

# prosiding\_IOP\_Conference\_Series\_\_Earth\_a\_5.pdf

*by*

---

**Submission date:** 02-Apr-2021 04:13AM (UTC+0700)

**Submission ID:** 1548532402

**File name:** prosiding\_IOP\_Conference\_Series\_\_Earth\_a\_5.pdf (413.55K)

**Word count:** 4065

**Character count:** 21330

PAPER • OPEN ACCESS

<sup>4</sup>First report of vascular streak dieback symptom of cocoa caused by *Ceratobasidium theobromae* in Barru District, South Sulawesi

<sup>3</sup>To cite this article: Muhammad Junaid *et al* 2020 *IOP Conf. Ser.: Earth Environ. Sci.* **486** 012170

View the [article online](#) for updates and enhancements.

4  
**First report of vascular streak dieback symptom of cocoa caused by *Ceratobasidium theobromae* in Barru District, South Sulawesi**

**Muhammad Junaid<sup>1,3</sup>, A Purwantara<sup>2</sup> and D Guest<sup>1</sup>**

<sup>1</sup>School of Life and Environmental Sciences, University of Sydney, Australia

<sup>2</sup>PT. EFFEM-Mars, Makassar, Indonesia.

<sup>3</sup>Cocoa Research Group Faculty of Agriculture, Universitas Hasanuddin, 90245 Makassar, Indonesia.

E-mail: muhammad.junaid@agri.unhas.ac.id

**Abstract.** The cause of vascular streak dieback disease, *Ceratobasidium theobromae* is an economic important cocoa pathogen wherever it attacks and therefore its spread poses a high attention to develop cocoa industry. One of National Islands facing the disease constraint is Sulawesi that many cocoa areas are infected by the pathogen. Understanding a source of pathogen infection in the cocoa farm by investigating specific signs of green chlorotic lesion followed by necrotic leaf and brown streaking within vascular layer is a common method. However, this method is not fully correct since the co-infection phenomenon, secondary fungal pathogens associated with the symptom, has occurred in the field and therefore, the need for detecting accurately by a molecular approach is a highly recommended. This paper will reveal the presence of VSD symptom and pathogen in the new cocoa areas, Barru district before suggestion way of limiting pathogen distribution to new areas was undertaken. Testing the presence of VSD disease pathogen was confirmed with PCR analysis using a pair of specific primers for *C. theobromae*; Than\_ITS1 (forward) and Than\_ITS2 (revers). The finding suggests that the signs of VSD disease symptom and pathogen were found to spread in nine villages in Barru district.

7 **Introduction**

Vascular streak dieback caused by *Ceratobasidium theobromae* is one of the most limiting factors behind the loss of production in developing cocoa industry [1-6]. The disease spreads through Southeast Asia, part of China and India and Pacific islands [1, 4, 7] and in Indonesia, a wider range of disease distribution was reported in Sulawesi [3-6]. Although VSD symptom disease poses in major cocoa plantation across Sulawesi, number of infected cocoa areas remains unclear. Apart of South Sulawesi region, Barru district has typical mountainous landscapes and coastal areas and is located near to districts of Pangkep, Pare-Pare and Soppeng (figure 4). Under national and local cocoa programs, Barru district has become one of important cocoa plantation areas to supply national cocoa production. Mostly cocoa is growing in the highlands and it seems to be isolated from VSD symptom spread. Due to adjacent Soppeng district, there may be potential risk of VSD disease spread to new areas since contributed factors in different way to support the disease spread are inevitable. It is known that Soppeng district contributed to supply seedling propagation for replacing from old tree rehabilitation under national cocoa revitalization scheme (Gernas), where VSD symptom exists.



Content from this work may be used under the terms of the Creative Commons Attribution 3.0 licence. Any further distribution of this work must maintain attribution to the author(s) and the title of the work, journal citation and DOI.

Therefore, the objective of this study was to reveal the presence of VSD disease symptom caused by *C. theobromae* and its spread to new areas of Barru district.

