In vitro growth induction of *Ceratobasidium theobromae*, the causal agent of cacao Vascular Streak Dieback disease

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Abstract. Vascular Streak Dieback (VSD) disease, caused by *Ceratobasidium theobromae*, has devastated cacao plants in Indonesia and other countries. However, there is limited information on the biology of the fungus, including mass propagation method. This study aimed to find a technique in inducing the growth of *C. theobromae* as inoculum sources. The stages of research were sampling the plant cacao tissue infected by *C. theobromae*; induction of mycelia growth on infected cacao tissue using coconut water, glucose, and sucrose; and confirmation *C. theobromae* by morphology, pathogenicity test on cacao seedling, and genetic. We found characteristic symptoms of VSD disease in cocoa plantations marked by chlorosis in cocoa leaves and blackening of the node on the leaf petiole. The growth induction of *C. theobromae* could be performed using a 2% glucose solution. The hyaline hyphae, perpendicular branching of hyphae, and a dolipare septate were shown in the hyphae structure. Pathogenicity test of mycelia *C. theobromae* from infected plant tissue showed typical symptoms of VSD. Genetic confirmation by PCR method successfully amplified specific target DNA of *C. theobromae* resulted in ± 550 bp amplicon. The result could be performed to propagate *C. theobromae* inoculum for various purposes in studying VSD disease.

1. Introduction
Cocoa (*Theobroma cacao* L) is a plantation crop with high economic value. World cocoa production is estimated to reach 4,232,000 tonnes in 2014/2015 [1]. Indonesia is one of the countries that contribute to world cocoa production. To date, Indonesia is the third largest cocoa producer after Ghana and Ivory Coast [2]. However, data shows that national cocoa production has decreased in the last three years, namely 767,280, 734,796, and 713,378 tons, respectively, between 2018, 2019, and 2020 [3].

The main obstacle in cocoa production is the attack of plant pests and diseases. Total yield loss due to pests and diseases is estimated at 198,000 tons/year [4]. One of the cacao diseases that causes substantial losses to cocoa production is cacao wood vascular disease or Vascular Streak Dieback (VSD) disease. VSD disease in cocoa plants is caused by infection of the obligate fungus, *Ceratobasidium theobromae* [5]. VSD disease can cause yield losses in the range of 15% in resistant cocoa clones, 100% in susceptible clones [6].

VSD was first reported in Papua New Guinea in 1960 [7, 8, 9] and continues to spread rapidly to Southeast Asia. In Indonesia, the first report related to VSD occurred in 1983 in Sebatik Island, East Kalimantan and in 2006 it was known to have spread in most parts of Indonesia [6]. In 2015 VSD disease was found in cocoa plantations in West Sumatera Province in several locations, with the disease incidence reached 100% and diseases severity of 24.29–44.71% [10]. In Yogyakarta Province, VSD...
disease was reported to attack cacao plantations in Gunung Kidul Regency with disease incidence of 30-100% and disease severity 7.5 -60%, while in Kulon Progo Regency, the incidence of disease was 0-70% with disease severity ranging from 0-25% [11].

Several VSD disease control techniques have been developed, including the use of resistant varieties, technical culture, botanical pesticides, biological control and the use of synthetic fungicides, induction of resistance, and others [12]. However, these control techniques are not effective enough to control VSD disease in the field. This is due to the lack of bioecological information on VSD disease caused by the causal agent is an obligate fungus \textit{C. theobromae}. The inability to reproduce \textit{C. theobromae} in the laboratory is one of the factors that has caused many studies related to the development of various VSD disease control techniques such as selection of resistant clones, development of biological control, evaluation of fungicide effectiveness, and others depending on natural infection in the field which is influenced by various complex environmental factors.

The purpose of this study was to study the growth induction method of \textit{C. theobromae} in infected cocoa twigs using liquid nutrition so that it can be used as a material to develop effective control techniques for VSD disease in cocoa plants.

