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The use of local endophyte fungus to control vascular streak dieback (*Ceratobasidium theobromae*) disease in cocoa

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Vascular streak dieback (VSD) is an important disease of cocoa in Indonesia because can kill a mature plant. One of solution to reduce VSD infection is by using endophytic fungi which can suppress the growth of *Ceratobasidium theobromae*. The aims of the study were to determine the effectiveness of local endophytic fungus in reducing the incidence of VSD, analyze the secondary production of metabolites produced by endophytic fungus and determining cocoa clones that were resistant to VSD. This research was carried out in a split plot design with the basic design of the Randomized Block Design (RBD). The main plot was a different type of clone, consists of 2 types: (K1) = Clone of Sulawesi 1 and (K2) = Clone of Sulawesi 2. Whereas in subplot were a different treatment of endophytic fungus, consists of: (C0) = Control without endophytic fungus, (C1) = fungicides, (C2) = *Paecilomyces* sp., (C3) = *Cladosporium* sp. and (C4) = *Nigrospora* sp.. Each treatment combination consists of 3 plants and repeated 3 times. The data obtained analyzed using a one-way ANOVA, then continue with Duncan's Multiple Range Test at the 95% level of confidence. This data showed the endophytic fungus can increase the vegetative growth of cocoa plants and control VSD disease. Endophytic fungi *Paecilomyces* sp. and *Nigrospora* sp. are recommended to use for control VSD disease in the field because can suppress VSD disease with percentage value of disease incidence respectively 0.00%. Endophytic fungi *Paecilomyces* sp. and *Nigrospora* sp. also have high concentrations of salicylic acid respectively 15.45 mg/L and 16.09 mg/L, and peroxidase respectively 1.706 units/ml and 1.473.

Keywords: Cocoa, *Paecilomyces* sp., *Cladosporium* sp., *Nigrospora* sp., Endophytic fungus, vascular streak dieback

INTRODUCTION

Cocoa (*Theobromae cocoa* L.) is a plantation crops that has high economic value and one of the important commodities in international trade. Some countries are trying to increase their cocoa production to meet the needs of world cocoa. Currently, Ivory Coast supplies 38% (1.5 million tons/year) of world cocoa needs, while Ghana is 19% (ICCO, 2013) and Indonesia is 13% (Binam et al., 2008; Iberemo et al., 2014). The increase in the amount of cocoa supply from Indonesia still has a very big opportunity considering the current national cocoa productivity is still not optimal.

According to data from the Plantation Office of Southeast Sulawesi in 2015, cocoa production was 821.1 kg/ha, while national cocoa production

was 854 kg/ha (Agency of Plantations and Horticulture Indonesia, 2015). The cocoa plantation area in Southeast Sulawesi was dominated by smallholder plantations covering 255350 ha, with cocoa production about 161514 tons in 2014 and decreasing production to 133708 tons in 2015. Productivity also decreased from 869 kg/ha to 733.8 kg/ha (Agency of Plantations and Horticulture Indonesia, 2015). This is very much different compared to the productivity of cocoa in Malaysia in the same year, which was 1500-1800 kg/ha (Azhar and Lee, 2004; Dormon et al., 2004) and in Papua New Guinea which reached 1200 kg/ha (CABI, 2016). The decline in cocoa production and productivity in Southeast Sulawesi will have an impact on cocoa production and productivity at the national level. This is because the area of cocoa plantations in Southeast Sulawesi is the second national rank in Indonesia, covering an area of 284125 ha, a level below Central Sulawesi, which is covering an area of 251730 ha (Directorat General of Plantations Indonesia, 2014).

Fungus *Ceratobasidium theobromae* Talbot & Keane is a pathogenic fungus that causes vascular streak dieback (VSD) disease. These pathogenic fungus are obligate parasites and live on cacao plant tissue. VSD pathogenic fungus becomes an important problem because it can cause a decrease in cocoa production by 40-50%, and even in vulnerable clones, this fungus can cause death in productive cocoa plants (Guest and Keane, 2007; Rosmana et al., 2005; Samuel et al., 2012; Taufik et al., 2015).

