Evaluating the ability of endophyte fungus to control VSD diseases in cocoa seeding

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Abstract. The use of endophytic fungus is one of the effective ways to control Vascular Streak Dieback (VSD)-*Ceratobasidium theobromae* in cocoa plants both preventively and curatively. The study aimed to evaluate the potential of several endophytic fungus isolates to reduce the incidence of VSD at the seedling level. This research was carried out in a completely randomized design (CRD) with eight endophytic fungus isolates as treatment, namely 1) *Paecilomyces* sp. EP1* isolate; 2) *Paecilomyces* sp. EP1 isolate; 3) *Cladosporium* sp.; 4) *Nigrospora* sp.; 5) *Paecilomyces* sp. EP isolate; 6) *Paecilomyces* sp. EP8 isolate; 7) Fungicide and 8) distilled water as a control. The results showed that endophytic fungi *Paecilomyces* Ep1* from cacao petiole was the best inhibitor of VSD disease (0%), and was able to increase the growth of plant height and stem diameter of cocoa plants.

1. Introduction
Cocoa is one of the important plantation crops in Indonesia because cocoa is a source of income for farmers and other communities and contributes to the country’s foreign exchange as well. Indonesia is one of the main cocoa producers in the world after Ivory Coast and Ghana. The area of cocoa plants in Indonesia is around 1727437 ha which consists of 98% of people's plantations and the rest are private and state plantations, with production reaching 728414 tons/year [1]. Southeast Sulawesi Province is the second largest cocoa producer in Indonesia after South Sulawesi. The area of cocoa plantations in Southeast Sulawesi is dominated by smallholder plantations covering 255350 ha, with 2014 cocoa production of 118320 tons/year, lower than in 2013 which reached 120240 tons/year [2].

Low production is influenced by many factors, including plant diseases. An important disease in cacao plants is Vascular Streak Dieback (VSD) caused by *Ceratobasidium theobromae* [3,4]. In Southeast Sulawesi, VSD disease has become a new problem for cocoa farmers because of its spread to almost all cocoa plantation centers in Southeast Sulawesi. In the report written by McMahon stated that VSD was found to have infected cacao plants since 1980 in District of Ladongi, Kolaka Regency (East Kolaka) [5]. It was also reported to have been found in Kolaka in 1989 [6].

Rosmana noted that losses due to VSD infection could reduce yield by 40% [3]. Keane reported that infection in the seed phase could cause plant death by 50% after the seed is moved to the field [7]. This disease can cause large losses in mature plants and seeds which planted adjacent to infected
plants because the spread of this pathogen occurs through windborne and is a pathogen that infects the vascular tissue of cacao plants [8].

The loss caused by VSD is very large, so it is necessary to control this VSD disease. Common control by farmers is using synthetic pesticides (fungicides). However, these controls have a negative impact, namely: 1) Immunity to target plant pest organisms; 2) The emergence of explosions or epidemics of secondary plant disturbing organisms that were previously less important; 3) Bad or deadly effects on the types of non-target organisms; 4) The danger of pesticide residues in protected products or the environment; and 5) Harmful directly to users/applicators and the environment. Based on these impacts, other control methods that are more appropriate and environmentally friendly are needed, such as the use of endophytic fungus biological agents.

Endophytic fungi have the potential as biocontrol agents because the presence of endophytic fungi is very diverse and abundant, and can be found both in agricultural crops and in grasses. Chaetomium globosum and Phoma sp. are endophytic animals isolated from wheat plants, both of which can reduce the severity of the disease caused by Puccinia triticina and Pyonophora sp. Endophytic fungi are microorganisms that can live and form colonies in plant tissues without causing symptoms of pain in their host.

The study aimed to evaluate the potential of endophytic fungi from the petiole of cocoa in inhibiting VSD growth in cocoa seedlings.

2. 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; from August to November 2016.

Materials used in this research included polybag, cocoa seeds, PDA media, water agar (WA) media, endophytic fungus isolates Paecilomyces sp. EP11, EP1, EP6, EP8 Cladosporium sp. and Nigrospora sp., NaCl, label paper, filter tissue, distilled water, 70% ethanol, plastic wrap, aluminium foil, spiritus. The tools used in this study include autoclave, Erlenmeyer, laminar air flow, cutter, scissors, measuring cup, Schott bottle, petri dish, Bunsen, needle ose, tweezers, string, ruler, calipers, camera and stationery.

