboratory, Faculty of Agriculture, Syiah Kuala University. The isolate was cultured aseptically by

cutting a small portion of *T. harzianum* inoculums growth on PDA media using a scalpel knife and then transferred it to a Petridis containing PDA, incubated for five days at room temperature (25 °C).

For a week, *Trichoderma* was propagated on PDA media and then in liquid media. Liquid media consisted of 4 L of rice washing water, 1 L of coconut water, and 75 g of granulated sugar. All ingredients were boiled for 40-60 minutes, then put into sterile plastic bottle (which have been washed with 70 % alcohol), each plastic bottle filled with 2.5 L of liquid media, closed tightly and cooled by soaking in a bucket. After being cool, *T. harzianum* and *T. virens* as much as 10 ml were inoculated into each plastic bottle aseptically on laminar air flow. The plastic bottle was tightly closed to avoid contamination by microorganisms from outside. Liquid media were shaken by a shaker (Eyeglass) device for three weeks at 150 rpm. The shaking function was to produce metabolites and reduce the formation of mycelium. *Trichoderma* liquid formulation was used after three weeks.

Trichoderma Secondary Metabolite Application

The application of suspension of *T. harzianum* and *T. virens* was carried out after the last harvest by spraying suspension metabolites which had been diluted. Suspension applied to cacao plantations on the stems, branches, leaves, flowers and evenly on the rhizosphere using sprayer/solo. The application of secondary metabolites was applied in the morning (from 7:00 to 10:00 a.m.) with three times and 14 days of application interval. The dilution formula was described as formula 1.

$$N1.V1 = N2.V2 \quad 1)$$

Where:

N1 = initial normality, namely the number of spore calculations on day 21 after multiplication = (c) x 25 x 10² x 10ⁿ

V1 = initial volume (L)

N2 = desired normality 1 x 10⁶

V2 = final volume (L)

Percentage of Affected Fruit

The attacked fruit that counted was cacao fruit infected by *P. palmivora*. The observation of the percentage of attacked fruit was carried out at 4, 6, 8, 10 and 12 WAA. The percentage of infected fruit was calculated based on the formula 2.

$$P = \frac{a}{b} \times 100\% \quad 2)$$

Where: P = percentage of fruit attacked; a = number of fruits attacked; b = number of whole fruits

Intensity of Affected Fruit

After calculating all fruits were classified to the affected fruit based on the percentage of the attack on each fruit (Fig. 1). The intensity of infected fruit was calculated based on the formula 3.

$$I = \frac{(\sum(n_i \times s_i))}{(N \times S)} \times 100\% \quad 3)$$

Where: I = the intensity of the fruit attacked; n_i = the number of pieces in a particular attack; s_i = the scale on certain attacks; N = the number of fruits observed; S = the highest scale value

Percentage of Reduction Area

The area of the initial spot was measured at 84 Days After Application (DAA) to determine the increase in the area of the spot in each treatment. The measurement repeated in the area of spotting at 87 DAA, by wrapping cacao fruit whose attack rates were 1-5 %, 6-20 % and > 20 % each one sample with plastic and given a broad marking of the initial spot, three days later a large area of spotting was added and marked. The plastic that has been marked was cut according to the size of the spot. Then the leaf area meter was inserted. The way to find out the area of the spot was the area of the spot on the 87th gauge DAA reduced by the area of measurement to 84 DAA. To get the percentage reduction in speckle area was calculated by the formula 2 (Asrul, 2009):

$$\text{PPLB}(\%) = \frac{(\text{Spot of Control} - \text{Spots of Treatment})}{(\text{Spots of Control})} \times 100\% \quad 4)$$

Where PPLB is Percentage of Reduction in Spots.

Data Analysis

Sigma Plot 12.0 versions was used to provide the graph and statistical analysis. The Analysis of variance was used to evaluate data. If there were found significances between each treatment, Duncan Multiple Range Test (DMRT) at 5 % of α is performed to check the significant differences between each treatment.