One control effort that can be done is using endophytic fungus. The results of previous studies have been successfully isolated from six local endophytic fungus derived from cocoa petiole, including three generas of endophytic fungus, *Paecilomyces* sp., *Cladosporium* sp. and *Nigrospora* sp. which has the potential to control VSD disease in-vitro and in-vivo (Assad et al., 2017). Rosmana reported that the use of *Trichoderma asperellum* fungus combined with plant residues was effective in controlling VSD disease. The control of VSD disease based on endophytic fungus is believed to be appropriate and comprehensive for controlling VSD disease in the field (Rosmana et al., 2015). This is because endophytic fungus work in cocoa plant tissues that can directly and indirectly against VSD pathogens that are also in the same niche. The mechanism for controlling endophytic fungus is through space competition, antibiosis or parasitation (indirectly inducing the resistance of cocoa plants). Segarra reported that endophytic fungus are able to produce salicylic acid, jasmonic acid and peroxidase, and can induce plant resistance to pathogens (Segarra et al., 2007). One of important strategy will be done in this study and that was to design the effectiveness of local endophytic fungus in reducing the incidence of VSD, analyze the secondary production of metabolites produced by endophytic fungus and determining cocoa clones that were resistant to VSD.

MATERIALS AND METHODS

This research was conducted in the Laboratory of Plant Protection Unit Phytopathology and Screen House, Faculty of Agriculture, Halu Oleo University, Kendari, Southeast Sulawesi; during February to July 2018.

Materials used in this research included two cocoa clone seeds, endophytic fungus isolates (*Paecilomyces* sp., *Cladosporium* sp. and *Nigrospora* sp.), VSD inoculum, alcohol, distilled water, bayclen, PDA media, agar media, soil, sand, manure, polybag, and NPK compound fertilizer (Nitrogen-Phosphorus-Potassium). The tools used in this study included laminar air flow, autoclave, PCR machine, spectrophotometer, Bunsen lamp, pruning shears, calipers, shovel, watering-can, beaker, petri dishes, shaker, micro tube, sprayer, drop pipettes, nursery tray, electric scales, cameras,and stationery.

This research was carried out in a split plot design with the basic design of the Randomized Block Design (RBD). The main plot was a different type of clone, consists of 2 types: (K1) = Clone Sulawesi 1 and (K2) = Clone Sulawesi 2. Whereas in subplot were a different treatment of endophytic fungus, consists of: (C0) = Control without endophytic fungus, (C1) = fungicides, (C2) = *Paecilomyces* sp., (C3) = *Cladosporium* sp. and (C4) = *Nigrospora* sp.. Each treatment combination consists of 3 plants and repeated 3 times.

The research procedure used in this study referred to the research procedure carried out by Taufik (Taufik et al., 2015; Taufik et al., 2016a; Taufik et al., 2016b) as follows:

Preparation of planting media.

The planting medium consists of top soil, manure, and sand with a ratio of 1: 1: 1. Planting media sterilized using an autoclave, then put into polybags measuring 20x25 cm with a thickness of 0.05 mm. The planting media fill in a polybag up

to 1-2 cm from the edge of the up-border. The filled polybags then arranged in a screen house according to the experimental design used and watered to saturation.

Preparation of endophytic fungus suspension.

Endophytic fungus isolates (*Paecilomyces* sp., *Cladosporium* sp. and *Nigrospora* sp.) were grown on PDA media by removing the pure culture of the endophytic fungus in 1 × 1 cm area into other petri dishes containing PDA media and incubated for 2 weeks until the conidia of endophytic fungus formed. Furthermore, endophytic fungus isolates were mixed with 100 ml of distilled water and applied to cocoa seeds.

Nursery, Transfer, and Maintenance of Cocoa Seeds.

Cocoa seeds that have been dried weres own on prepared seeding media. After reaching a sufficient age, it was transferred to a polybag to be treated according to the design that had been prepared. Plant maintenance included watering, fertilizing and controlling pests and diseases. Watering was done twice a day in the morning and evening. Fertilization done two times used NPK compound fertilizer with a dose of 5 g/plant. The first stage of fertilization done at the age of the plant 2 weeks after the plant transferred to the polybag with a dose of 2.5 g/plant. The second stage of fertilization done at the age of 4 weeks with a dose of 2.5 g/plant. Pest and disease control were carried out mechanically and cleaning weeds that grow inside the polybag.