This research was carried out in a Completely Randomized Design (CRD) consisting of 8 treatments which were repeated three times: (A) = Application of endophytic fungi Paecilomyces sp. EP11 isolate, (B) = Application of endophytic fungi Paecilomyces sp. EP1 isolate, (C) = Application of endophytic fungi Cladosporium sp., (D) = Application of endophytic fungi Nigrospora sp., (E) = Application of endophytic fungi Paecilomyces sp. EP6 isolate, (F) = Application of endophytic fungi Paecilomyces sp. EP8 isolate, (G) = Application of Fungicide and (H) = Control.

2.1. Preparation of planting media
The soil that has been used as a planting medium was the soil originating from the experimental garden of Agriculture Faculty, Halu Oleo University Kendari. Subsequent soil is mixed with sand and cow manure with a comparison of sand, cow manure and soil are 1: 1: 1 (v/v) and steam sterilization were carried out to kill the possibility of soil-borne microorganisms. Then the soil was put in a polybag with a size of 20 × 25 cm to be used as a planting medium.

2.2. Preparation of endophytic fungi isolate
The endophytic fungus isolate used the result of a study by Taufik et al. 2016 [9]. Endophytic fungus used in this research were Paecilomyces sp. EP11, EP6, EP8, EP1 isolates, Cladosporium sp. and Nigrospora sp. which were previously stored at low temperatures in the refrigerator, therefore it was needed to be refreshed by growing on PDA media. Isolates of 7-day endophytic fungi were suspended by inserting 10 mL of sterile aquades into endophytic fungi isolates on PDA media and then spore harvesting was carried out, then put into Erlenmeyer until the endophytic fungi suspension reached 100 mL. This procedure is done for each endophytic fungus.
2.3. **Seed preparation**

The cocoa seeds used in this study were the Sulawesi-1 Clone originating from the Cocoa Research Sub-Station of the Plantation and Horticulture Office of Southeast Sulawesi Province, Alebo Jaya Village, Konda District, South Konawe Regency. The cocoa beans cleaned from the sticky mucus in the following way: mixing rubbing ash on the slimy seeds then squeezing by hand. After the seeds separate from the mucus, the seeds were washed with clean water and dried for one day. Then the seed disinfectant was carried out by washing it using 70% ethanol for ± 1 minute and NaCl solution for ± 3 minutes. Then the seeds were rinsed with sterile aquades for ± 1 minute and repeated three times. The seeds were soaked in a previously prepared fungus suspension according to each treatment and put in a shaker for 2 hours. Next, the seeds were removed from the fungus suspension and dried on sterile filter paper.

2.4. **Cacao seed nursery**

The dried cacao seeds were then neatly sorted in the seedling tray that has contained media growing with a ratio of sterile soil, sand and cow manure each of 1: 1: 1.

2.5. **Moving to plant**

The 14-day-old cacao seedlings were planted into polybags that had been prepared; the transfer was carried out carefully using the help of a bamboo spoon so that the taproot did not break, then the seeds were planted into poly bags.

2.6. **Preparation of VSD inoculum**

VSD inoculum was obtained from the Cocoa Research Sub-Station of the Plantation and Horticulture Office of Southeast Sulawesi Province, Alebo Jaya Village, Konda District, South Konawe Regency. VSD inoculum was propagated on WA media.

2.7. **Endophytic fungus application**

The application of endophytic fungi was carried out in 3 stages namely (a) Performed when the seeds nursed by means of the seeds soaked in the suspension of endophytic fungi according to the treatment; (b) Performed when transferring sprouts into polybags by spraying endophytic fungi on the entire surface of the seed using a 10 mL/plant sprayer; and (c) Performed 2 weeks after planting by dipping a 2-3 cm leaf tip into a beaker containing an endophytic fungus suspension at a dose of 10 mL/plant and the remaining suspension splashed on the roots of the cocoa plant.

2.8. **Pathogen inoculation in plants**

Pathogen inoculation was carried out two weeks after transplanting. VSD mycelium that had been grown in the WA media than printed using cork holes and placed on the leaf surface. In each plant, there were two leaves inoculated and placed two pieces of *C. theobromae* mycelium with a distance of 5 cm between mycelium.

2.9. **Plant maintenance**

Plant maintenance included watering, weeding, fertilizing, and controlling pests. Watering was done twice a day in the morning and evening. Weeding was done at any time when there were weeds in a polybag. Fertilization was done by making a hole around the plant. Pest control was carried out physically by taking the dangerous pests found in the crop.

