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PELLET FORMULATION OF *Trichoderma* ISOLATED FROM ACEH WITH POTENTIAL FOR BIOCONTROL OF *Phytophthora* sp. ON CACAO SEDLINGS

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Abstrak. *Molleculler study was conducted to identify several species of Trichoderma isolate from several plant (Pine, Cacao, Gliceria, Nutmeg, Bamboo, Coffee, Potato). The growth of eight species Trichodermaafter pelleting formulation has been observed. Pellet Trichoderma harzianum have good ability to growth on PDA medium after 4 weeks storage. Base on their mycelium diameter growth on PDA, T. harzianum have selected as potential species on pellet formulation growth. Several dose of pellet formulation have been applied for controlling Phythopthora disease. The application of T. harzianum pellets in the form of a 2 g / 100 ml (S1) suspension effective in inhibiting the development of Phytophthora sp in cacao seedlings, when the higher concentrations of T. harzianum pellets applied to cacao seeds,the disease severity increase. Pellet Trichoderma could be use as biological control agent of cacao seedling in certain dosage.*

Keywords: *Trichoderma harzianum, T.virens, T.hamatum, T. asperellum, T. atroviride,.*

1. Introduction

Trichoderma sp. it has been widely used in biological control agent, especially the soil pathogens. From biological control perspective, *Trichoderma* species of indigenous plantation is needs more exploration, especially in Indonesia, Aceh region. The ability of mycoparasite of *Trichoderma* sp. works by wrapped around the pathogenic hyphae, then followed by the production of lysis enzymes that can penetrate the cell wall and produce antibiotics gliotoxin and viridian (Khaledi & Taheri, 2016; Lumsden, 1989; Sriwati et al., 2015). Applications of *Trichoderma* sp. in the form of substrates was less practical because it requires a lot of containers, a lot of labor, and often have problems to be brought and applied in the field. Therefore, it is necessary to look for the formulation of *Trichoderma* sp. which is more practical, effective, and efficient (Soekarno et al., 2014). One of the contributing factors to the development of *Trichoderma's* active pellet is the

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shelf life. The shelf life of biological control agent products in a weeks, months and even earlier years depends on the type, material and purpose of the biological agent product.

Previous studies have been done to evaluate the effectiveness of *Trichoderma* pellet formulation on controlling dumping of disease on cucumber seedling (Soekarno et al., 2014). Some *Trichoderma* fungi have been isolated from leading commodities in Aceh such cacao plantation(Sriwati et al., 2015),the result show that *Trichoderma* can inhibit growth of *Phytophthora* spon laboratory or on cacao seedling scale.

Recently, study about formulation of *Trichoderma* pellets is limited and still needs more investigate. Basic material of pellets*Trichoderma*formulation were the ingredients source of carbohydrates required by *Trichoderma* sp. or fungus in general. Bran and agro-wastes were a source of nutrients used to stimulate growth and development of fungi. agro-wastes such sawdust, corncob and sugarcane bagasse were added to improve the nutrition of planting media, as a source of nutrition and mineral composition of muchrom(Hoa et al., 2015). In addition, bran also contains protein, vitamins, minerals, and crude fiber. The composition of each component will differ depending on the type of rice. (Shafie & Esa, 2017).Whilesauropus leaf flour require for ash, protein, crude fiber and nitrogen free extract (Santoso et al., 2015).Therefore, all those nutrition can be added as a source of nutrition for *Trichoderma* sp.

Based on previous description, it is necessary to identify local *Trichoderma*s pecies that have potential used as*Trichoderma* pellets formulation, as well as increase the long-term growth and storage capacity, the highest potential *Trichoderma* species that could store for long time under pellet formulation could be apply to cacao seedlings to determine the effective dose for controlling a leaf blight disease on cacao seedlings.

2. Materials And Methods

Isolation and Identification *Trichoderma* Species

The molecular identification process was carried out at the Virology Laboratory of the Lembang Vegetable Research Institute, with 0.1 gram DNA extraction samples crushed using pistil and mortar until smooth then put in a 1.5 ml micro tube and added 400 µl AP1 buffer and 4 µl of RNaseA stock solution then cortex to homogenize the solution. Furthermore, the DNA extraction process was conducted out using a procedure (White et al., 1990). The primary used is the primary pair ITS1 (5 '-TCCTCCGCTTATTGATATGC-3') and ITS4 (5 '-TCCGTAGGTGAACCTGCG G- 3 ') which will amplify the DNA internal transcribed spacer (ITS) ribosomes (rDNA) (White