Measurement of Secondary Metabolites.

Analysis of the content of salicylic acid was using a method that refers to Warrier (Warrier et al., 2013). Extraction of plant leaves was done by mixing small pieces of leaves with 50% ethanol solution as much as 10 mL, then shaking for 15 minutes at 150 rpm and filtered. The leaf filtrate was placed into eppendorf and centrifuged for 10 minutes at a speed of 12000 rpm at 25^{o}C, then added 1% $FeCl_3$ solution. The absorbance was measured at a wavelength of 525 nm using a UV-Vis spectrophotometer.

Analysis of the peroxidase enzyme activity was using a method that refers to Patra and Mishra (Patra and Mishra, 1979). A mixture of solutions consisting of 1 mL phosphate buffer (pH 6.5), 3 mL pyrogallol, 2 mL 1% H_2O_2 and 1 mL plant leaf extract (resulted by grinding of 1:10 g/v leaf samples) were inserted into a UV-Vis Spectrophotometer tube. The absorbance was measured at a wavelength of 420 nm.

Observation Variable.

Observations were made on sample plants with the following observational variables: plant height (cm), number of leaves (strands), stem diameter (cm), the incidence of VSD disease and measurement of secondary metabolites, carried out by observing external symptoms in plants. Observations were made every 2 and 6 weeks after inoculation (WAI). The rate of disease incidence calculated using the following formula:

$$KP = \frac{N}{N} \times 100\%$$

Where: DI = Disease incidence (%)
N = Number of leaves with VSD symptoms
N = Number of leaves observed

The data obtained were analyzed using a one-way ANOVA, if between treatments have a significant effect, then continue with Duncan's Multiple Range Test at the 95% level of confidence.

RESULTS

Response to the Increase in Height of Cocoa Plants

The results of ANOVA showed that clone Sulawesi 2 had a higher plant height compared to clone Sulawesi 1, both in the 2 and 6 WAI.

The results of Duncan's Multiple Range Test at the 95% level of confidence were as follows: At the age of 2 WAI, *Paecilomyces* sp. gave a response to the increase in height of cocoa plants which was not significantly different with *Nigrospora* sp., but significantly different with *Cladosporium* sp., fungicide, and control (without the application of endophytic fungus and fungicide). The control gave the lowest plant height response, but it was not significantly different with the application of fungicide (Figure 1).

At the age of 6 WAI, *Nigrospora* sp. gave a response to the increase in height of cocoa plants which was not significantly different with *Paecilomyces* sp., *Cladosporium* sp. and application of fungicides, but significantly different with controls. The control gave the lowest plant height response, but it was not significantly different with the application of fungicide (Figure 1).

Table 1: The independent effect of cocoa plants clone on the increase of cocoa plant height at the age of 2 and 6 WAI

Cocoa Clone	Plant height (cm)	
	2 WAI	6 WAI
Sulawesi 1	2.22[b]	4.09[b]
Sulawesi 2	2.74[a]	5.10[a]
DMDT (0.05)	0.29	0.37

*The numbers followed by letters were significantly different in Duncan's Multiple Range Test at the 95% level of confidence