2.10. **Data analysis**

The data obtained were analyzed by analysis of variance (ANOVA), and if the treatment had a significant effect based on the F test, then a further test was carried out by comparing the mean value of treatment with Duncan Multiple Range Test (DMRT) at $\alpha = 0.05$. For the incubation period and standard tabulation disease events.
3. Results and Discussions

3.1. Plant height

Treatment of *Paecilomyces* sp. EP 11, *Cladosporium* sp., *Nigrospora* sp. and *Paecilomyces* sp. EP 6, gave the best plant height in observations 2, 4, 6, and eight weeks after inoculation (WAI). In the treatment of *Paecilomyces* sp. EP 11 (A) age 8 WAI, average plant height was 44.20 cm which was not significantly different with B, C, D, E, F, and G, but it was significantly different from the control treatment (H) where the average of plant height was only 31.07 cm. The results of the average plant height difference test are presented in Table 1.

Table 1. The effect of the application of endophytic fungus to the average plant height of cacao at 2, 4, 6 and 8 WAI

| Treatment                | Average plant height (cm) in observation |
|--------------------------|------------------------------------------|
|                          | 2WAI  | 4WAI | 6WAI | 8WAI |
| **A = Paecilomyces sp. EP 11** | 29.93 | 39.37 | 43.63 | 44.20 |
| **B = Paecilomyces sp. EP 1** | 23.27 | 30.30 | 37.13 | 37.53 |
| **C = Cladosporium sp.** | 27.47 | 37.20 | 39.03 | 39.30 |
| **D = Nigrospora sp.** | 26.43 | 35.00 | 40.27 | 40.57 |
| **E = Paecilomyces sp. EP 6** | 26.60 | 38.27 | 43.07 | 43.30 |
| **F = Paecilomyces sp. EP 8** | 24.33 | 31.00 | 36.83 | 37.07 |
| **G = Fungicide** | 26.33 | 36.43 | 39.40 | 39.50 |
| **H = Control** | 22.67 | 28.70 | 31.00 | 31.07 |

Note: The numbers followed by the same letters in the same column are not significantly different at the 95% confidence level.

Observations on the variable of cocoa plants height showed that the application of endophytic fungi *Paecilomyces* sp. EP11 produces better plant height responses than other treatments from 4 to 8 WAI, based on the ability of endophytic fungi to produce hormones that can trigger an increase in plant growth. Wahab reported that the application of biological agents independently produced more than 32 cm cocoa plant height, whereas in control without a biological agent produced only 22 cm, both were inoculated with the pathogen *C. theobromae* [10]. The ability of endophytic fungi to increase plant growth depends on its ability to produce several growth metabolites. Growing agents such as gibberellins and auxins are produced by endophytic fungi [11].

3.2. Number of leaves

Treatment of *Paecilomyces* sp. EP 11, *Nigrospora* sp., *Paecilomyces* sp. EP 6 and *Paecilomyces* sp. EP 8, gave the highest number of leaves in observations 2, 4, 6, and 8 WAI. In the treatment of *Paecilomyces* sp. EP 11 (A) age 8 WAI, the average number of leaves of the plant was 22.67 strands, not significantly different from B, C, D, E, F, and G, but significantly different from the control treatment (H) where the average number of leaves was only 18.87 strands. The results of the test different mean number of leaves are presented in Table 2.

Table 2. The effect of the application of endophytic fungus on the average number of leaves of cocoa plants at 2, 4, 6 and 8 WAI
The results of DMRT on the observation of the average number of leaves of the cocoa plants showed that the application of Fungicides produced a better response of the number of leaves of the plant compared to other treatments, ranging from 2 to 8 WAI and significantly different from the control treatment.

### 3.3. Stem diameter

Treatment of *Paecilomyces* sp. EP 11, *Nigrospora* sp., *Paecilomyces* sp. EP 6 and *Paecilomyces* sp. EP 8, gave the largest stem diameter in observations 2, 4, 6, and 8 WAI. In the treatment of *Paecilomyces* sp. EP 11 (A) at 8 WAI, the plant stem diameter was 0.93 cm which was not significantly different from B, C, D, E, F, G, and H, but significantly different from the control treatment where the plant height was only 0.77 cm. The results of further tests on the average stem diameter of plants are presented in Table 3.

