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Research Article

Fungal Pathogens Associated with Vascular Streak Dieback (VSD) Disease on Cacao in Special Region of Yogyakarta Province

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ABSTRACT

Cacao is one of the pre-eminent crops plantation with high economic value. Indonesia's cacao beans production is the third largest in the world after Ivory Coast and Ghana. Vascular Streak Dieback (VSD) is one of the important diseases of cacao which caused a decreased yield either in quantity or quality. The disease is caused by the basidiomycete fungus *Ceratobasidium theobromae* (syn. *Oncobasidium theobromae*). Spores are carried by wind to spread, infect young leaves and penetrate through natural openings and colonize xylem vessel which could inhibit the transportation system in the plant tissue. There are several fungal pathogens associated with VSD diseases on cacao. This research aimed to study the fungi associate with VSD diseases on cacao in Special Region of Yogyakarta Province. Survey and sampling were conducted in cacao plantations in regencies of Gunungkidul, Kulon Progo, Bantul and Sleman. The severity of VSD disease in the regencies of Gunungkidul and Kulon Progo were high, while in the regencies of Sleman and Bantul were moderate. Eighty eight fungal isolates were isolated from infected petiole and stem. The in vitro pathogenicity test screened 32 fungal isolates causing necrotic and chlorotic symptoms on young healthy cacao leaves with and without wounding. The first symptoms appeared at 8-12 days after inoculation and fungal mycelium could grow at 1−3 days after inoculation. Those isolates collected showed a high diversity of colony morphology. *Lasiodiplodia* sp., *Fusarium* sp., *Colletotrichum* sp., and *Pestalotiopsis* sp. had been identified based on conidial morphology.

Keywords: associated, cacao, fungal, VSD, Yogyakarta

INTRODUCTION

Cacao (*Theobroma cacao* L.) is one of the most important estate crops in Indonesia. Based on The International Cocoa Organization (ICCO), Indonesia is the third largest cacao producer in the world after Ivory and Ghana which has contributed around 325,000 tonnes or 7.7% from the total world production (ICCO, 2017). Indonesia's cacao productivity is still low with production reached 700−800 kg/ha. According to Directorate General of Estate Crops Ministry of Agriculture, the national cacao production of Indonesia in 2015 reached 593,331 tonnes with cacao growing areas amounted to 1,709,284 ha (Ditjenbun, 2016).

Java Island is an area that has the potential to increase the productivity of cacao in Indonesia. Special Region of Yogyakarta Province is one of the center of cacao production in Java which has contributed to the national cacao production of Indonesia about 1,121 tonnes with the area plantations of 5,516 ha in the year 2015 (BPS, 2017). Some majors areas of cacao plantations in Yogyakarta province were Kulon Progo (3,597.59 ha), Gunungkidul (1,403 ha), and Sleman (101.4 ha) (BPS Prov. DIY, 2016).

Pests and diseases are the major constraints to cacao productivity in Indonesia. One of the major diseases on cacao was Vascular Streak Dieback (VSD) caused by basidiomycetes fungus *Ceratobasidium theobromae* (syn. *Oncobasidium theobromae*, *Thanatephorus theobromae*) (Samuels *et al*. 2012; McMahon & Purwantara, 2016). *C. theobromae* is a near-obligate pathogen. The fungus could not be isolated from infected stems, leaves, or petioles and growth of the fungus on agar media were hampered. The fungus cannot be sub-cultured from the initial hyphae that emerged from infected stems onto agar media (McMahon & Purwantara, 2016).

VSD disease was firstly discovered in Papua New Guinea and recognized in the 1960s. The disease later spread to other Asian countries and now occurs in South India, Hainan Island-China, Burma, Thailand, Malaysia, the Philippines and a number of islands in Oceania (Guest & Keane 2007). VSD was newly encountered disease which cause serious problem on c in Asia and Melanesia (Ploetz, 2016). In Indonesia, VSD has spread to several areas such as Papua, Sulawesi, Kalimantan, East Java, Bali, West Sumatra (Rosmana, 2005; Samuel *et al*., 2012; Trisno *et al*., 2016). However, information about bioecology and morphology of pathogenic causal agent of VSD is still limited.

In some cacao genotypes and areas, the causal agents of this disease were recognized as different fungi. Some species of *Fusarium* were identified as agents of dieback on cacao i.e. *F. chlamydosporum, F. solani, F. oxysporum* and *F. proliferatum* (Adu-Acheampong & Archer, 2011). *F. decemcellulare* and *Lasiodiplodia theobromae* caused dieback disease on cacao genotypes in Ghana (Adu-Acheampong *et al*., 2012). Dieback disease caused by *L. theobromae* was a new constraint to cacao production in Cameroon (Mbenoun *et al*., 2008). It caused a damaging dieback of cacao in India (Kannan *et al.*, 2010). This fungus was also reported become causal agent of vascular streak dieback (VSD)–like symptoms of cacao in Davao Region, Philippines (Alvindia & Gallema, 2017). Samuels *et al.* (2012) has identified the presence of *C. ramicola* on VSD-infected cacao leaves in Java using molecular methods.