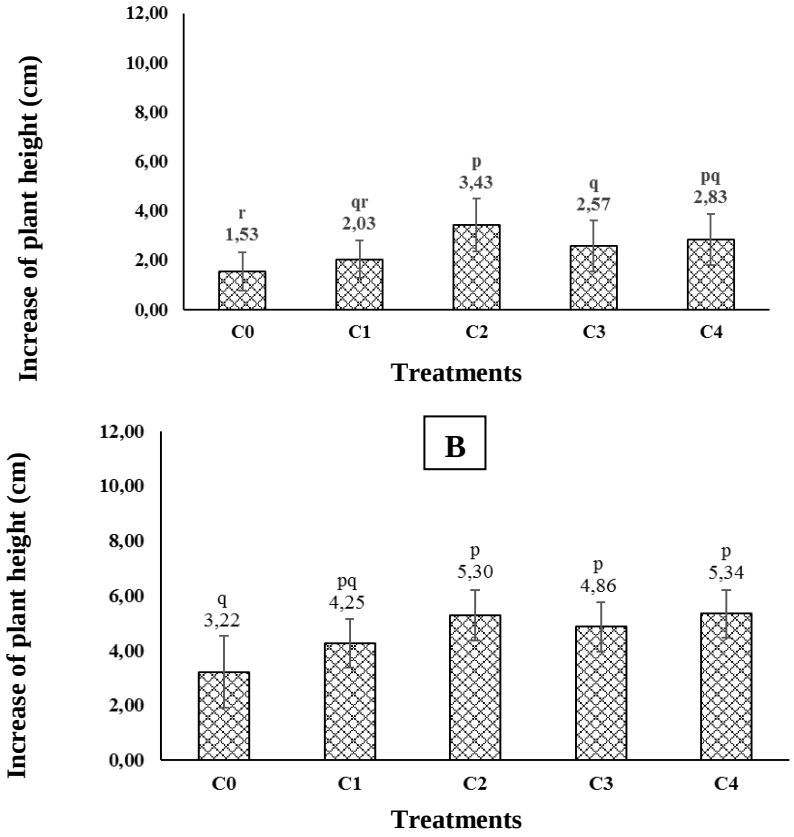

Figure1: The independent effect of endophytic fungus on the increase of cocoa plant height, where A = Increase of cocoa plant height 2 WAI, B = Increase of cocoa plant height 6 WAI, (where: C0 = Control, C1 = Fungisida, C2 = *Paecilomyces* sp., C3 = *Cladosporium* sp., C4 = *Nigrospora* sp.).

Number of Leaves of Cocoa Plants

The results of ANOVA showed that there was no significant difference between the clone Sulawesi 1 and clone Sulawesi 2 in all observations independently.

The results of Duncan's Multiple Range Test at the 95% level of confidence were as follows: At the age of 2 WAI, *Paecilomyces* sp. gave a response to the number of leaves of cocoa plants which was not significantly different with *Cladosporium* sp. and *Nigrospora* sp., but significantly different with control and application of fungicide. The application of fungicide gave a response to the number of leaves of cocoa plants which was not significantly different with control.

At the age of 6 WAI, *Paecilomyces* sp. gave a response to the number of leaves of cocoa plants which was not significantly different with *Nigrospora* sp., *Cladosporium* sp. and application of fungicide, but significantly different with control. The control gave a response to the number of leaves of cocoa plants which was not significantly different with the application of fungicide (Figure 2).

Table 2: The independent effect of cocoa plants clone on the increase of cocoa plant number of leaves at the age of 2 and 6 WAI

Cocoa Clone	Number of leaves	
	2 WAI	6 WAI
Sulawesi 1	2.87	3.01
Sulawesi 2	2.67	3.54
DMDT (0.05)		