et al., 1990). The PCR process is carried out through a denaturation process at 94 ° C for 5 minutes 1 time, then annealing at 56 ° C for 2 minutes, then extention at 72 ° C for 2 minutes and elongation of DNA (extention) at temperature 72 ° C for 10 minutes, and repeated for 35 replications. The electrophoresis amplification results using 1.2% agarose gel with 0.5x TBE buffer at 100 volts for 30 minutes. DNA bands are seen above UV transilluminator. Producing DNA fragments measuring under ± 600 bp. The process of reading DNA nucleotide strands is carried out with the shipment sample to PT MacrogenInc Korea to do the nucleotide base tracing to find out the identity of the fungus. Fungi isolates that have been subcultured, identified until the taxa level of Genus uses by the bioinformatics method Basic Local Alignment Search Tool (BLAST) online at the website address http://blast.ncbi.nlm.nih.gov/Blast.c

The Growth and Storage Time Test of Pellet *Trichoderma*

Making of this pellet formulation refers to the study of Soekarno et al. 2014and modified 2016. The basic ingredients in making pellets used saoruposleaf, bran and molasses. Each base material (bran, saoropus leaf flour) was weighed according to the predetermined composition, 31.0 g bran and 10.5 g sauropus leaves. Then 15 ml of molasses and 42 ml of sterile water were added to the mixture and stirred until homogeneous, then put in heat-resistant plastic and possessed by autoclaving at 121 ° C for 30 minutes.One ml suspension of *T. harzianum* put into the dough material that has been sterilized and stirred.The material of pellet then prepared for processed to making pellet by using pellet machine. Pellet incubated in an incubator at 30 ° C for 48 hours until the pellet mixture dried.

Growth testing of in vitro pellet formulations was conducted every week with different storage periods of 0, 1, 2, 3, 4 weeks. Each pellet formulation was grown on PDA media in a petri dish, then incubated at room temperature for 7 days and observed with predetermined parameters. Each treatment was repeated 3 times. The best result of species *Trichoderma* on colony crowing after periods of storing were used for seedling application test.

Application of Several Dose Pellet *T harzianum* on Cacao Seedling

Pellet *Trichoderma* species that have been known as long life and good on growth performance will be used on seedlings test. Seven day old of cacao seed germination were planted into polybags with a depth of 5 cm planting holes and placed in the screen house. The application of *T. harzianum* pellet suspension was carried out by diluting the pellets into 100 ml of distilled water and shaking for 3 days to obtain a homogeneous.

Application pellets according to each treatment were 2 g / 100 ml, 4 g / 100 ml, 6 g / 100 ml, 8 g / 100 ml and 10 g / 100 ml. Furthermore, the suspension of *T. harzianum* pellets was sprayed onto the entire surface of the plant leaves using a sprayer tudor (3 weeks after planting). 1 week after pellet suspension applied, *Phytophthora* sp was inoculate. *Phytophthora* sp inoculums from cacao fruit infected were obtained from Pulo Hagu Village, Padang Tiji Subdistrict, Pidie Regency, Aceh. Infected fruit was sliced or cut and crushed using a blender with 100 ml of distilled water. Then the results filtered using a filter and the suspension was taken.

The variable observed were disease severity was carried out on 28 HSI, observing each leaf showing symptoms and inserting into the scale of the attack on the leaf. With the formula:

$KeP = (\sum [(n \times v)]) / (N \times Z) \times 100\%$

Information :

KeP = Severity of the disease

n = Number of leaves in each attack category

Z = Value of the highest category scale

N = Number of leaves observed

v = The scale value of each attack category

The category value used according to Abadi (2003).

The number of leaves calculated at 7, 14, 21 and 28 HSI. while the area of the affected leaves was calculated using a Leaf Area Meter (LAM).

3. Result and Discussion

Isolation and Identification *Trichoderma* species

Eight *Trichoderma* species have been isolated from several high plantations in Aceh Region, Sumatera Indonesia. The *Trichoderma* isolates come from several plants (Pine, Cacao, Gliceria, Nutmeg, Bamboo, Coffee, Potato). Based on the molecular identification there are the primary pairs of ITS1 (5'-TCCTCCGCTTATTGATATGC-3 ') and ITS4 (5'-TCCGTAGGTGAACCTGCGG-3'). DNA PCR amplification was done to duplicate fragments located in the Internal Transcribed. Based on the primers used in this identification are the primary pairs of ITS1 (5'-TCCTCCGCTTATTGATATGC-3 ') DNA PCR amplification is done to duplicate fragments located in the Transcribed Spacer (ITS) Internal area consisting of areas of ITS1. The position of the amplification target

with the primer used refers to result of White et al (1990).This experiment results can be seen in the sequence of fragments on the Table 1 bellow.