2. Methods

2.1. Sampling the VSD symptomatic cacao twigs
Twigs of cacao plants with VSD disease symptom characteristics were taken from cocoa plantations in Cianjur Regency, West Java from September to December 2017.

2.2. Growth induction of \textit{Caeratobasidium theobromae} in VSD symptomatic cacao twigs
Induction of growth of the fungus \textit{C. theobromae} on plant tissue infected with pathogens using liquid media with various nutritional contents. Twigs were taken from plants that showed symptoms of VSD disease, such as leaves with chlorosis with a combination of yellow and green, three brown dots on the leaf petioles, and rough-textured cacao tree bark [5]. Cocoa twigs were selected in medium size with a diameter of 1-2 cm and cut to a length of 15 cm. The twigs were surface sterilized by immersing them in 1% sodium hypochlorite (NaOCl) solution for 2 minutes and rinsing with sterile water 3 times and air dried on sterile tissue paper.

Treatment of liquid nutrient solution for growth induction consisted of: (1) control (K), (2) coconut water (AK), (3) 2% glucose, (4) 5% glucose, (5) 2% sucrose, and (6) 5% sucrose. The liquid nutrient solution were prepared and sterilized and put as much as 30 ml in sterilized bottle jar. Twigs that have been prepared at one end are put in a bottle jar that already contains a solution and is submerged for approximately 3 cm. Each treatment consisted of 5 twigs and was incubated in an incubation room at room temperature with and without lighting. Observations were made every day and the observed variables on the growth of mycelia of the fungus were qualitative with the categories of hyphae growth being thin, thick, and very thick.

2.3. Morphological observations of \textit{Ceratobasidium theobromae}
Microscopic observation of the morphology of the fungus is part of the confirmation of \textit{C. theobromae} which causes VSD disease from mycelium as a result of growth-induced growth on cocoa twigs with liquid nutrient solution. Microscopic observations included observations on the morphology of the mycelium of the fungus and spores growing on cocoa twigs.

2.4. Pathogenicity test
The pathogenicity test was carried out based on Koch's postulates, that is, isolates of fungi that were successful in growing on cocoa twigs were inoculated onto the leaves of cacao plant seedling. Fungal isolates inoculated on cocoa seedling leaves were isolates that successfully grew on twigs induced with coconut water, 2% glucose solution, 5% glucose, 2% sucrose, 5% sucrose. Inoculation was done by cutting the tip of the twig that was overgrown with \textit{C. theobromae} mycelium for 2 cm. The twigs and mycelium are attached to the lower surface of the young cocoa leaves (young cacao leaves that have
fully opened), covered with thin sterile cotton wool moistened with sterile water and attached with plastic tape. As a control, cocoa seedlings were inoculated with cocoa twigs which were sterilized by immersing in 2% NaOCl solution for 10 minutes and rinsed with sterile water 3 times. The inoculated cocoa seedling was covered with clear plastic to maintain moisture for 3 days. Observations were made once a week until symptoms appeared indicating a VSD disease on the infected seedling.

2.5. Genetic confirmation of Ceratobasidium theobromae by PCR technique
Genetic confirmation of C. theobromae was initiated by harvesting the mycelium that had successfully grown on twigs that were overgrown with mycelium as a result of growth induction. Extraction of total DNA from the successfully harvested mycelium using the method of Dellaporta [7] and a DNA purification protocol with a modified purification kit. The obtained fungal DNA was used for molecular identification using the polymerase chain reaction (PCR) technique. The primers used were specific primers for C. theobromae, namely the forward primer ITS1 (5’-GAGTCTTGGCAGTTGCTG-3’) and reverse primer ITS2 (5’AGAAGCGGTACATCTGTA – 3’) [5]. PCR reactions were carried out at a total volume of 25 µL consisting of 2x Dreamtaq Green PCR Master Mix (Thermo Scientific) 12.5 µL, primers ITS1 1 µL (10 pmol) and ITS2 (10 pmol), DNA template 1 µL, and nuclease-free water 9.5 µL. The PCR process was initiated by predenaturing 92 °C for 2 minutes. The PCR reaction was continued with 35 reaction cycles including; denaturation 94 °C, 30 seconds; annealing 51.8 °C, 30 seconds; extension 72 °C, 1 min. The PCR reaction was terminated by adding a final extension of 72 °C for 10 min.