#### Table 3. The effect of the application of endophytic fungus on the average stem diameter of cocoa plants at 2, 4, 6 and 8 WAI

| Treatment                  | Average stem diameter (cm) in observation |
|----------------------------|------------------------------------------|
|                            | 2 WAI  | 4 WAI  | 6 WAI  | 8 WAI  |
| A = *Paecilomyces* sp. EP11| 0.61 a  | 0.67 A  | 0.88 a  | 0.93 a  |
| B = *Paecilomyces* sp. EP1 | 0.52 cd | 0.62 A  | 0.78 A  | 0.85 a  |
| C = *Cladosporium* sp.     | 0.60 ab | 0.68 A  | 0.81 a  | 0.87 a  |
| D = *Nigrospora* sp.       | 0.55 abcd| 0.61 A  | 0.83 a  | 0.88 a  |
| E = *Paecilomyces* sp. EP6 | 0.57 abc| 0.66 A  | 0.82 a  | 0.89 a  |
| F = *Paecilomyces* sp. EP8 | 0.54 bcd| 0.60 ab | 0.80 a  | 0.87 a  |
| G = Fungicide              | 0.56 abcd| 0.64 A  | 0.81 a  | 0.86 a  |
| H = Control                | 0.50 d  | 0.53 B  | 0.68 b  | 0.77 b  |

Note: The numbers followed by the same letters in the same column are not significantly different at the 95% confidence level.
Observations on stem diameter of plants at the age of 2 WAI showed that the treatment of \textit{Paecilomyces} sp. EP11 was better than other treatments. At the age of 4 WAI, the treatment of \textit{Cladosporium} sp. was the best treatment compared to other treatments. At the age of 6-8 WAI, the treatment of \textit{Paecilomyces} sp. EP11 showed the highest stem diameter compared to other treatments caused by genetic factors. Susilo stated that one indicator of the good growth of plants is stem diameter to predict the ability of roots to absorb plant nutrients [12]. The difference in stem diameter can be a plant selection criterion and high inheritance. Dai stated that plants applied by endophytic fungi caused better plant growth in all observed variables, plant biomass was twice as high and indole acetic acid (IAA) and gibberellins accumulated higher than control plants [13].

3.4. Leaf area
Treatment of \textit{Nigrospora} sp., \textit{Paecilomyces} sp. EP11, \textit{Paecilomyces} sp. EP 6 and \textit{Paecilomyces} sp. EP 8, gave the best leaf area on observations 2, 4, 6, and 8 WAI. In the treatment of \textit{Nigrospora} sp. (D) age 8 WAI, the average of leaf area was 88.41 cm$^2$, not significantly different from A, B, C, E, F, and G, but significantly different from control treatment where the average of leaf area of the plant was only 70.10 cm$^2$. The results of further tests on the average leaf area of plants are presented in Table 4.

Table 4. The effect of the application of endophytic fungus on the average of leaf area of cocoa plants at 2, 4, 6 and 8 WAI

| Treatment               | Average of leaf area (cm$^2$) in observation |
|-------------------------|-----------------------------------------------|
|                         | 2 WAI   | 4 WAI   | 6 WAI   | 8 WAI   |
| A = \textit{Paecilomyces} sp. EP11 | 69.34 b | 71.94 bc | 73.63 ab | 74.66 ab |
| B = \textit{Paecilomyces} sp. EP 1 | 66.03 b | 67.40 c  | 69.33 b  | 70.59 b  |
| C = \textit{Cladosporium} sp. | 84.74 a | 84.69 ab | 84.70 ab | 84.71 ab |
| D = \textit{Nigrospora} sp. | 86.38 a | 87.07 a  | 87.66 a  | 88.41 a  |
| E = \textit{Paecilomyces} sp. EP 6 | 86.23 a | 86.66 a  | 86.92 a  | 87.34 a  |
| F = \textit{Paecilomyces} sp. EP 8 | 72.94 ab| 73.47 abc| 73.48 ab | 73.49 ab |
| G = Fungicide           | 78.46 ab| 78.96 abc| 79.30 ab | 79.64 ab |
| H = Control             | 68.74 b | 70.08 c  | 70.10 b  | 70.10 b  |

Note: The numbers followed by the same letters in the same column are not significantly different at the 95% confidence level

3.5. Incubation period
The incubation period in cocoa plants which did not apply fungicides and endophytic fungus gave a faster response to the incidence of VSD. Control (H) and \textit{Paecilomyces} sp. EP 1 (B) gave the fastest disease response where the average of incubation period was 15.43 and 4.37 days after inoculation, significantly different from \textit{Paecilomyces} sp. EP11 (A), \textit{Cladosporium} sp. EP11 (C), \textit{Nigrospora} sp.EP4 (D), \textit{Paecilomyces} sp. EP 6 (E), \textit{Paecilomyces} sp. EP8 (F) and the application of fungicide (G). The results of further tests on the average incubation period of VSD disease in cocoa plants are presented in Table 5.

