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4 Endophytic fungi associated with cacao branch and their potential for biocontrol vascular streak dieback disease on cacao seedling

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Abstract. Endophytic fungi are one of the agents to control phytopathogen both bacterial or fungi including dieback disease on cacao such as Vascular Streak Dieback (VSD). Ten fungal isolates that associated with a healthy branch of VSD resistant clone was applied to cacao seedling, the fungi were a *Curvularia*-like colony, a *Fusarium*-like colony, a *Geotrichum*-like colony, an *Aspergillus*-like colony, a *Gliocladium*-like colony, a *Colletotrichum*-like colony and four isolates indicates as mycelia sterilia fungi. Those fungi were applied at 2.5-old months cacao seedling of moderate to susceptible clone against VSD, and 30 days after inoculation of those fungi, the seedling was applied with a bunch of mycelia emerged from infected branch that mix with water using mixer appliance and then the seedling was exposed at open area surrounded by infested cacao tree. The disease incidence was recorded started at 30 and then 60, 90 and 120 days after inoculation of the pathogen and exposed. The result of study shows that the disease appeared increasing gradually and 120 days fungus *Curvularia*-like colony exhibited lower incidence followed by Copper fungicide, Mycelia sterilia 1, *Gliocladium*-like colony, *Fusarium*-like colony, *Geotrichum*-like colony, Mycelia sterilia 3, *Colletotrichum*-like colony, *Aspergillus*-like colony, control (no application), Mycelia sterilia 2, Mycelia sterilia 4 with percentage of 3.0, 8.3, 9.5, 11.3, 15.8, 18.0, 19.0, 19.3, 21.3, 26.5, 30.5, respectively. The result indicates that some fungi associated with VSD resistant clone were able to prevent VSD to develop and causing symptoms.

8 1. Introduction

16 Vascular streak dieback (VSD) is an important disease affecting Southeast Asian cocoa production, including Indonesia. 27 Indonesia is the world's third largest producer of cacao [1], where mostly produced in Sulawesi Island. Estimates of production losses due to VSD is about 774 million US \$ [2]. The disease has devastated some of the cacao 20 plantations and then threaten cacao sustainability, particularly plants belong to smallholder farmer. The disease caused by the basidiomycete fungus *Ceratobasidium theobromae* (formerly *Oncobasidium theobromae*) [3, 4].

The disease has been spread out dramatically at cacao plantation area and seems difficult to control. The pathogen is wind-borne, leaf-penetrating, and xylem 2 infecting [5]. Basidiospores of the pathogen occurs mainly after midnight into the early morning [3]. Basidiospores germinate and hyphae penetrate unhardened leaves at branch termini by growing directly through the cuticle, above leaf veins [6]. Infected leaves do not show symptoms for 3 to 5 months, by which time the pathogen has ramified



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through the xylem in the adjacent stem [3]. Due to its systemic infection characteristic, the VSD disease is difficult to control. Control tools that needed are able to protect young leaves from germination of basidiospores and capable to ramify through the xylem vessel. Even though few fungicides had high fungitoxic activity against the pathogen under experimental condition such as the triazole compounds triadimefon, triadimenol, and propiconazole [7]. However, these fungicides only a few applied by farmers due to its high cost. Currently, cultural practices including regular pruning, fertilizing, and pruning of diseased plant material still remain the most prevalent applied in the diseased tree.

Biocontrol is an option to encounter losses caused by VSD. Endophytic fungi [4] a good alternative to suppress the disease, they proved to be effective against cacao pathogen such as black po[6] rot, frosty pod rot, witches' broom, and vascular streak dieback [8, 9, 10, 11, 12, 13, 14, 15,16, 17]. Endophytic fungi can colonize their host[6] without causing disease symptoms [18], and could isolated both stem and leaves [15, 8, 18, 11, 12, 19]. In the present study, we used endophytic fungi from stems of VSD resistant [9] cacao plants to further identify possible biocontrol agent for vascular streak d[21]ack disease and the possibilities of preventing VSD to develop and penetrate on young leaves using endophytic fungi.

