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Microbial community occupying cocoa branch and petiole with vascular streak dieback disease symptoms

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Abstract. Vascular Streak Dieback (VSD) disease caused by *Ceratobasidium theobromae* is the most severe emerging cocoa disease in Southeast Asia. Once the tree is infected, tree defoliation will occur as leaf senescence followed by leaf falls. VSD to have associated with endophytes. However, a fundamental question is whether a similar microbe inhabiting vascular tissue of branch and petiole is entire or patchy? The aim of research was to track and quantify microbial diversity associated with VSD symptoms occupying branches and petiole throughout the stem in different clones. The study focused on VSD symptoms expressing in local (Sulawesi 02), susceptible (RCC 70), and resistant (MCC 02) clones to VSD disease. Sample collection was conducted in Lili Riaja, Soppeng District. The 30 cm-branch with the attached petiole of interest was cut into small pieces 1 cm and transferred into water agar medium before submersion into 2.5% NaOCl for 3 minutes, 70% alcohol for 2 minutes and sterilized water for 1 minute. Morphological characterization followed by microscope check was determined to microbial genera. Eight different fungal communities occupying branch and/or petiole consisted of *Cladosporium* sp., *Fusarium* sp., *Colletotrichum* sp., *Penicillium* sp., *Aspergillus* sp., *Moniliophthora* sp., *Lasioidiplodia* sp., *Curvularia* sp. and one Oomycete *Phytophthora palmivora*. Among occupants in branch and petiole, *Fusarium* sp. and *Colletotrichum* sp. were the most frequent. Only one Oomycete *Phytophthora palmivora* occupying clone samples was found. The most diverse microbes were found to associate with Sulawesi 02 and RCC 70 clones, while fewer microbes were detected with MCC 02 clone.

1. Introduction

Vascular streak dieback (VSD) disease has widely spread across Sulawesi. Not just in cocoa development regions but also in hinterland areas where cocoa has been extensively grown [1]. The VSD symptom and its association [2, 3, 4, 5], pathogen isolation obtained from cocoa tissue [6] and its sequence of pathogen DNA [7] has been clearly explained, threatening to develop the national cocoa industry [8, 9]. The infection causes disruption of nutrient and water transportation that leads to loss of leaf development. Due to leaf defoliation, the disease also affects leaf photosynthesis [10]. Finding VSD symptoms in the environment is sometimes misunderstood. Since a complex factor affecting the lesions, a corrected check through sufficient molecular characterization is necessary [11].

The interaction between the fungal endophytic community and healthy cocoa tissues in the environment is quite common. Several endophytes associated with healthy cocoa stems of single



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environmental clone were previously reported [12]. In many cocoa tissues expressing symptomatic lesions, however, the endophytic community association is not fully understood yet. In the current study, tracking and quantifying microbial community associated with cocoa tissues infected by *C. theobromae* VSD is focused, explaining and aligning the VSD symptom and microbe-associated insights.

2. Methods

2.1. Sample collection

Three selected clones were chosen, namely local clone (Sulawesi 02), susceptible (RCC-70) and resistant (MCC-02) clones. Sample collection was a destructive method in which the tissue was separated from the tree. The branch (together with the attached leaf) was transversely cut 30 cm long. In each clone, six branches and six petioles with VSD symptoms were collected as follows figure 1.



Figure 1. An illustration of sample collection (branch and petiole) obtained from the tree with VSD disease symptom in cocoa orchard

2.2. Microbial isolation and characterization

2% (g/mL) WA medium was dissolved and stirred into 1 L Erlenmeyer container and sterilized under 121°C for 2 hours with autoclave. Stock solution was initially cooled down at 30-50 °C before 10 g chloramphenicol was mixed and diluted. All mixes were dissolved and stirred. Every 100 mL WA medium was poured into a 100 x 15mm petri dish.

The 30 cm- branch of interest with attached petiole was cut into small pieces 1 cm long and then transferred for rapid surface sterilization by submersion of 2.5% NaOCl solution for 3 minutes, 70% alcohol for 2 minutes, and sterilized water for 1 minute, respectively [11]. The branch and petiole were then placed on the sterilized filter paper for 5 minutes to dry out. Soon after, dried tissues were gently laid onto the surface agar layer (WA) for further observation.

Microbes were characterized through morphological and microscopic-based analysis. For morphological analysis, septation, hyphal branching pattern, spore shape, and colony color were the main focus. The microscopic check was undertaken to strengthen the morphological analysis [13].

2.3. Microbial quantification

Every single microbe successfully growing out from the branch and or petiole either in the WA medium was quantified as '1' (one) individual. If no microbe growing out from each tissue was marked '0' (zero). In each clone, the maximum WA medium preparation to grow isolates was 12 Petri dishes, including six pieces for 1 cm-branches and six pieces for 1 cm- petioles.

3. Results and discussion

Quantifying microbes occupied branch and petiole associated with VSD symptom in 3 different clones is shown in table 1.

Table 1. Number of microbes occupying cocoa branch and or petiole with VSD symptom in different clone in cocoa farm in Soppeng District.