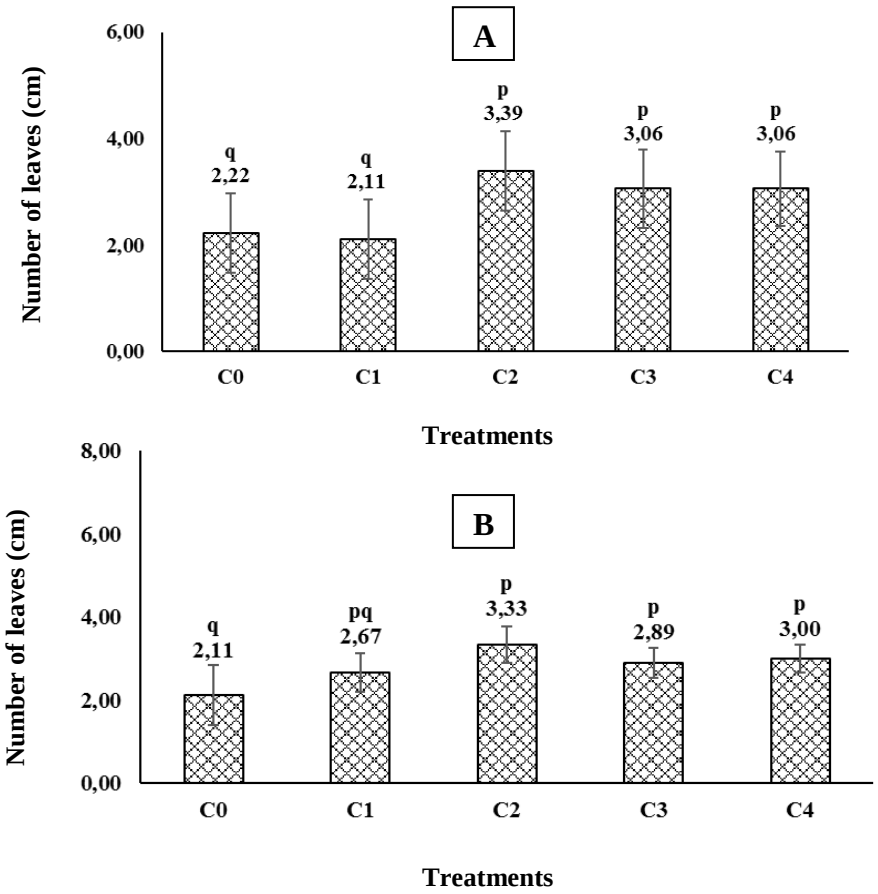

Figure 2: The independent effect of endophytic fungi on the number of leaves of cocoa plants, where A = number of leaves 2 WAI, B = number of leaves 6 WAI, (where: C0 = Control, C1 = Fungicide, C2 = *Paecilomyces* sp., C3 = *Cladosporium* sp., C4 = *Nigrospora* sp.)

Stem Diameter of Cocoa Plants

Based on the observation of stem diameter, clone Sulawesi 1 and clone Sulawesi 2 showed significant differences, both at observations 4 and 8 WAI, whereas observation resulted in 2 and 6 WAI showed that there was no significant difference between the clones. Clone Sulawesi 2 had a wider diameter result.

The results of Duncan's Multiple Range Test at the 95% level of confidence were as follows: At 2 WAI, *Paecilomyces* sp. gave a response to the stem diameter of cocoa plants which was not significantly different with *Cladosporium* sp. and *Nigrospora* sp., but significantly different with control and the application of fungicides. The application of fungicide gave a response to the stem diameter of cocoa plants which was not significantly different with the control and *Nigrospora* sp.

At 6 WAI, *Paecilomyces* sp. gave a response to the stem diameter of cocoa plants which was not significantly different with *Cladosporium* sp. and *Nigrospora* sp., but significantly different with the application of fungicides and control.

Table 3: The independent effect of cocoa plants clone on stem diameter of cocoa plants at the age of 2 and 6 WAI

Cocoa Clone	Stem Diameter (cm)	
	2 WAI	6 WAI
Sulawesi 1	0.33	0.49
Sulawesi 2	0.35	0.51
DMDT (0.05)		