2. Materials and methods

2.1. Endophytic fungi application

Isolates of endophytic fungi were isolated from VSD resistant clone [19]. The Seedling was developed from VSD susceptible[6] to moderate clone. The seedling was 2.5 months old, and before the seedling was treated, it maintained in a nursery building with UV plastic as roof and the seedling growth assisted with fertilizer. Twelve plants for each isolate [11] the controls were used. The treatments are no application (Control), Copper fungicide, *Curvularia*-like colony, *Fusarium*-like colony, *Geotrichum*-like colony, *Aspergillus*-like colony, *Gliocladium*-like colony, *Colletotrichum*-like colony, Mycelia Sterilia Isolate 1, Mycelia Sterilia Isolate 2, Mycelia Sterilia Isolate 3, Mycelia Sterilia Isolate 4

The fungi were formulated in powder form use rice medium, after colony of fungi was well distributed throughly in rice medium, the medium grinded until turn to powder form. Two grams of fungi powder applied to each seedling that already sprayed with 0.7% CMC before. Application of fungi powder to seedling using hand sprayer and the seedling was wounded on stem use needle. For Copper fungicide is using Cuprous Oxide fungicide with concentrae 0.2 g/100 ml.

During 30 days after application of endophyte fungi powder, the seedling was kept under a nursery building with UV plastic as roof, after 30 days the seedling was applied with the disease inoculum and placed at open area surrounded by older high infested cacao tree, seedling placed near infested older cacao.

2.2. Evaluation of Endophytism status

This activity conducted through isolation of treated seedling before and after the seedling applied by Disease inoculum and placed at the open area. the isolation was carried out 30 days after inoculation (DAI) and 150 DAI. Part of seedling that isolated were leaf, stem, and root.

Initially, samp[15] of leaf, stem, and root were rinsed with tap water, and then surface sterilized with NaOCl solution (2.5%) for three minutes and Ethanol (70%) for two minutes and sterilized water for one minute. and then the samples were dried [9] ove sterilized filter paper. Approximately 4 mm diametre pieces of plant materials were placed on Potato-Dextrose Agar (PDA) supplemented with Chloramphenicol and incubated at 25 °C.

2.3. Disease Record and Statistical Analysis

The incidence of the disease was recorded visually on leaf of the seedlin[8] The data regarding the incidence of the VSD was collected 30, 60, 90, 120 after the last application. Aggressivity of fungi was [12] ermined using analysis of variance (ANOVA) and standard error. When significant difference detected, means were separated using Duncan test at 5% probability level.

3. Results and Discussion

3.1. Results

3.1.1. *Incidence of Vascular Streak Dieback.* Evaluation of ten different fungal isolates on seedling under field condition on VSD incidence are presented in table 1 and figure 1. The result of the effect of ten different fungal isolate and controls on 60 DAI observation, where *Curvularia*-like colony, Mycelia Sterilia Isolate 1, *Geotrichum*-like colony, Copper fungicide, *Fusarium*-like colony, *Aspergillus*-like colony, *Colletotrichum*-like colony, Mycelia Sterilia Isolate 3 shows the best result in suppressing the VSD incidence and significantly different with control. Meanwhile, on 90 DAI recording incidence of the disease was lower on *Curvularia*-like colony, Mycelia Sterilia Isolate 1, *Geotrichum*-like colony, Copper fungicide, *Aspergillus*-like colony, those treatments shows significantly different with control.

Table 1. Incidence of Vascular Streak Dieback on 60 and 90 days after inoculation and exposed on cacao seedling.