## 2. Methods

The study focused on mountainous and isolated areas in Barru district (about 100 miles from Makassar City). Collecting samples with three leaves of interest in a tree per orchard (total 200 leaves) were undertaken in three subdistricts of Pujananting, Taneteriaja and Barru. All infected leaves with VSD symptom appearance were carried to the lab in Universitas Hasanuddin. In this method, there were sample collection and plant DNA extraction and isolation, DNA quantification and PCR analysis and sequencing. To assess genetic variability of the pathogen, total DNA extracts of infected plant tissue were prepared and the internal transcribed spacer (ITS) region amplified with fungal and Basidiomycete specific primer pairs [11], and *C. theobromae* specific [3] primer pairs, followed by sequencing. The ITS region provides sufficient variability for most fungal population genetic studies and is a useful technique for grouping *Rhizoctonia* spp. [8].

### 2.1. Collecting samples and plant DNA extraction and isolation

All infected leaves of interests collected from cocoa orchards in three subdistricts were carried to Lab of Department of Plant Pest and Disease, Universitas Hasanuddin, Makassar. Infected leaves every village were extracted and isolated by using Kit (Bioline) and a modified CTAB lysis buffer [9]. To obtain sufficient heterogeneous and robust total DNA samples, the most critical step was in maintaining tissue freshness during the trip and this was achieved by using RNAlater® (Sigma-Aldrich). For more detail to obtain total DNA, the protocol is underlined in table 1.

**Table 1.** The modified sample collection and DNA extraction protocol of infected leaf analysis

Stage	Protocol for sample collection and DNA extraction
1	Field sample preserved in RNAlater buffer for stabilization solution (Sigma-Aldrich). Ensure samples indicating VSD symptom lesions are not mislabeled or misplaced. Record the location of the sample collected and the symptom. Remove infected petiole (the sample unit) from the tree with a sterilized blade (scalpel).
2	Transfer petiole into 500 µL RNAlater solution in a 2.0 mL cryovial. If the petiole size is unsuitable for the tube, it can be cut into two pieces using a sterile blade. For short term storage, the petiole is kept at room temperature for a week and for long term storage kept between -5°C and -20°C.
3	In the laboratory, prepare the infected petiole that was stored in RNAlater buffer solution. Remove the petiole and blot with dry paper towel.
4	Blot dry the plant material with a sterile tissue towel or blotting paper to ensure no residual RNAlater buffer solution remains. The dry sample is then returned to a dry 2 mL cryovial before vacuum freeze drying.
5	Freeze dry tissue under vacuum at -50°C for 48 h. If the sample is still moist, continue freeze drying for a further 72 h.
6	Pre-DNA isolation and extraction: Cut the vacuumed-dried petiole into small pieces and place into a mortar that has been washed with dilute HCl. Add about 0.01 g silica sand. Grind the dried petiole with a pestle until it becomes soft and fine with a floury appearance.
7	Transfer product to a 2 mL microcentrifuge tube and add 700 µL preheated lysis CTAB buffer.
8	Vortex lysate for 60-90 seconds to homogenize sample and lysate buffer solution and transfer into 65°C water bath for 100 to 150 minutes. The original CTAB protocol [1] suggested an incubation time that was relatively short (about an hour) probably as the type of specimen tested differs from