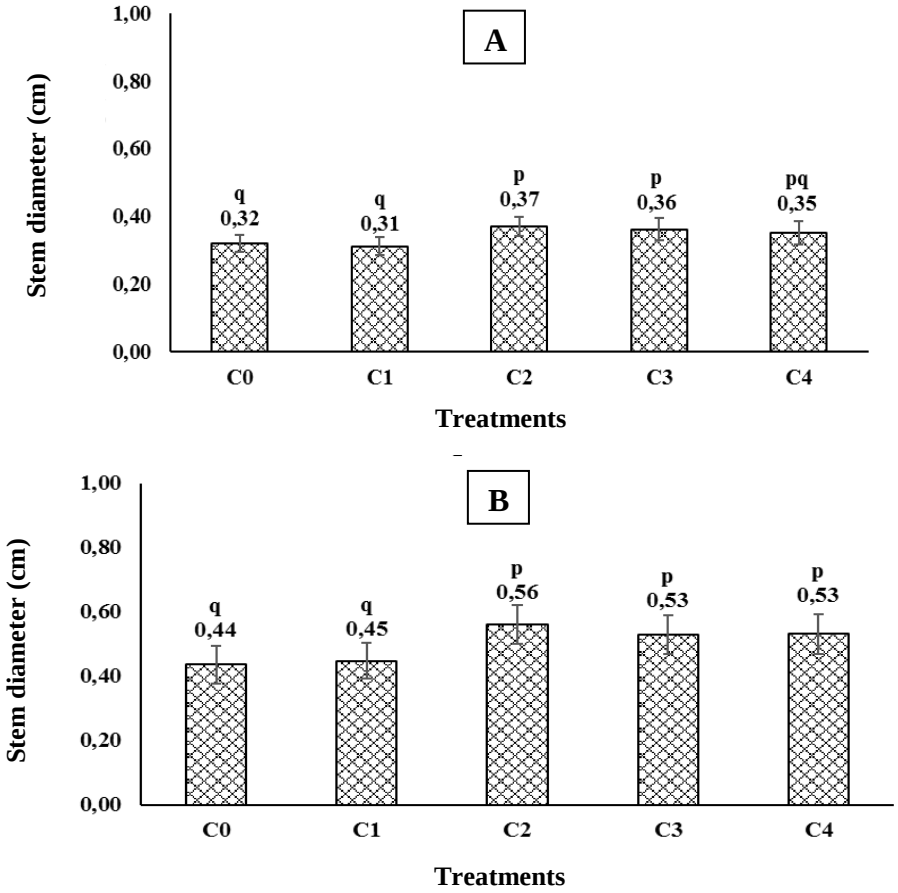

Figure 3:The independent effect of endophytic fungus on the stem diameter of cocoa plants, where A = stem diameter of cocoa plants 2 WAI, B = stem diameter of cocoa plants stems 6 WAI, (where: C0 = Control, C1 = Fungicide, C2 = *Paecilomyces* sp., C3 = *Cladosporium* sp., C4 = *Nigrospora* sp.)

The control gave a response to the stem diameter of cocoa plants which was not significantly different with the application of fungicide (Figure 3).

The incidence of VSD Disease in Cocoa Plants

The results of Duncan's Multiple Range Test at the 95% level of confidence were the control gave a response to the incidence of VSD disease in cocoa plants which was significantly different with the application of fungicides, *Cladosporium* sp., *Paecilomyces* sp. and *Nigrospora* sp.. *Paecilomyces* sp. and *Nigrospora* sp. gave a response to the incidence of VSD disease in cocoa plants which were significantly different with *Cladosporium* sp. and the application of fungicides (Figure 4).

Measurement of Secondary Metabolites

In general, the absorbance value of salicylic acid in Lambda 525 nm increased compared to plants that were not given endophytic fungus.

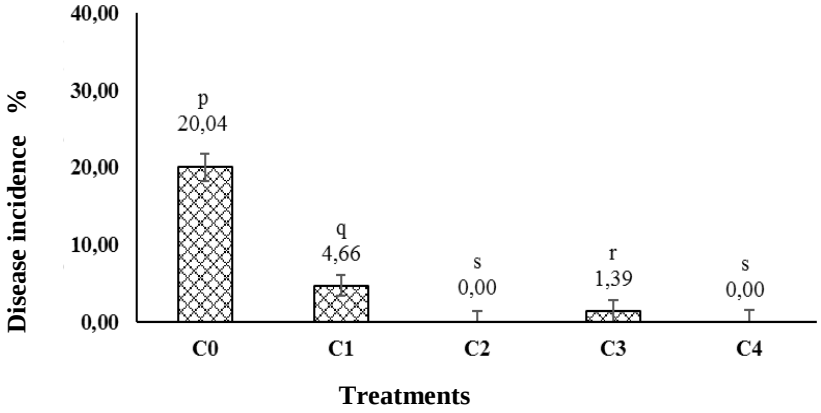

Figure 4: The independent effect of endophytic fungus on the incidence of Vascular Streak Dieback (VSD) disease in cocoa plants (where: C0 = Control, C1 = Fungicide, C2 = *Paecilomyces* sp., C3 = *Cladosporium* sp., C4 = *Nigrospora* sp.)