Visualization of the amplified DNA was carried out on 1% agarose gel by the electrophoresis process. The results of the electrophoresis were then visualized with an ultraviolet transilluminator to observe the DNA fragments formed and documented using a digital camera.

3. Result and discussion
3.1. Symptoms of VSD disease in cocoa plants
Based on observations in cocoa plantations in Cianjur Regency, West Java Province, several cocoa plants were found that have symptoms similar to typical symptoms of VSD infection. These symptoms include chlorosis on cocoa leaves which consists of a combination of yellow color with green spots (Figure 1A), there are three blackish-brown dots on the part of the former sticking of the leaves (Figure 1B), and the surface of the bark of cocoa twigs infected with VSD has a rough texture (Figure 1C). The characteristics of these symptoms are the same as those described by previous researcher [13], that in cacao plants infected with C. theobromae have typical symptoms such as the presence of chlorosis on cocoa leaves, generally, on the second or third leaf of leaf flus, there are three dots of blackish brown color in the area of the twig tissue where there is chlorosis of the leaves, when the twig is split there is a blackish brown line along the xylem tissue of the twig and when the fungus has spread in the internal tissue, the plant will be bare and die.

Based on the results of field observations, the spread of VSD disease in the cocoa plantation area in Cianjur Regency spreads in groups with an irregular distribution pattern in one planting area. This occurrence is becaused the spores of the fungus C. theobromae can be carried by the wind and infect the surrounding cocoa plants. In addition, cocoa cultivation techniques using stem grafting techniques carried out by the company can also affect the spread of disease inoculum, so that VSD infection can spread throughout the cocoa plantation area. This is in line with the previous research [14], in addition to the inoculum of pathogens contained in plant tissue, cultivation techniques with side grafting techniques using young cacao stalks can affect the spread of VSD infection.
3.2. Effect of liquid nutrient solution on growth of Ceratobasidium theobromae on VSD symptomatic cacao twigs

Growth induction with liquid nutrient solution was carried out to stimulate the growth of the obligate mycelium of the fungus *C. theobromae*. Fungal mycelium that has successfully grown from the induction of growth nutrients can be used as material for various studies related to VSD disease in cocoa plants. The use of liquid nutritional induction of growth with coconut water, glucose 2%, glucose 5%, sucrose 2%, and sucrose 5% can stimulate the growth of the mycelium of the fungus *C. theobromae* from the growing media on infected plant tissue compared to the control treatment. This is indicated by the presence of the fungal hyphae. As an illustration, the following are the differences in the growth of hyphae on cocoa twigs as a result of the growth of *C. theobromae* (Figure 2).

**Figure 2.** Mycelium growth on cacao twig samples. a) mycelium grows very thick (+++) on 2% G-induced twig, b) mycelium grows thick (+) on S 2% induced twigs, c) mycelium grows thin (+) on S 5% induced twigs.
Table 1. Mycelium growth quality in cacao twigs induced by various types liquid nutrition solution.

| No | Liquid Nutrient Solution | Lighting | Without Lighting |
|----|--------------------------|----------|-----------------|
| 1  | K (control)              | +        | +               |
| 2  | AK (coconut water)       | ++       | +++             |
| 3  | G 2% Glucose 2%          | +++      | +++             |
| 4  | G 5% (Glucose 5%)        | +        | ++              |
| 5  | S 2% (Sucrose 2%)        | +        | ++              |
| 6  | S 5% (Sucrose 5%)        | +        | +++             |