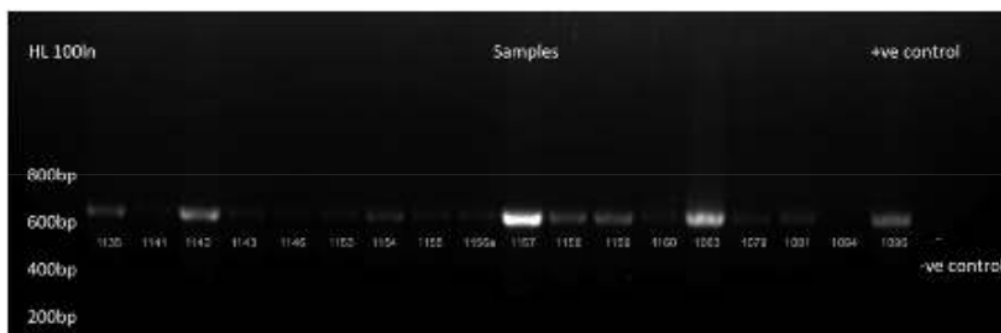
cocoa petioles. Therefore, this method was modified to enable DNA extraction from petioles. Invert the tube every 10 minutes. Add 200  $\mu$ L sodium acetate for a second inversion. If lysate evaporated during preheating, add more 200-300  $\mu$ L mixed lysis CTAB buffer and return to preheated treatment. Adding lysis solution to avoid dried lysate will lessen the loss of a significant volume of lysis buffer. Therefore, when lysis buffer preparation is made, the volume should reach up to 2 mL, more than sample size that is expected in extraction. A transparent aqueous lysate indicates a successful extraction. Adding more incubation time at 65°C to lyse the specimen is the main key to obtaining a good lysate for further work, while leaving lysate with too little lysis buffer leads to poor results.

Once an obvious visible layer separating lysate and waste plant material is seen, carefully pipette the lysate into clean microcentrifuge tubes while avoiding contaminating the sample with waste material.

- 9 Add 400  $\mu$ L chloroform: isoamyl alcohol (24:1) (*Chilco*) to dissolve lysate and vortex thoroughly for 30-60 seconds until the lysate and solution mix, with a resulting milk white-like to light yellow emulsion. Centrifuge the lysate solution for 15 min at 12,000 – 14,000 rpm (room temperature).
- 9 Carefully pipette the aqueous phase into a sterilized 2 mL tube. Once an obvious visible separate layer is seen in the organic phase at the bottom, with an interphase in the middle and an upper aqueous transparent phase, collect the nucleic acids. In this part, a modified pipette tip is important to pull out the nucleic acids gently avoiding uptake of the organic and interphase layers.
- 10 Add 400  $\mu$ L chilled isopropanol and 200  $\mu$ L sodium acetate 3 M (NaOAc), invert the tube for 10-20 seconds and incubate overnight at 29°C for the nucleic acid precipitation.
- 11 Centrifuge the sample at 13,000 – 14,000 rpm in room temperature to separate nucleic acids and waste. Discard waste. Invert the tube on a 60°C heating block over a sterilized tissue towel for 5 min to ensure no residual liquid remains.
- 12 Wash the nucleic acids by adding 400  $\mu$ L 70% chilled ethanol and centrifuge for 60 seconds at 14,000 rpm. Decant the liquid gently so that the precipitate remains. Dry tubes by inversion for 10 min on sterilized tissue towel. Add 300  $\mu$ L 96% EtOH again and centrifuge for 3 min at 14,000 rpm. Visible nucleic acids are retained at the bottom of the vial.
- 13 Dry DNA by inverting tube over a heating block (65°C) for 5-10 min, or by leaving in normal atmosphere for 1-2 h. Do not over dry DNA.
- 14 Add 50  $\mu$ L TE buffer to preserve nucleic acids followed by rapid centrifugation for 15 seconds at 13,000 rpm to ensure all nucleic acid is in solution. Store at -20°C.
- 15 Prior to PCR, the molecular weight and quality of the nucleic acids was analyzed using spectrophotometry (NanoDrop™ 2000/2000c Spectrophotometers).

## 2.2. DNA quantification and PCR analysis

All DNA samples were quantified using Nanodrop spectrophotometry following UV/VIS protocol prior to quantifying with initial PCR amplification for primers ITS 1&4 (forward: CTTGGTCATTTAGA GGAAGTAA and reverse: CAGACTT(G/A) TA(C/T) ATGGTCCAG) [10] before using primers Than ITS 1&2 (forward: GAGTCTTGGCAGTTGCTG and reverse: AGAAGCGGTCATCTGTA) [4]. In the ITS 1& 4 primers, thermal cycler is set into three stages namely denaturation, annealing and extension. For denaturation stage, thermal cycler is aligned earlier for initial denaturation with temperature 94°C; 2 minutes and continuing to 94°C; 30 seconds with cycling repeat 35 times. For annealing stage, temperature is decreased to 55°C; 1 minute for cycling repeat 35 times and in the extension stage, the temperature is then increased to 72°C; 1 minute with cycling repeat 35 times and is added 10 minutes extra times to finalize the thermal cycling program.