Treatments	Days After Inoculation (DAI) and Exposes	
	60	90
Control	15.00 ^a	17.50 ^{ab}
Copper fungicide	2.50 ^{cd}	8.25 ^{bcd}
<i>Curvularia</i> -like colony	0.00 ^d	0.00 ^d
<i>Fusarium</i> -like colony	1.50 ^{cd}	14.75 ^{abc}
<i>Geotrichum</i> -like colony	0.00 ^d	8.25 ^{bcd}
<i>Aspergillus</i> -like colony	5.00 ^{bcd}	9.75 ^{bcd}
<i>Gliocladium</i> -like colony	6.50 ^{bc}	10.50 ^{abc}
<i>Colletotrichum</i> -like colony	4.50 ^{bcd}	18.25 ^{ab}
Mycelia Sterilia Isolate 1	0.00 ^d	6.50 ^{cd}
Mycelia Sterilia Isolate 2	15.75 ^a	21.00 ^a
Mycelia Sterilia Isolate 3	10.50 ^{bcd}	18.00 ^{ab}
Mycelia Sterilia Isolate 4	0.41 ^{ab}	18.75 ^{ab}

Numbers in same column followed by same letters are not significantly different (P=0.05, Duncan test).

Incidence of Vascular Streak Dieback on 120 DAI (figure 1) was consistently suppressed by a number of fungal viz *Curvularia*-like colony, Copper fungicide, *Gliocladium*-like colony, Mycelia Sterilia Isolate 1 with significantly different with control.

3.1.2. *Endophytism status of fungi isolated.* Evaluation of endophyte fungi presence on seedling are presented in table 2. the result shows that fungi were reisolated only on the leaf, whereas on stem and root were not found. All fungi except *Colletotrichum*-like colony was found only on 30 DAI while on 150 DAI was not found.

3.2. Discussion

Two months after exposes and inoculation of disease inoculum of cocoa seedling a number of endophytic fungi viz *Curvularia*-like colony, Mycelia Sterilia Isolate 1, *Geotrichum*-like colony, Copper fungicide, *Fusarium*-like colony, *Aspergillus*-like colony, *Colletotrichum*-like colony, Mycelia Sterilia Isolate 3 able to inhibit VSD to develop, but after three months number of fungi were reduced remained four fungi including *Curvularia*-like colony, Mycelia Sterilia Isolate 1, *Geotrichum*-like colony,

Aspergillus-like colony as effective fungi, However, after four months only three fungi were effective that was *Curvularia*-like colony, *Gliocladium*-like colony, Mycelia Sterilia Isolate 1.

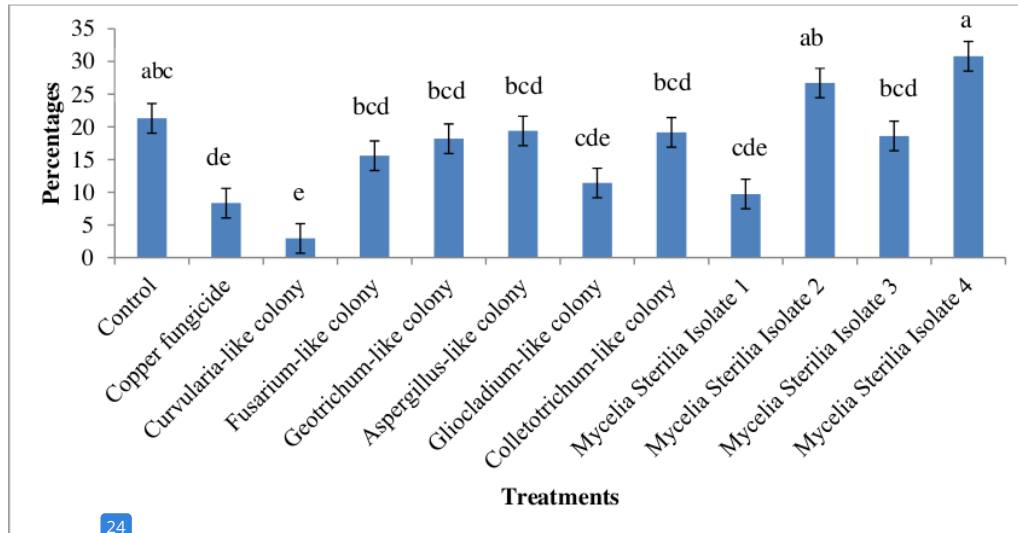


Figure 1. The incidence of Vascular Streak Dieback on 120 days after inoculation and exposed on cacao seedling. Numbers in the same color bar followed by same letters are not significantly different (P=0.05, Duncan test).