RESULTS AND DISCUSSION

Phytochemical Test

The content of secondary metabolites in each species of *Trichoderma* spp. after the phytochemical test is shown in Table 1. Phytochemical test results show the presence of secondary metabolite content in *Trichoderma* spp. species (Table 1). However, the five species of *Trichoderma* spp. tested, there were no species containing terpenoids. This is indicated by the absence of red color when the five species were reacted with the Liebermann-Burchad reagent. Likewise Steroids, no fat-soluble complex molecules that are shown by four rings are joined together (Kumaresan, Karthi, Senthilkumar, Balakumar, & Stephen, 2015). While flavonoid compounds were also not found which marked by the absence of pink color after adding magnesium metal and a drop of hydrochloric acid in the final process of flavonoid tests.



Remarks: A = Health (0 %), B = Low (1-5 %), C = Medium (6-20 %), D = High (> 20 %)

Fig. 1. Intensity of affected fruit

Table 1. Composition of secondary metabolite compounds in *T. harzianum* and *T. virens* based on the phytochemical test

Trichoderma Species	Secondary Metabolite Compounds Group				
	Terpenoid	Steroid	Alkaloid	Flavonoid	Saponin
<i>T. harzianum</i>	-	-	+	-	-
<i>T. virens</i>	-	-	+	-	-

Remarks: + (positive) = there is a secondary metabolite content, - (negative) = no secondary metabolite content

However, alkaloid compounds were found in all *Trichoderma* species tested and was marked by the appearance of orange color after reacting with Dragendorff reagent, and the color was white after reacting with Mayer reagent. Alkaloids are secondary metabolite organic compounds found in nature. In fungi (especially endophytic fungi), alkaloids have properties such as antifungals and antiviral activity (Zhang et al., 2012).

Trichoderma Field Application Test Percentage of Affected Fruit

The average percentage of infected fruit due to the application of *T. harzianum* and *T. virens* suspension on observations 4, 6, 8, 10 and 12 WAA can be seen in the Table 2. The treatment without the application of *Trichoderma* suspension (T0) has the highest percentage of infected fruit in each observation (Table 2). The highest percentage of infected fruit in T0 treatment was 56.04 % at 12 WAA and the lowest percentage of infected fruit in T1 treatment was 7.47 % at 12 WAA. The application of *T. harzianum* suspension could reduce the percentage of infected fruit at each observation by 15.17 % at 6 WAA, 27.29 % at 8 WAA, 41.98 % at 10 WAA and 48.57 % at 12 WAA while the suspension treatment of metabolite *T. virens* reduced the percentage of infected fruit by 11.76 % at 6 WAA, 22.19 % at 8

WAA, 38.45 % at 10 WAA and 40.61 % at 12 WAA. The data of the percentage of infected fruit showed that the suspension treatment of *T. harzianum* was the best treatment in suppressing the percentage of infected fruit caused by *P. palmivora*. Biofungicides with active ingredients *T. harzianum* applied have the advantage of being able to stick to the soil and roots, protect the roots and are not dissolved by water, because *Trichoderma* produces different kinds of enzymes which play a major role in biocontrol activity like degradation of cell wall, tolerance to biotic or abiotic stresses, hyphal growth etc. (Waghunde, Shelake, & Sabalpara, 2016). It was also suspected that the metabolite suspension of *T. harzianum* and *T. virens* which was applied to the flower, leaves and roots of cacao could prevent pathogenic infection of *P. palmivora* from the beginning of flowering so that the fruit had been colonized with suspension of *Trichoderma* metabolites. Efficacy of foliar spray of *T. harzianum* and *T. viride* isolates against *Fusarium graminearum* causing head blight (head scab) of wheat (Panwar et al., 2014). Among the different species and isolates of *Trichoderma* evaluated worldwide for control of foliar diseases, one such as grey mold disease cause by *Botrytis cinera* (Sawant, 2014) and monilia infection on cocoa (Seng, Herrera, Vaughan, & McCoy, 2014).

The ability of *Trichoderma* to reduce the percentage of infected fruit is due to its ability to suppress pathogenic populations directly or indirectly. Directly through the mechanism of antibiotics, competition and mycoparasites, while indirectly through increasing plant resistance (Aneja, Gianfagna, & Hebbar, 2005; Benítez, Rincón, Limón, & Codón, 2004). *Trichoderma* sp. also has the ability to remove hydrolytic enzymes, a class of hydrolytic enzymes whose very important role in the process of mycoparasitism of some pathogenic fungi is chitinolytic enzymes, which consist of chitinase.