Table 4: Salicylic acid concentration in cocoa plants 2 WAI

Treatment	AB	ppm (mg/L)	Mg (mg)
Co⁺	0.152	13.45	1.21
C2	0.174	15.45	1.39
C4	0.181	16.09	1.44
C3	0.174	15.45	1.39

*C0⁺= inoculated plants (Clones1), C2 = Application of *Paecilomyces* sp., C3 = Application of *Cladosporium* sp., C4 = Application of *Nigrosporium* sp.

Table 5: Peroxidase concentration in cocoa plants 2 WAI

Treatment	Initial measurement value (unit/L)	Final measurement value (unit/L)
CO⁺	0.260	1.361
C2	0.458	1.706
C4	0.633	1.473
C3	0.896	2.038

*C0⁺= inoculated plants (Clones1), C2 = Application of *Paecilomyces* sp., C3 = Application of *Cladosporium* sp., C4 = Application of *Nigrosporium* sp.

The highest concentration of salicylic acid was found in cocoa plants which given endophytic fungi *Nigrospora* sp., *Paecilomyces* sp. and *Cladosporium* sp..

Peroxidase concentrations tended to be higher in cocoa plants that were applied to endophytic fungus than plants that were not applied to endophytic fungus. The highest peroxidase value occurred in cocoa plants which given *Nigrospora* sp. (Table 5).

DISCUSSION

The ability of endophytic fungus to increase plant growth depend on it ability to produce a number of growth-promoting metabolites. Growth agents such as gibberellins and auxins are produced by endophytic fungus (Khan et al., 2012). *Paecilomyces* formosus LHL10 endophytic fungus are able to produce gibberellin phytohormones reaching 32 µg/mL and indole acetic acid 1.21µg/mL. Increasing of plant growth by endophytic fungus is thought to be caused also by an increase in the number of hair roots, branching of hair roots, and lateral roots, therefore the roots of plants will be wider and deeper. Vasudevan reported that root length in IR 24, IR 50 and Joythi rice varieties increased after endophytic fungus were applied. Thus, the absorption of nutrients will be more and plant growth will be better, more vigorous and more resistant to disease (Vasudevan et al., 2002).

In terms of cocoa plant height increase, the

application of endophytic fungi *Paecilomyces* sp. produced a height plant response that was better than other treatments. The height increases of cocoa plants that given *Paecilomyces* sp. can be caused by the ability of endophytic fungi to produce hormones that trigger an increase in plant growth. The results of research by Wahab stated that the application of biological agents independently produced more than 32 cm height of cocoa plant whereas in the control without biological agents only produced more than 22 cm height of the cocoa plant, both were inoculated with C. *theobromae* (Wahab et al., 2016).

The response of the number of leaves in cocoa plants was in line with the results of plant height observation. The cocoa plant that was applied endophytic fungus gave a significantly different number of leaves with control, especially at the end of the observation. Application of *Cladosporium* sp. produces an average number of leaves higher than other treatments. This difference thought to be caused by genetic differences in each endophytic fungus. But in general, both fungus have the ability to increase plant biomass. Rodriguez reported that some endophytic fungus might be able to increase plant ecophysiology and increase the ability of plants to counter stress due to biotic factors and increase plant biomass (Rodriguez et al., 2009). Endophytic fungus have the ability to secrete the growth hormone gibberellin as a mechanism to promote plant growth.

The result of observation of cocoa stem diameter in nurseries showed an increase. Clone Sulawesi 2 has a larger stem diameter compared to clone Sulawesi 1. It was thought that these two clones respond differently to stem diameter increase caused by genetic factors. One indicator of the good growth of plants is stem diameter which can be used to predict the ability of roots to absorb plants nutrients (Susilo and Suhendi, 2001). Differences in stem diameter could be a criterion in crop selection. Plants which was applied an endophytic fungus caused a better plants growth in all observed variables, including plants biomass that was twice as high as control plants, and the content of indole acetic acid (IAA) and gibberellin accumulated higher than control plants (Dai et al., 2008). Lu reported that endophytic fungi *Colletotrichum* sp. on the Artemisia annua plants produces IAA to encourage the growth process (Lu et al., 2000).