+++ : mycelium grows very thick
++ : mycelium grows thick
+ : mycelium grows thin

The average mycelium growth quality on cocoa twigs that grew as a result of growth induction with various types of nutrient solutions is presented in Table 1. The results of observations of mycelium growth at 10 days after incubation showed that growth induction with the nutrient solution could improve the growth quality of *C. theobromae* mycelium on incubation period twigs in a liquid solution medium when incubated without lighting (dark). Incubation under lighting conditions with nutrient solution treatment, both glucose, and 5% sucrose indicated the mycelium growth quality was not different from that of the control (Table 1). On the other hand, the treatment of 2% glucose nutrient solution incubated both with and without lighting was able to induce the growth quality of *C. theobromae* mycelium on cocoa twigs.

3.3. Morphological characterization of *Ceratobasidium theobromae*

Microscopic observations were carried out to confirm that the mycelium grown from the growth induction was the mycelium of *C. theobromae*. Microscopic observations included morphological observations of hyphae and spores of *C. theobromae*. Based on observations that the hyphae of the fungus *C. theobromae* are hyaline in color and have a dolipore septum on the hyphae, hyphae branching is perpendicular, and the spores are oval in shape with one side of the spore slightly flat (Figure 3). This is in line with a previous report [15] that *C. theobromae* has morphological characteristics such as hyaline hyphae, perpendicular branching with irregular branching grooves, there is a dolipore bulkhead, and *C. theobromae* spores are egg-shaped, with one side of the spore horizontal.

![Figure 3](image-url)

**Figure 3.** The morphology of *C. theobromae* was based on microscopic observations at a magnification of 40x10. (A, B, C) morphology of hyphae and spores of *C. theobromae* as a result of growth induction, (D, E, F) morphology of hyphae and spores from previous research [5, 15].
3.4. Pathogenicity test
The pathogenicity test of the fungus *C. theobromae* was carried out on cacao seedlings aged 60 DAP (days after planting) with an incubation period of 70 DAI (days after inoculation). The results of the fungal pathogenicity test showed that the fungal isolates inoculated with mycelium-covered twigs growing on the twigs induced growth with a nutrient solution were able to cause typical symptoms of the disease. Symptoms of the disease in the form of chlorosis on cocoa leaves with a combination of yellow color with green spots (Figure 5B), while the control treatment did not show any symptoms of chlorosis (Figure 4A). This is in accordance with the results of research [16], that the initial symptoms of VSD in young cocoa plants can be seen from the presence of chlorosis and can develop into a characteristic symptom in the form of green stripes on a yellow background.

![Figure 4. Pathogenicity test of *C. theobromae* on cacao seedlings; (A) inoculation of control media without symptoms of chlorosis, (B) inoculation cacao seedling with cacao twig covered with mycelium induced by glucose 2% showed symptoms of chlorosis (indicated by arrows).](image)

3.5. Genetic confirmation of *Ceratobasidium theobromae* causes VSD with PCR technique
Total DNA extraction of the fungus *C. theobromae* was carried out using a conventional method [17] and followed by a DNA purification method following the DNA purification kit protocol (Thermo scientific). Target DNA amplification using specific primers ITS1 and ITS2 [5, 12]. PCR amplification using specific primers ITS1 and ITS2 resulted in DNA fragments of approximately 550 bp in size. There is no contradiction with the result from researcher [5] that amplification using ITS1 and ITS 2 primers on *C. theobromae* chromosomal DNA will produce specific DNA fragments measuring approximately 550 bp as shown in Figure 5, where the amplicon is in the size of 500-700 bp in both sample 1 and sample 2 taken from the mycelium fungi on cocoa twigs induced using a 2% glucose liquid nutrient solution (Figure 5).