Tabel 1. BLAST Results Sequence of Nine Fragments DNA Isolate

Sample code	Descriptions	Primers	Similarity	Accession Number
T1 (Bamboo)	*Trichoderma asperellum* isolate IIRRCK1	C1_ITS1	99 %	MH825714.1
T2 (Pine)	*Trichoderma atroviride* strain TR5	C2_ITS1	99 %	KP211542.1
T3 (Cacao)	*Trichoderma longibrachiatum* strain QTYC46	C3_ITS1	99 %	KM103308.1
T4 (Gamal)	*Trichoderma hamatum* strain THM2 18S	C4_ITS1	95 %	KC403948.1
T5 (Potato)	*Trichoderma sp*. isolate SDAS203680	C5_ITS1	99 %	MK871066.1
T6 (Nutmeg)	*Trichoderma harzianum* voucher USM ED6	C6_ITS1	99 %	KU882056.1
T7 (Coffee)	*Trichoderma virens* strain CEN835	C7_ITS1	99 %	KC576738.1
T8 (Coffee)	*Trichoderma harzianum* strain ACCC32808 18S	C8_ITS1	99 %	MF669728.1
T9 (Coffee)	*Trichoderma hamatum* strain LXM1	C9_ITS1	98 %	GQ220703.1

The universal primers (internal transcribed spacer, ITS) were used for the amplification of 18S rRNA gene fragment and strains were thus characterized with the help of ITS marker. Based on phylogenetic tree analysis together with the 18S rRNA gene sequence search in Ribosomal Database, small subunit rRNA and large subunit rRNA databases, all species fungi has be assigned as the type of a species of genus *Trichoderma*, respectively as *Trichoderma asperellum*,*Trichoderma atroviride*, *Trichoderma longibrachiatum*, *Trichoderma hamatum*, *Trichoderma* sp., *Trichoderma harzianum*, *Trichoderma virens*, *Trichoderma harzianum*, *Trichoderma hamatum*. The sequence was deposited in GenBank with the accession numbers as shown on the Tabel 1. Ribosomal RNA (rRNA) sequence analysis has been well-documented as a means of determining phylogenetic relationships in all of the major organism domains, the *Gliocladium* species founded to be closer to all *Trichoderma* species identified (Fig. 1).(Rehner and Samuels, 1994) stated that *Gliocladium*is polyphyletic and that *G. penicillioides*, *G. roseum*, and *Trichoderma*virens (= *G. virens*), are generically distinct.