In terms of observing the resistance level of cocoa clones, there was a difference in the level of resistance of these cocoa plants to VSD disease. Based on the symptoms of VSD disease incidence, clone Sulawesi 2 were more resistant than clone Sulawesi 1, however based on the incubation period, clone Sulawesi 1 showed VSD symptoms 14.48 days slower than clone Sulawesi 2. This difference might be due to genetic differences of each clone.

VSD disease in cocoa plants is estimated to cause 100% yield loss in vulnerable clones, and 15% yield loss in resistant clones. The use of resistant cocoa clones is the cheapest and most effective way to control VSD disease (Susilo and Suhendi, 2001). Halimah and Sukamto reported that the genetic differences (clones) of cocoa produce different resistance responses indicated by high or low loss of yield (Halima and Sukamto, 2006). The mechanism of plant resistance that may occur is the structural resistance of plants because plants have a physical barrier such as wax layers, the thickness of the epidermal layer (cell wall) and cellulose or stomata form.

The result of observation of salicylic acid and peroxidase showed a tendency for both endophytic fungi *Paecilomyces* sp. and *Nigrospora* sp. to accumulate the higher of salicylic acid and peroxidase compared to control. It is suspected that the endophytic fungus uses a resistance induction mechanism to fight infection with VSD pathogens through the accumulation of salicylic acid. The increase of concentration of salicylic acid not always indicate an increase in peroxidase concentration, as seen in the application of *Nigrospora* sp. with a concentration of salicylic acid 16.09 mg/L whereas the peroxidase value of 1.473 unit/L, the lowest compared to the application of other endophytic fungus. Peroxidase is one of the PR-proteins that play a role in plant resistance to disease. The study result indicated that an increase in the concentration of salicylic acid in cocoa plants with the application of *Nigrospora* sp. did not affect the increase in peroxidase enzyme concentration. This was contrary to the statement of Murphy that salicylic acid is a signal transduction in which one branch activates PR-proteins, including peroxidise (Murphy et al., 2001). Activation of the PR-protein gene not always coincide with an increase in the content of salicylic acid. The effect of induction by an agent has the specificity of the type of PR-protein it induces (Molina et al., 1998).

From the result of the observation, it showed that the symptoms of disease in the cocoa plants that applied endophytic fungi *Cladosporium* sp. amounting to less than 1.39%, this indicates a better level of resistance compared to controls

that showed symptoms of the disease by 20.04%. The possibility is there was a resistance mechanism developed by plants through the mechanism of localized cell death (Gao et al., 2010). It is important to note that the direct effects of endophytic fungus on pathogens are complex, possibly due to specific species antagonism sensitivity (Arnold et al., 2003) or other factors that cannot be explained from this study. However, the research has proven that cocoa plants applied endophytic fungus, generally had a better growth and more resistant to inoculation of VSD disease than control, even better than the use of fungicides.

Based on this study, the use of endophytic fungus is a good way to control VSD disease, because pathogens are in the same tissue as endophytic fungus. Eventhough *Cladosporium* sp. have highest peroxidase value, but the result obtained showed that the endophytic fungi *Paecilomyces* sp. and *Nigrospora* sp. potentially used to control VSD disease during nursery. Further studies are needed in the field to prove the consistency of the results of the study.

CONCLUSION

Endophytic fungus can increase the vegetative growth of cacao plants even though it has been inoculated with VSD disease. The use of Endophytic fungi *Nigrospora* sp. and *Paecilomyces* sp. are recommended to control VSD disease in the field, both fungi are able to produce salicylic acid and peroxidation which functions to build a resistance mechanism in cocoa plants.

CONFLICT OF INTEREST

The authors declared that present study was original and performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

MT designed and ferformed the experiments, collected sample, obsevation of plant growth, research paper editor and reviewed the manuscript. DB collected the data, prepared cocoa clone and maintanaced of cocoa seed. S collected samples and measurement of secondary metabolic of plant. DNY preparation of soil for planted, collected data, research paper editor and reviewed the manuscript. NPP preparation of samples, isolation and preparation of endophyte fungi and fungus suspension, research paper editor and reviewed the manuscript.

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