Table 5. The observation of the incubation period of VSD in cocoa seedlings
Treatment | Average of incubation Period (day)
--- | ---
A = *Paecilomyces* sp. EP11 | 4.37
B = *Paecilomyces* sp. EP 1 | -
C = *Cladosporium* sp. | -
D = *Nigrospora* sp. | -
E = *Paecilomyces* sp. EP 6 | -
F = *Paecilomyces* sp. EP 8 | -
G = Fungicide | -
H = Control | 15.43

Note: - = there is no incidence of VSD disease in cocoa plants

### 3.6 Disease occurrence

The incidence of VSD disease in the control treatment (without biological agents) showed the highest percentage of disease incidence compared to all other treatments with the average disease incidence at 4, 6 and 8 WAI at 58.51%, 60.02%, and 64.67%. The results of further testing of the average incidence of VSD disease in cocoa plants are presented in Table 6.

**Table 6.** The average percentage of VSD disease incidence in cocoa plants according to treatment

| Treatment | Average disease incidence (%) |
| --- | --- |
|  | 4 WAI | 6 WAI | 8 WAI |
| A = *Paecilomyces* sp. EP11 | 0 | 0 | 0 |
| B = *Paecilomyces* sp. EP 1 | 7.37 | 16.78 | 16.78 |
| C = *Cladosporium* sp. | 0 | 0 | 0 |
| D = *Nigrospora* sp. | 0 | 0 | 0 |
| E = *Paecilomyces* sp. EP 6 | 0 | 0 | 0 |
| F = *Paecilomyces* sp. EP 8 | 0 | 0 | 0 |
| G = Fungicide | 0 | 0 | 0 |
| H = Control | 58.51 | 60.02 | 64.67 |

Note: 0 = there is no incidence of VSD disease in cocoa plants.

The results of the observations on the incidence of VSD showed that the highest incidence of disease occurred in the control treatment at 60.02%. This could be known through the inoculation of VSD pathogens which were placed on the leaf surface. Symptoms that arise were the occurrence of damage to leaf tissue with chlorosis between leaf bones, where the pathogen VSD infects cocoa leaves by entering the natural hole in the stomata. The results of previous studies have reported that the stomata in cocoa leaves can function as a hole in the infection of the *Crinipellisperniciosa* pathogen witches-broom disease [14]. The role of endophytic fungi in plants expresses various mechanisms to fight stress, both biotic and abiotic. The expression mechanism shown can be a direct effect or an indirect effect. The direct effect is the occurrence of interactions between endophytes and pathogens, while the indirect effect is an increase in plant resistance. The direct effect shows the ability of endophytic fungi to produce degrading antibiotics or enzymes, while the indirect effects are related to
the mechanism of systemically induced resistance. The level of pathogenicity is associated with plant resistance systems.

Morphological and biochemical changes including cell necrosis, hypersensitivity to response reactions and production of phytoalexin are rapid forms of plant defense against environmental stresses or pathogens. The results showed that the incidence of VSD was also linear with a very low disease incubation period in cocoa plants given endophytic fungi and inoculated with pathogens that cause VSD disease. The incidence of VSD and the incubation period only occur in the application of fungi, controls, and Paecilomyces sp. EP1, while other endophytic fungi are Paecilomyces sp. EP11, Cladosporium sp., Nigrospora sp., Paecilomyces sp. EP6, Paecilomyces sp. EP8, and the Fungicide does not show symptoms of VSD disease and symptoms after inoculation. In contrast to Paecilomyces sp EP1, the control treatment without endophytic fungi showed the incidence of VSD.

The results of this study indicate the opportunity for the use of endophytic fungi as one of the proper ways to control VSD disease, considering that pathogens are in the tissues and endophytic fungi are also in the same tissue. The results obtained give hope that the endophytic fungus Paecilomyces sp. EP11, Cladosporium sp., Nigrospora sp., Paecilomyces sp. EP6, Paecilomyces sp. EP8 potentially used to control VSD disease in nurseries.

4. Conclusion
Based on the results of this study, it can be concluded that endophytic fungus from cacao petiole can increase the growth of cocoa plants and inhibit the incidence of VSD disease. Endophytic fungus Paecilomyces sp. isolate EP11, Nigrospora sp., Paecilomyces sp. isolate EP 6, Fungicide and Paecilomyces sp. isolate EP 8, showed the best treatment on plant height, number of leaves, stem diameter, and leaf area, and was able to inhibit the incidence of VSD by 64.67%.

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