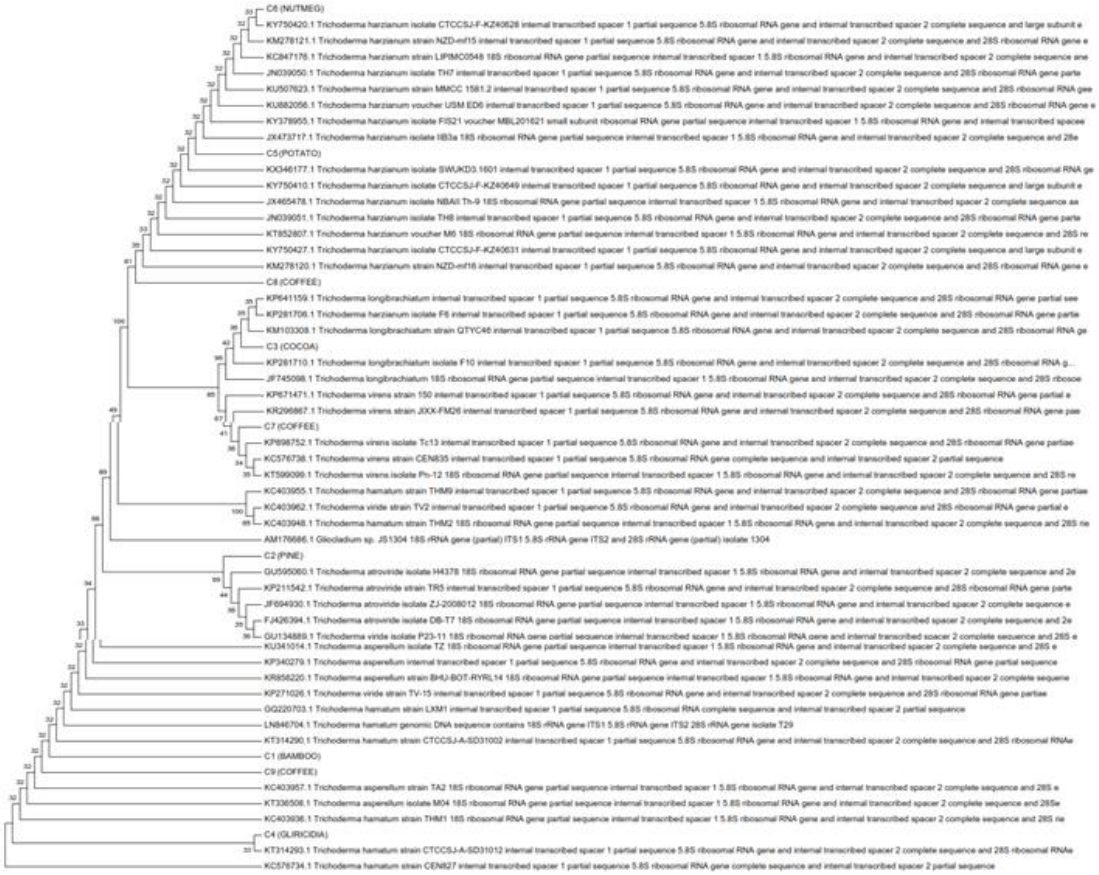

Figure. 1.Philogenetic of *Trichoderma* (Maximum Parsimony Joining Tree)

The Growth and Storage Time of Pellet *Trichoderma*

The results of observation on the diameter of fungal colony growth from *Trichoderma* pellet formulation can be seen in Table 2. Pellet *Trichoderma* of several species isolated from root of plant grown on PDA medium then stored on several storage period streatments from 0 to 4 week storage and looking their ability for growth again after each store. There were interaction between species *Trichoderma* and storage time after 3 DAI. Average diameter colony of *Trichoderma* species due to interaction of the period of storage and species as shown on Table 2.below.

Table 2.Average Colony Diameter of *Trichoderma* Species Due to Interaction of the Period of Storage and Species of *Trichoderma*at 3 DAI.

Interaction	Diameter colony SpesiesTrichoderma (mm)								
Storage Time (Weeks)	*T. asperelum* (Bamboo)	*T. atroviridae* (Pine)	*T. longibrachiatum* (Cacao)	*T. hamatum* (Gamal)	*T. harzianum* (Potato)	*T. harzianum* (Nutmeg)	*T. virens* (Cofee)	*T. harzianum* (Cofee)	BNT 0.05
W0	90.00 (9.51) C c	29.33 (5.37) B a	90.00 (9.51) C c	53.67 (6.03) C b	79.23 (8.92) B c	90.00 (9.51) B c	90.00(9.51) C c	84.33 (9.21) B c	
W1	48.33 (6.75) B c	12.50 (3.59) AB a	90.00 (9.51) C b	36.00 (6.03) BC b	83.63 (9.17) B d	90.00 (9.51) B d	22.83(3.24) B ab	85.33 (9.26) B d	
W2	32.13 (5.49) B ab	17.00 (4.13) AB	36.67 (6.01) B ab	17.67 (3.69) AB	90.00 (9.51) B	74.83 (8.68) AB	43.67 (5.64) B	65.77 (8.10) B cd	22.17

		a		a	e	de	bc	
W3	0.00 (0.71) A a	6.67 (1.98) A ab	0.00 (0.71) A a	0.00 (0.71) A a	87.73 (9.39) B c	67.17 (8.23) A c	21.67 (4.67) AB ab	27.50 (5.37) A b
W4	0.00 (0.71) A a	0.00 (0.71) A a	0.00 (0.71) A a	0.00 (0.71) A a	38.17 (5.20) A b	71.67 (8.46) AB c	0.00(0.71)A a	11.90 (3.08) A a

Description: The numbers followed by letters which are similar to column (small letters) and the same line (capital letters) are not different from the5% BNT test, (n): this is the form of transformation with √x+0,5.