Table 2. The average percentage of infected fruit due to the application of *T. harzianum* and *T. virens* suspension in cacao plants

Treatment	Average percentage of fruit attacked (%)				
	4 WAA (x̄±SD)	6 WAA (x̄±SD)	8 WAA (x̄±SD)	10 WAA (x̄±SD)	12 WAA (x̄±SD)
T0	31.07±4.30	35.93±2.44 c	41.23±3.87 c	51.26±3.72 c	56.04±3.27 c
T1	26.65±3.45	20.76±1.76 a	13.94±2.84 a	9.78±1.25 a	7.47±1.46 a
T2	28.09±2.89	24.17±2.43 b	19.04±3.97 b	12.81±2.40 b	15.43±3.46 b

Remarks: T0 = Without *Trichoderma* suspension, T1 = Suspension of *T. harzianum*, T2 = Suspension of *T. virens*, WAA = Week After Application, x̄ = Average, SD = Standard Deviation. The numbers followed by the same letters in the same column are not significantly different at the level of 5 % (DMRT test)

Chitinase is the name for a group of enzymes that are capable of hydrolyzing the B--1.4 bonds in chitin and chitin oligomers (Kullnig, Mach, Lorito, & Kubicek, 2000; Viterbo et al., 2002).

Intensity of Affected Fruit

The highest attacked fruit intensity was found in treatment K0 which was 51.44 % at 12 WAA and the lowest was in the treatment of *T. harzianum* suspension 5.40 % at 12 WAA (Table 3). Metabolite *T. harzianum* suspension application reduced the intensity of infected fruit by 6.18 % at 4 WAA, 16.73 % at 6 WAA, 25.11 % at 8 WAA, 40.34 % at 10 WAA and 46.04 % at 12 WAA while the suspension treatment of metabolite *T. virens* reduced the intensity of infected fruit by 5.08 % at 4 WAA, 13.65 % at 6 WAA, 19.88 % at 8 WAA, 37.16 % at 10 WAA and 38.02 % at 12 WAA, the data was obtained from the difference between treatment and control.

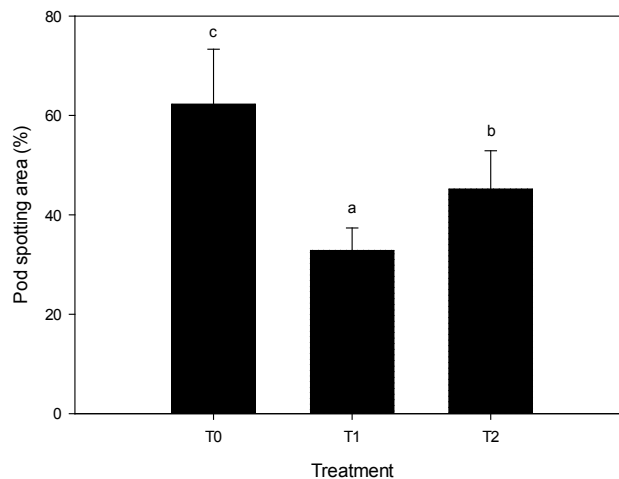
The concentration of *T. virens* 1×10^6 could reduce the severity of *P. palmivora* disease on cacao seedlings by 18 % and the concentration of *T. virens* 1×10^8 decreased the severity of *P. palmivora* on the leaves of cacao seeds by 20 % (Sriwati et al., 2015). The application of formulations proved stable when diluted in water with 1 % and 0.5 % of sedimentation respectively after 24 hours showed an improved efficacy of *Trichoderma* spp. to control the cacao pods disease (Mbarga et al., 2012).

The effectiveness of *Trichoderma* in controlling cacao pod rot is due to the metabolites in the form of Alkaloid and other antibiotic produced by *Trichoderma*. Howell (2003) studied the molecular mechanism of lytic enzymes involved in *T. harzianum* activity and stated that *T. harzianum* could degrade fungal cell walls due to chitinase, gluconase and protease.