Table 2. Re-isolation of fungi from treated seedling on 30 DAI and 150 DAI.

Treatments	Leaf		Stem		Root	
	30 DAI	150 DAI	30 DAI	150 DAI	30 DAI	150 DAI
<i>Curvularia</i> -like colony	+	-	-	-	-	-
<i>Fusarium</i> -like colony	+	-	-	-	-	-
<i>Geotrichum</i> -like colony	+	-	-	-	-	-
<i>Aspergillus</i> -like colony	+	-	-	-	-	-
<i>Gliocladium</i> -like colony	+	-	-	-	-	-
<i>Colletotrichum</i> -like colony	-	-	-	-	-	-
Mycelia Sterilia Isolate 1	+	-	-	-	-	-
Mycelia Sterilia Isolate 2	+	-	-	-	-	-
Mycelia Sterilia Isolate 3	+	-	-	-	-	-
Mycelia Sterilia Isolate 4	+	-	-	-	-	-

* (+) : Found; (-) : Not Found;

These findings revealed that fungi associated with healthy tissue of cacao provide a potential role in suppressing VSD disease and could be used as biocontrol agents. The result supported by previously studies regarding endophytic fungi were used for helping tree from the infestation of pathogen in cacao [8, 12, 15, 16, 13, 14].

Curvularia-like colony and mycelia Sterilia Isolate 1 appeared consistently has low incidence of the disease. The fungi were able to protect seedling from VSD infestation. Obviously, the fungi should not be considered risk-free because some strains of the fungi found as pathogen at other crops [20, 21, 22]. Some of the fungi were used in this study found as pathogen at cacao viz *Fusarium* [23], *Colletotrichum* [24, 25]. Further, even though they did not induce disease symptom we should be careful when we decide to use them as biocontrol agents.

The fungi were able colonized of seedling leaves, then the pathogen could not penetrate the leaves tissue, this verified by re-isolation of the fungi. The endophytic fungi protect plants from pathogen through the mechanisms of competition, induced resistance, antagonisms, and mycoparasite [9, 12]. The result of re-isolation of the fungi indicates that the fungi only found in leaf at 30 days after inoculation of endophytic fungi, this result suggested that the fungi should be re-inoculation for longer protection from VSD disease. Application frequency of antagonist fungi has significant role reduce cacao disease incidence [26].

Colonized and protecting of cacao leaves, especially young leaves were one of an important step to prevent VSD pathogen landed and germinate on leaf surface due to VSD pathogen, *Ceratobasidium theobromae*, where it basidiospores germinate and hyphae penetrate unhardened leaves [3].

Copper fungicide showing as one of the best treatment, cuprous oxide applied on leaf thoroughly. the fungicide is contact fungi and provides longer protection against fungal infection [27]. Copper-based treatments are effective against a wide spectrum of fungal pathogen. Their action is fungistatic and prevents spore germination [28]. However, application of this compound should be accurate since copper is toxic to plants, it must be used at low levels or in the insoluble form [27] and cuprous oxide is classified as FSC highly hazardous due to its acute toxicity to aquatic organisms.

4. Conclusion

Endophytic fungi was able to prevent VSD penetrating young leaves and slow down the pathogen to develop. However, some of endophytic fungi isolated found as pathogen at other crops. Therefore, those of endophytic fungi should not be considered risk-free. In addition, for longer protection, the endophytic fungi should be apply repetitive.

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