**Figure 1.** Gel electrophoresis amplification by touchdown thermal cycler program. HL100ln is marker with hyper ladder 100 lanes; Samples mean infected leaves are tested; +ve control is positive control (sample was sequenced to have similar *C. theobromae* in NCBI website) and -ve control refers to negative control (PCR mixture -DNA template).

A PCR master mix was prepared by adding 7.6  $\mu\text{L}$   $\text{dH}_2\text{O}$ , 7  $\mu\text{L}$   $5\times$  MyTaq Red reaction buffer, 2  $\mu\text{L}$  primer forward (Than\_ITS 1 10mM), 2  $\mu\text{L}$  primer Reverse (Than\_ITS2 10 mL), and 0.4  $\mu\text{L}$  MyTaq Red DNA polymerase (Bioline#Bio-2118). Every 1.5  $\mu\text{L}$  of PCR master mix was combined with about 0.5-1  $\mu\text{L}$  DNA template in PCR tubes. For a negative control, a 2  $\mu\text{L}$  PCR mixture without DNA template, and for positive control a 1.5  $\mu\text{L}$  PCR mixture with 0.5  $\mu\text{L}$  DNA template. The DNA template obtained from infected plant extracts and sequenced with similar *C. theobromae* in National Center for Biotechnology Information (NCBI) <https://www.ncbi.nlm.nih.gov/> were included. Thermal cycle is aligned with touch down program as following table 2.

**Table 2.** The touchdown PCR program for the Than\_ITS 1 and 2 primers

Step	Phase	Time	Temperature
11			
1	Initial denaturation	2 min	94°C
2	Denaturation	30 sec	94°C
3	Annealing	30 sec	65°C -1/ cycle
4	Extension	1 minute	72°C
Go to step 2 $\times 14$			
5	Denaturation	30 sec	94°C
6	Annealing	30 sec	48°C
7	Extension	1 min	72°C
Go to step 5 $\times 34$			
8	Final extension	10 min	72°C
9	End program		4°C
Total time 2 hours 33 min			

All PCR products obtained from primers ITS 1 and 4 were analysed using gel electrophoresis in a 1.5% agarose gel. The DNA fragments shown in the gel by PCR amplification of primers ITS 1&4 were continued to test with touchdown thermal cycler PCR program for specific primers Than\_ITS 1&2. Once DNA samples was successfully amplified, the sample was counted as 1 population unit. If not, sample DNA was negative.

### 3. Results and discussion

To answer the fundamental question why VSD symptom and pathogen spread in the new and remote areas, the study was undertaken with systematically method and the results revealed that all subdistricts of interest to collect samples were found VSD symptomatic lesions and pathogen. It is acknowledged that unnecessary secondary pathogens were also found to have a noticeable association with the symptoms after many attempts were done. From this, hypothesis of co-infection disease is put forward.



Figure 2. Symptom diversity of VSD in District of Barru South Sulawesi

Figure 2 shows that infected leaves expressing typical VSD symptomatic lesions varied in nine villages of Barru district. Two characteristics of VSD symptoms were discovered including chlorotic lesion with green spots (5-6; black arrowhead) and bright yellowing before commencing the necrotic tissue in veins and expressing marginal leaf necrosis followed by dry leaf before fallen leaf. Number of leaves with emerging spores from cracking midrib and petioles (1-4; red arrowhead) was found as well.