![Figure 5. Visualization of the results of DNA amplification of *C. theobromae* using specific primers ITS1 and ITS2 on 1% agarose gel with a marker (M) 1 kb DNA ladder (Thermo Scientific), (1) amplification results using DNA template from sample 1, and (2) amplification results using DNA template sample 2 isolated from mycelium growing on cocoa twigs induced using a 2% glucose liquid nutrient solution, replicates 1 and 2.](image)

4. Conclusion
Symptoms of VSD found in the field have special characteristics, such as chlorosis on the leaves of the cocoa plant with a mixture of yellow and green spots, three black-brown dots on the petiole of the leaf,
the surface of the bark of infected cocoa twigs is rough with small nodules. Induction of mycelium growth of *C. theobromae* can be done with coconut water, glucose solution 2 and 5%, and sucrose solution 2 and 5% can stimulate the growth of fungi from infected plant tissue, which is characterized by the presence of white mycelium with the best result is the use of glucose solution 2%. Confirmation of morphology, pathogenicity test, and amplification of target DNA with specific primers ITS1 and ITS2 showed that the fungus that was induced by growth on cocoa twigs with VSD symptoms was *C. theobromae*, the causal agent of VSD disease.

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**References**
[1] [ICCO] International Cacao Organization 2015 *Quarterly Bulletin of Cocoa Statistics* 40 (4), Cocoa year 2014/15.
[2] [DEPPERIN] Departemen Perindustrian 2007 [http://www.bbp2tp.litbang.pertanian.go.id/images/stories/budidaya/kakao.pdf](http://www.bbp2tp.litbang.pertanian.go.id/images/stories/budidaya/kakao.pdf)
[3] [DITJENBUN] Direktorat Jenderal Perkebunan 2020 [http://ditjenbun.pertanian.go.id](http://ditjenbun.pertanian.go.id)
[4] Topae, F N H, Lakani, I and P Panggesa (2016) *Agrotekbis* 4(2) 134–141.
[5] Samuel G J, Ismaiel A, Rosmana A, Junaid M, Guest D, Mcmahon P, Keane P, Purwantara A, Lambert S, Rodrigues-carres M and Cubeta M A. 2012. *Fung biol.* 116:11-23.
[6] Halimah and Sri-Sukamto (2006) *Warta Pusat Penelitian Kopi dan Kakao Indonesia* 22(3):107-119
[7] Bridgland L A, Richardson I M and Edward I L 1966. *S. Pac. Planter* 1(6), 13–20, 28.
[8] Shaw D E 1962 *Papua New Guinea Agr. J*. 15, 79–90.
[9] Keane P J, Flentje N T and Lamb K P 1972 *Aust J Biol Sci* 25: 553-564.
[10] Trisno J, Reflin and Martinius 2016 *J Fitopatol Indones*. 12(4):142-147.
[11] Widiastuti A, A Wibowo, A B Prakoso and Hendra 2017 *Proceeding of the 2nd International Conference on Tropical Agriculture* (26-27 October 2017. Hal 133-140.
[12] Harni R, Wahyuno D and Trisawa I M 2019 *Perspective*. 18(2):120-134
[13] Guest D and Keane P (2007) *J Phytopathol*, 97, 1654–1657.
[14] Wahab A, Sulle A. 2008 [http://sultra.litbang.pertanian.go.id/ind/phocadownload/vol5_tahun_2008/penyakitvascularstreakdieback](http://sultra.litbang.pertanian.go.id/ind/phocadownload/vol5_tahun_2008/penyakitvascularstreakdieback)
[15] Lisnawita, Lubis L and Dhana N P 2013 *J On Agro.* 2(3): 288-293.
[16] Rosmana A 2005 *Prosiding Seminar Ilmiah dan Pertemuan Tahunan PEI dan PFI XVI Komda Sul-Sel; 2005 Mei 23*; Makasar. Indonesia (ID): Makasar. BALITSEREAL Marros. Hlm 1-6.
[17] Dellaporta S L, Wood J and Hicks J B 1983 *Plant Molecular Biology Reporter* 1: 19-21.