From Table 2 above indicated that 0 weeks storage treatment (W0) gives the highest average value of the diameter of the fungal colony, founded on *T. asperellum, T. longibrachiatum*, *T. Harzianum* and *T. virens*, while when continue to the 4-week storage treatment the diameter colony become decrease until 0 mm. Only several Pellet *Trichoderma* which species of *T. harzianum* remained highest in diameter colony. Yetti et al, (2013) founded that *Trichoderma* pellet biofungicide is good act in controlling Ganoderma disease and Soekarno et al., (2014) also explained that formulation Pellet of *T. harzianum* very good action for controlling *hytium* disease on cucumber seedling.

The diameter of fungal colonies observed on all of species*Trichoderma* have been shown that the combination of 4 week storage time and species of *T. harzianum* could be grown on PDA medium. To analysis how their ability tobe good biological control agent of Plant should be shown on next exsperiment. *T. harzianum* (T1) on 4 week storage still growth in the colony of71.61 mm, while for the lowest average diameter fungi colony was found in the treatment of species*T. virens*ie00 mm, T. *atroviridae, T hamatum, T longibrachiatum, T. virens* and *T asperellum*. The results indicate that *T. harzianum* isolate nutmeg was the best *Trichoderma* species in the pellet formulation because it was able to grow back with the highest average diameter comp here with other species treatments.

Application of Pellet *T harzianum* on Cacao Seedling

The results on the analysis of disease severity on the application dosage of *T. harzianum* pellet treatment showed that the treatment without *T. harzianum* pellet (S0) had the highest disease severity compared to other doses treatments. The highest disease severity in the treatment without *T. harzianum* pellet was 41.19% and the lowest disease severity was found in the treatment of *T. harzianum* pellet dosage 2 g mL-1 of sterile water with 11.97% disease severity (Figure 2.).

Cacao seedlings inoculated with *Phytophthora* sp shown specific symptoms. *Phytophthora palmivora* species is the main species of cocoa attacks throughout the

Southeast Asia, in favorable conditions, it is capableinfect young seedlings (causing seeds blight) (Drenth et al., 2004). Doses of pellets 2 g / 100 mL-1 of sterile water (S1) have been able to suppress the incidence of *Phytophthora* leaf blight, compared to other pellet doses. Leaves applied with *T. harzianum* pellets or colonized with antagonistic fungi *Trichoderma* have ability to prevent pathogens from infecting plants, and delay the occurrence of *Phytophthora* disease (Sriwati et al., 2015). In the tomato, *Trichoderma* could induce systemic mechanisms of plant host, the symbiotic interaction between plant and *Trichoderma* have been explained byTucci et al., 2011. *Trichoderma* as a biological agent has ability to produce chitinase enzymes which can inhibit the development of plant pathogens. *Trichoderma* has an antagonistic mechanism to inhibit the development of pathogenic fungi, competition for growth and nutrition, antibiosis and hyphae system interactions (Khaledi & Taheri, 2016). Finally, *Trichoderma* can acting the following ways as :a) Colonizing the soil and / or parts of the plant, occupying a physical space and avoiding the multiplication of the pathogens; b) producing cell wall degrading enzymes against the pathogens; c) producing antibiotics that can kill the pathogens; d) promoting the plant development and e) inducing the defensive mechanisms of the plant. Antifungal formulations based on Trichoderma strains, as in the case of chemical fungicides, require an expensive registration process before commercialization (Mohammad et al., 2014). Although *Trichoderma* species are known as Biological Control Agent (BCA), the selection of host-pathogen specific *Trichoderma* is essential for the successful field application (Saravanakumar et al., 2017).

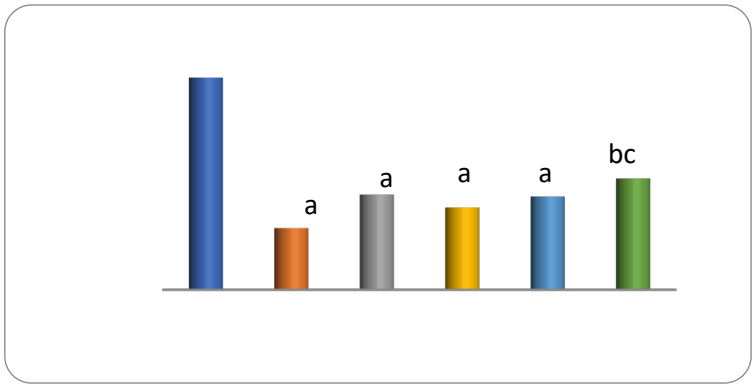

Figure 2. Average disease severity (%) of leaf blight of 14 HSI cocoa seedlings due to Trichoderma pellet treatment The numbers followed by the same letters are not significantly different based on the LSD test at the 0.05 level. Data is transformedArcsin √x