Table 3. The average intensity of fruit affected by the application of the metabolites of *T. harzianum* and *T. virens* on cacao plants

Treatment	Average intensity of attacked fruit (%)				
	4 WAA ($\bar{x} \pm SD$)	6 WAA ($\bar{x} \pm SD$)	8 WAA ($\bar{x} \pm SD$)	10 WAA ($\bar{x} \pm SD$)	12 WAA ($\bar{x} \pm SD$)
K0	29.00±5.41 b	33.98±1.98 c	36.92±2.80 c	48.16±4.17 c	51.44±3.47 c
K1	22.82±3.70 a	17.25±2.04 a	11.81±2.49 a	7.82±1.58 a	5.40±1.13 a
K2	23.92±1.94 a	20.33±2.64 b	17.04±3.15 b	11.00±2.18 b	13.42±3.76 b

Remarks: K0 = Without secondary metabolites, K1 = Secondary metabolites *T. harzianum*, K2 = Secondary metabolites *T. virens*, WAA = Week After Application, \bar{x} = Average, SD = Standard Deviation. The numbers followed by the same letter in the same column are not significantly different at the 5 % level (DMRT test).



Remarks: T0 = Control, T1 = *T. harzianum*, and T2 = *T. virens*

Fig. 2. Average area of pod spotting on cacao fruit. The numbers followed by the same letter in the figure are not significantly different at the 5 % level (DMRT test)

In addition *T. harzianum* can produce volatile (alkyl pyrones) compounds that are anti-fungal. While secondary metabolites produced by *T. virens* are pyrones, Isocyno Metabolites, Peptaibols, exoglyconase enzymes and endoglyconase, selobiase and chitinase (Vinale et al., 2014). *Trichoderma virens* is able to produce gliotoxins and gliovirin antibiotics that are specific in inhibiting oomycote (Howell & Puckhaber, 2005). *Trichoderma* metabolite have displayed beneficial effects to plants, increasing plant growth and development an inducing defense responses to abiotic stresses and pathogens (Cardoza et al., 2005). By seeing on metabolite compound group of *T. harzianum* and *T. viren* result (Table 1.), both contain only alkaloid compound, the point of mechanism role of antagonistic also working together with metabolite compound in ability to inhibited pathogen growth and development.

Percentage of Pod Spotting Area

The reduction percentage in spotting area between the metabolite suspension treatment of *T. harzianum* and the control was 47.24 % while the *T. virens* suspension treatment was 27.46 % (Fig. 2). This was confirmed with the research (Hanada, Pomella, Soberanis, Loguercio, & Pereira, 2009) states that the application *Trichoderma* spp. tended to decrease progressively on the surface of cacao pods, with a concomitant increase in the severity of pod disease. Sriwati et al. (2015) stated that *T. harzianum* had the highest level of colonizing cacao fruit compared to *T. virens* and *T. asperellum*.

Cacao plants that have been applied for suspension *T. harzianum* dan *T. virens* have been colonized by *Trichoderma* so that the area of the spot on the cacao fruit can be reduced. In addition *Trichoderma* is able to produce secondary metabolites in the form of viridine and trichomidine, and form of alkaloid both compounds capable of producing hydrolytic enzymes β 1,3glucanase, chitinase and cellulase which can actively degrade other fungal cells; some of the mechanisms by which pathogenic microbes and various abiotic stresses as well as specific plant responses to pathogen attack, the genetic control of host-pathogen interactions (Mazid, Khan, & Mohammad, 2011). The biocontrol ability of *Trichoderma* is due to multiple factors, as they have the ability to produce a variety of extracellular lytic enzymes and many secondary metabolites (Cardoza et al., 2005).

CONCLUSION AND SUGGESTION

The application of *T. harzianum* suspension

suppresses the percentage of infected fruit and the intensity of attacked fruit for 48.57 % and 46.04 % at 12 WAA respectively. In addition, based on the percentage reduction in the area of spot between control and *T. harzianum* suspension and *T. virens* are 47.24 % and 27.46 % at 87 WAA respectively. In addition, *T. virens* suppressed the percentage of infected fruit and the intensity of infected fruit for 40.61 % and 38.02 % at 12 WAA. Further research is needed on the effect of secondary metabolite combinations of various *Trichoderma* species in controlling cacao pod rot.

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