Microbes	Sulawesi 02 (Local clone)		RCC 70 (Susceptible clone to VSD)		MCC 02 (Resistant clone to VSD)		Total
	Branch	Petiole	Branch	Petiole	Branch	Petiole	
a Fungi							
<i>Cladosporium</i> sp.	1	0	0	0	0	0	1
<i>Fusarium</i> sp.	1	1	1	1	5	1	10
<i>Colletotrichum</i> sp.	1	2	0	1	2	2	8
<i>Penicillium</i> sp.	2	0	0	0	0	0	2
<i>Aspergillus</i> sp.	0	1	0	2	0	0	3
<i>Moniliophthora</i> sp.	1	1	0	0	0	0	2
<i>Lasiodiplodia</i> sp.	0	0	1	2	0	0	3
<i>Curvularia</i> sp.	0	0	2	1	0	1	4
b Oomycete							
<i>Phytophthora palmivora</i>	0	0	1	0	0	0	1
Total	6	5	5	7	7	4	

Table 2. Microbes occupying cocoa branch and petiole or either with VSD symptom expression.

Microbes	Sulawesi 02 (Local clone)			RCC 70 (Susceptible Clone to VSD)			MCC02 (Resistant Clone to VSD)		
	Both	Only branch	Only petiole	Both	Only branch	Only petiole	Both	Only branch	Only petiole
a Fungi									
<i>Cladosporium</i> sp.	no	yes	no	no	no	no	no	no	no
<i>Fusarium</i> sp.	yes	no	no	yes	no	no	yes	yes	no
<i>Colletotrichum</i> sp.	yes	no	no	no	yes	no	yes	no	no
<i>Penicillium</i> sp.	no	yes	no	no	no	no	no	no	no
<i>Aspergillus</i> sp.	no	no	yes	no	no	yes	no	no	no
<i>Moniliophthora</i> sp.	yes	no	no	no	no	no	no	no	no
<i>Lasiodiplodia</i> sp.	no	no	no	yes	no	no	no	no	no
<i>Curvularia</i> sp.	no	no	no	yes	no	no	no	no	yes
b Oomycete									
<i>Phytophthora palmivora</i>	no	no	no	no	yes	no	no	no	no
Total	3	2	1	3	2	1	2	1	1

In the environment, there is a complex interaction among microbe-microbe and microbe-cocoa host that can be seen as competition, parasitism, or mutualism [14]. The current study has discovered that VSD symptoms in branches of resistant (MCC 02) and local (Sulawesi 02) clones shown to be occupied by many more microbes. In contrast to susceptible clone (RCC 70), VSD symptom in the petiole tissue was found to have a more dominant microbial living than the branch. The contribution of microbes inhabiting branch or petiole tissue either in three clones infected by VSD disease pathogen is still yet to be clearly understood, whether it has a negative or positive impact for the symptom development. Further study on understanding its roles, therefore, is necessary to be undertaken.

Fungal Fusarium sp. and *Colletotrichum* sp. were the most frequent occupants associated with VSD symptomatic branch and petiole in both local and resistant clones (table 1 and 2). In contrast to susceptible clone (RCC70), only *Phytophthora palmivora* was discovered to inhabit the petiole tissue. Regarding the role of microbes to associate with the cocoa-host, *Phytophthora palmivora* is the

pathogen of black pod and stem cancer diseases [5], whose virulent variation to cause pod damage level [8,9,16]. For *Fusarium* sp. and *Colletotrichum* sp. in cocoa farming, their role is a partly mutually beneficial 'endophyte' association with the host [11]. In some cases, *Colletotrichum* sp. is a known cocoa pathogen that causes leaf necrotic and pod malformation 'anthracnose' symptoms. Similarly, *Lasiodiplodia* sp. is such a common opportunistic fungus that damages the host when facing severe growth. *L. theobromae* was found to occupy in the tissue of susceptible clone only.

A microbe in the cocoa stem, branch, and petiole has a vital interaction role. Cocoa pathogen whose persistent living within a wide tissue can threaten the host with a significant yield loss. *Phytophthora palmivora*, for instance, besides infecting cocoa stem causing cancer disease, also causes pod disease and leaf necrosis. If endophytic microbes can live persistently within the tissues, the host will earn benefits from protection service against the pathogen. But, finding microbes as a living opportunist is exclusion. In Table 2, most of the microbes were found to occupy both branch and petiole altogether in three different clones, but *Fusarium* sp. and *Colletotrichum* sp. were very dominant. They are consistent with inhabiting both tissues of all clones.

4. Conclusions

Eight (8) different fungal communities occupying stem (both branch and petiole and branch or petiole either) of clone samples consisted of *Cladosporium* sp., *Fusarium* sp., *Colletotrichum* sp., *Penicillium* sp., *Aspergillus* sp., *Moniliophthora* sp., *Lasiodiplodia* sp., *Curvularia* sp. and one (1) Oomycete *Phytophthora palmivora*. Among other occupants in both tissues, *Fusarium* sp. and *Colletotrichum* sp. were the most frequent, and only one Oomycete *Phytophthora palmivora* occupying a branch of susceptible clone was found. The most diverse microbes in the stem (branch and petiole) were found to associate with Sulawesi 02 and RCC 70 clones, while fewer microbes were detected in MCC 02 clone.

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