Trichoderma pellets can be influenced by several factors, such as environmental conditions, pathogen conditions and conditions of biological agents. The treatment of a

high dose of antagonistic fungi inoculums gives initial athe biological agent have a high population. The treatment of *Trichoderma* pellet doses had a significant effect on the width of the leaves of the cacao seedlings. The average leaf width of the cacao seedlings increases every week, this can be seen in the average leaf width of the cacao seedlings at 7.14, 21 and 28 HSI (Figure 3), the treatment without the *Trichoderma* pellet treatment was significantly different from the other treatments, with the smallest average width leaf of cacao seedlings in the treatment of *Trichoderma* pellet doses of 10 g mL-1 of sterile water was 3.87 cm while the largest leaf width of the cacao seedlings in the treatment of *Trichoderma* pellets was 2 g mL-1 of sterile water with can affect plant pathogens. This is because *Trichoderma* able to produce growth hormones, including the production of auxin hormones in the form of IAA (Contreras-Cornejo et al., 2009). At low concentrations, IAA functions in plant growth, but auxin at high concentrations can inhibit plant growth, because excessive IAA production will stimulate the formation of excessive ethylene hormones which will inhibit the development / extension of plant root cells, especially in nurseries, are affected by the environmental conditions (Duca et al., 2014; Nieto-Jacobo et al., 2017). This is what underlies the higher the *Trichoderma* pellet dosage given to cacao seedlings, which can be proven to be stunted growth with a lower leaf area than the cacao seedlings given *Trichoderma* pellets at a dose of 2 g / 100 mL-1. The results of this study indicate that the effective dose of *Trichoderm*a pellets in suppressing the development of leaf blight is at a dose of 2 g mL-1.

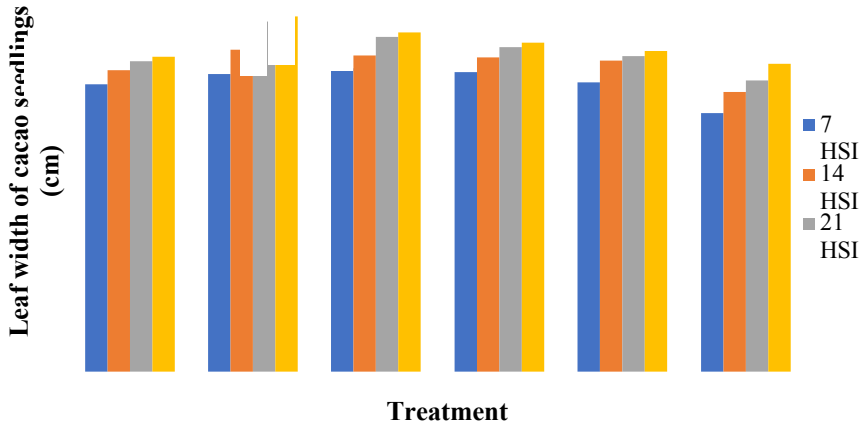

Figure 3. Averageleaf width of cacao seedlings due to *Trichoderma* pellet treatment at 7, 14, 21 and 28 HSI The numbers followed by the same letters are not significantly different based on the LSD test at the 0.05 level. Data transformed Arcsin √x

4. Conclusions

Eight *Trichoderma* species have been isolated from several high plantations in Aceh Region, Sumatera Indonesia (Pine, Cacao, Gliceria, Nutmeg, Bamboo, Coffee, Potato)

respectively a *Trichoderma asperellum*, *Trichoderma atroviride*, *Trichoderma longibrachiatum.* All species of *Trichoderma* have been tested for Pelet formulation, base on their mycelium diameter growth on PDA, *T. harzianum* have selected as potential species on pellet formulation growth with storage periods maximum in 4 weeks. *Trichoderma* harzianum in pellets formulation then were applied with several dose by spraying to cocoa seedling treatments, the result indicating that the dosage 2 g / 100 ml of sterile water is effective in suppressing the development of *Phytophthora* leaf blight compared to other doses. However, in the higher dosage treatment, which is at doses of 4 g to 10 g / 100 mL-1 sterile water has inhibited plant growth which the severity of the disease is higher.

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