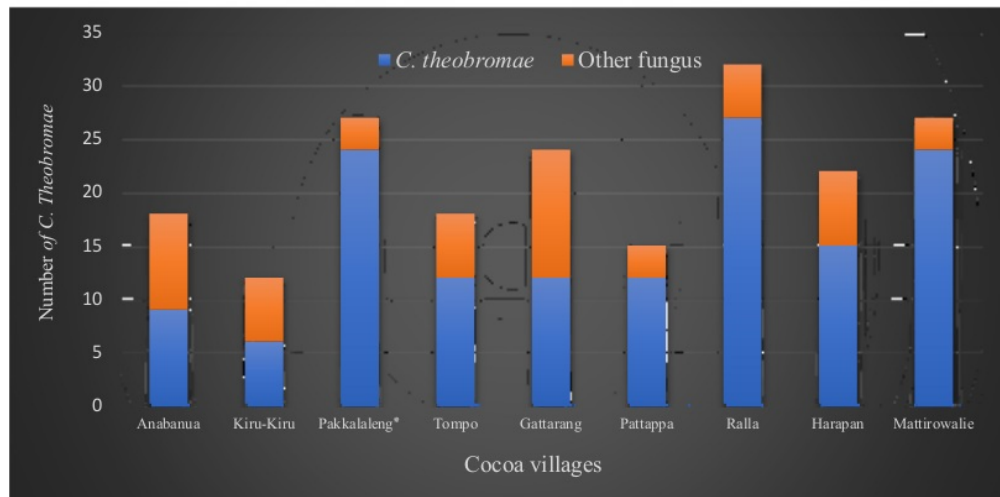


Figure 3. The presence of *C. theobromae* in the Barru district. [\*] One sample sequenced had a similar nucleotide sequence to *C. theobromae* and has been deposited in <https://www.ncbi.nlm.nih.gov/>.

Cocoa plantation in nine villages in Barru district was observed and the result shown to discover VSD disease symptom and its pathogen. Due to testing one infected leaf for one pathogen population, the greatest pathogen population occurred in Ralla, Pakkalaleng and Mattirowalie villages. As a positive control of VSD pathogen, one sample from Pakkalaleng was sequenced to assure species similarity. The findings suggest that the lowest population was found in Kiru-Kiru while the other six villages were almost the same population. During the test of VSD pathogen with specific primer pairs

of Than ITS1&2, number of samples were not amplified indicating the other fungal pathogens as well associated with the symptoms.

Ascomycete fungal pathogen *Fusarium* spp., *Colletotrichum* spp. and *Lasiodiplodia* spp. also associated with the symptom lesions which their role are secondary pathogen on cocoa. Nuramin et al. [11] isolated these typical fungi from resistant and susceptible clones and the preliminary study suggested that of many attempts to culture putative VSD basidiomycete pathogen in the solid media, contamination was major constraint to achieve pure culture. Ascomycete fungus usually interferes the medium and it is such a pirate fungus since it grows very fast to colonize surface medium and does not give a chance of putative *C. theobromae* to grow. The finding suggests that co-infection seems to fit for the association. Expressing symptomatic lesion is a consequence of a complex situation i.e. caused by multiple pathogen infections. VSD pathogen originally attacks vascular tissue causing brown streaking alongside the tissue before followed by tip dieback. Once the host resistant breaks down due to ageing tree, the subsequent infection is from secondary pathogens which occupy bark and phloem



tissues.

**Figure 4.** (Left) A current spot associated with VSD symptoms caused by *C. theobromae* was identified in Barru district (black arrow-head). (Right) The tree foliage facing severe VSD disease symptom in Pakkalaleng village

Figure 4 depicts a new spot found in Barru district surrounded by infected areas (red spots without blue circle). A fundamental question arises why such an isolated area farer from source of infection is infected by *C. theobromae*. One of the most possible factors is by transport of plant materials due to high demand of cocoa seedling in many villages during national cocoa program. It is known that in Gernas period 2009-2013 (national cocoa revitalization program aiming to increase cocoa production), a massive seedling propagation constructed to supply plantation in most districts of South Sulawesi has played important role of VSD disease spread to new areas in Barru district. Based on investigation during the program, one of central seedling propagation areas was in Soppeng district where it was previously suspected from VSD disease symptom. The method of top-grafting on chupons of mature trees is omnipresent since old trees are rehabilitated to introduce resistant material. This method has been disseminated a wider range by farmers in different location in South Sulawesi. To succeed this method, there was a significant demand of tremendous bud woods, rootstocks and chupons collected from mature plant materials and from VSD disease endemic areas i.e. Soppeng, Luwu and Pinrang. Consequently, an accidently disease distribution through plant material propagation is inevitable. Lack of seedling certification of top-grafting method during period of Gernas has triggered to increase the risk of disease distribution. One of example case is a VSD disease distribution in Bantaeng district (South Sulawesi). Prior to Gernas, the cocoa orchards were used to be free from VSD symptom but currently the VSD symptom has become rampant. No doubt about infection in Bantaeng district occurred since the investigation together with Philip Keane (VSD expertise) in 2011 and molecular check were undertaken to assure its presence. Eventually, disease and pathogen transmission through

fresh plant materials is suitable for typical an obligate or fastidious parasite i.e. *C. theobromae* since the pathogen retains within the tissue during the trip and it subsequently completes its life cycle if supported by the environment. Cocoa orchards in nine villages were infected by *C. theobromae* and an interesting future project is to monitor the disease spread in remote areas because many potential factors involved in the distribution are still unknown. Furthermore, this constraint is such an alarm of plant protection and plant quarantine as once the disease extensively spreads into new areas, the cost of VSD control is significant increase to cease pathogen life cycle.

Another important factor behind VSD disease spread into nine remote areas in Barru is due to a typical new encounter disease. The disease arises once indigenous pathogen commences to recognize introduced host i.e. cocoa. It is known that far before cocoa is cultivated in Barru district, basidiomycete pathogen perhaps exists on the vegetation. Once the tree was introduced in new lands due to economical important crop for farmers and national and local cocoa development programs, the pathogen commenced to associate with introduced tree and recognized it as a host without significant barrier.

Aerial medium to spread disease is a potential factor but this medium seems to be less effective way since the wind speed is only able to carry inoculum sources up to 100 m length [7]. Disease distribution from infected sources i.e. Soppeng district to near areas in Barru district is unlikely way due to tough physical barriers. In between Soppeng and Barru areas, number of high mountains exist and no access to open the forest due mainly to very slope lands and protected forest regulation. In the native forest, dense vegetation canopies contribute to limit its distribution and once spores fly, direct sunlight exposure and heavy rain can damage them too prior to landing into new host.

#### 4. Conclusion and recommendation

Presence of VSD disease symptom and the cause of VSD disease, *C. theobromae* paralleled to be discovered at nine villages in Barru district in mountainous areas. Number of pathogen population varied in different location but the greatest population of *C. theobromae* was in Pakkalaleng, Ralla and Mattiowalie. One of samples was convincingly similar with the pathogen deposited in Genbank. In the future, disease distribution should be paid attention to limit its spread. The most effective VSD disease spread in cocoa farms is through planting material movement for seedling propagation by top grafting on chupons. Therefore, to reduce disease spread we need to focus on planting material transmission. It is highly suggested that plant quarantine regulation is needed to limit its distribution and parallelly, educating farmer community is necessary to cease the use of infected plant materials as chupons for seedling propagation.

#### Acknowledgments

We acknowledged that the study was financially supported <sup>12</sup> Australian Centre for International Agricultural Research scheme and we also thanked to head of Department of Plant Pest and Disease, Faculty of Agriculture, Hasanuddin University for Lab equipment for research activity.

#### References

- [1] Ploetz R 2016 The impact of diseases on cacao production: Aglobal overview. In: Bailey A B and Meinhardt W L, eds. *Cacao Diseases: A History of Old Enemies and New Encounters* (New York: Springer International Publishing) p 307-35
- [2] Guest D and Keane P 2007 Vascular-streak dieback: A new encounter disease of cacao in papua new guinea and southeast asia caused by the obligate basidiomycete *Oncobasidium theobromae* *Phytopathology* **97** 1654-1657
- [3] Guest D, Samuels G J, Carres M R, Cubeta M, Ismaiel A, Rosmana A, Muhammad Junaid et al. 2012 VSD *Ceratobasidium* spp. associated with Vascular Streak Dieback (VSD) disease of Cacao

- [4] Samuels G J, Ismaiel A, Rosmana A, Junaid M, Guest D, McMahon P, Keane P, Purwantara A, Lambert S, Rodriguez-Carres M and Cubeta M A 2012 Vascular streak dieback of cacao in Southeast Asia and Melanesia: in planta detection of the pathogen and a new taxonomy *Fungal Biology* **116** 11-23
- [5] Purwantara A, Parawansa A, Laila N, Vandermark E, Junaid M, Rosmana A, et al. 2012 Vascular streak dieback (VSD) of cocoa in South East Asia and Melanesia – a strange new-encounter disease undergoing a region-wide change in symptoms *International Cocoa Seminar, Bali*
- [6] Marelli J P, Guest D I, Bailey B A, Evans H C, Brown J K, Junaid M, Baretto R B, Lisboa D O and Puig A S 2019 Chocolate under threat from old and new cacao diseases *Phytopathology* **109** 1331-1343
- [7] Keane P J 1992 *Diseases and Pests of Cocoa: An Overview. Cocoa Pest and Disease Management in Southeast Asia and Australasia* (Rome: FAO)
- [8] Carling D E, Kuninaga S and Brainard K A 2002 Hyphal anastomosis reactions, rDNA-internal transcribed spacer sequences, and virulence levels among subsets of *Rhizoctonia solani* anastomosis group-2 (AG-2) and AG-BI. *Phytopathology* **92** 43-50
- [9] Doyle J J 1987 A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull.* **19** 11-5
- [10] White T, Bruns T, Lee S and Taylor J 1990 *Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics* (San Diego: Academic Press)
- [11] Amin N, Salam M, Junaid M, Asman and Baco M S 2014 Isolation and identification of endophytic fungi from cocoa plant resistant VSD M.05 and cocoa plant susceptible VSD M.01 in South Sulawesi, Indonesia *Int. J. Curr. Microbiol. App. Sci.* **3** 459-467

ORIGINALITY REPORT

---

9%

SIMILARITY INDEX

9%

INTERNET SOURCES

8%

PUBLICATIONS

6%

STUDENT PAPERS

---

PRIMARY SOURCES

---

1	<a href="http://itp.agritech.unhas.ac.id">itp.agritech.unhas.ac.id</a> Internet Source	3%
2	<a href="http://www.researchgate.net">www.researchgate.net</a> Internet Source	1%
3	<a href="http://es.scribd.com">es.scribd.com</a> Internet Source	1%
4	Febrilia Nur'aini. "Control of Vascular Streak Dieback Disease of Cocoa with Flutriafol Fungicides", Pelita Perkebunan (a Coffee and Cocoa Research Journal), 2014 Publication	1%
5	<a href="http://www.cellgenetics.ru">www.cellgenetics.ru</a> Internet Source	1%
6	<a href="http://bmcmmedgenomics.biomedcentral.com">bmcmmedgenomics.biomedcentral.com</a> Internet Source	<1%
7	Asman Asman, Mohd. Hussin bin Purung, Smilja Lambert, Andi Amiruddin, Ade Rosmana. "Effect of rootstock and scion on resistance of cocoa clones to vascular streak dieback caused	<1%

by *Ceratobasidium theobromae*", *Annals of Agricultural Sciences*, 2021

Publication

---

8	<a href="http://doaj.org">doaj.org</a> Internet Source	<1%
9	<a href="http://iopscience.iop.org">iopscience.iop.org</a> Internet Source	<1%
10	<a href="http://mafiadoc.com">mafiadoc.com</a> Internet Source	<1%
11	<a href="http://pt.scribd.com">pt.scribd.com</a> Internet Source	<1%
12	<a href="http://purl.umn.edu">purl.umn.edu</a> Internet Source	<1%
13	"Abstracts of Presentations at the 2014 APS-CPS Joint Meeting", <i>Phytopathology</i> , 2014 Publication	<1%
14	Submitted to Universitas Diponegoro Student Paper	<1%

---

Exclude quotes On

Exclude matches < 5 words

Exclude bibliography On