VSD is a difficult disease to control because the fungal infection is systemic, and it is often not possible to remove the entire inoculum source from the plantation. An integrated disease management strategy is probably the best approach for managing VSD disease control. The VSD disease management has been categorized which including such as cultural practices (Prawoto, 2013; Anita-Sari *et al.,* 2017), clones resistant (Susilo, 2012; Prawoto, 2013), biological control (Vanhove *et al.,* 2016; Wahab *et al.,* 2016; Susiyanto *et al.,* 2017), chemical control (Susilo, 2012; Harni & Baharuddin, 2014; Nur 'Aini, 2014), and quarantine (Prior, 1985). The objectives of research were to detect and to identify fungi associated with VSD symptomatic cacao in Special Region of Yogyakarta Province.

MATERIALS AND METHODS

Survey and Sample Collection

Survey and sampling on symptomatic parts of cacao plants were conducted in several areas of cacao plantations in Special Region of Yogyakarta Province such as Kulon Progo, Gunungkidul, Sleman and Bantul regencies. The survey was carried out directly by observed the cacao trees which showed symptoms of VSD and sampling based on purposive sampling method. VSD-infected plant parts such as leaves, stems and branches were cut used cutters which a certain size with approximately 10-15 cm long then were put into the envelopes to be brought to the laboratory.

Isolation and Culturing of Fungi

Isolation was conducted in the Laboratory of Plant Disease, Faculty of Agriculture, University of Gadjah Mada Yogyakarta. Cacao petioles and stems with VSD-symptoms were cut into small pieces with approximately 1−2 cm^2 in size. The bark was removed aseptically and the segments of stem end were cut into small pieces to expose the vascular tissue. Those pieces were surface-sterilized, soaked into 70% ethanol for 3 min, then put into a solution of 5.25 % NaOCl for 5 min. The petioles and stems were retransferred into a 70% ethanol for 2 min, then rinsed for 3 times with sterile distilled water (SDW) and dried on tissue paper. The pieces of petiole and stem were placed in Petri plates containing potato dextrose agar (PDA) medium, sealed with plastic wrap and incubated for 3−7 days at room temperature. Pure culture of fungal isolate was obtained by transferring the active growing mycelium to PDA media.

Pathogenicity Test

Pathogenicity test was conducted to find out the ability of the VSD associated fungal isolates in infecting and causing symptoms. Fungal isolates were tested on young Lindak cacao leaves obtained from the collection of the Center for Agrotechnology Innovation (Pusat Inovasi Agroteknologi-PIAT) UGM-Yogyakarta. The leaves were washed under tap water, surface-sterilized in 5.25% sodium hypochlorite for 1 min, rinsed in running water for 5 min, air-dried for 30 min and transferred to tray covered with wet tissue paper to keep the moisture. The fungal isolates were grown on PDA at room

temperature for 4 days prior to inoculation. Each young leaf was inoculated with a 3-mm block agar of mycelium and was placed on the middle of the undersurface of leaves. The leaves for inoculation were treated with and without wounding, uninoculated leaves were considered as the control.

Colony Morphology

Fungal isolates causing VSD symptom on leaves were observed for their colony morphology. Mycelia of each fungal isolates was transferred using a sterile cork borer and placed at the centre of a PDA plate, three replicates were made for each isolate. The plates were incubated under dark condition at room temperatures and observed every day. The macroscopic characters (surface color, pattern, edge, mycelium, and texture) and mycelia growth were recorded at 24-h interval until one week. The observations of fungal microscopic morphology such as spore or conidia were conducted using binocular microscopes.

RESULTS AND DISCUSSION

Field Survey and Sampling

Survey and sampling were conducted at 16 cacao plantations in Special Region of Yogyakarta province (Table 1). The result showed that VSD disease on cacao was detected in all cacao plantations in surveyed region with various disease incidences. Disease incidences in Gunungkidul and Kulon Progo regencies were high, while in Sleman and Bantul regencies were moderate.

In the field, symptoms of VSD disease were seen from the infected leaves and stems (Figure 1). The characteristic VSD symptoms have described by Guest & Keane (2007). The initial symptom of VSD was the chlorosis on a single leaf, usually on the second or third flush behind the shoot apex but some parts remained green (known as "green spot island"). In some areas and on some cacao genotypes, necrosis also appeared on tip of symptomatic leaves. Lenticels on infected stem became enlarged and there were three blackening spots on the branch after leaves were fallen. The browning cambium could be seen if the bark of infected stem was peeled.

Pathogenicity Test

Eighty-eight isolates were obtained from cacao stems and petioles with VSD infected from all regencies in Special Region of Yogyakarta (Table 2). All isolates had specific morphological characteristics corresponding to several genus fungi and only 32 isolates were selected for further morphological identification. They were screened according to their ability in causing symptoms on young healthy cacao leaves with and without wounding treatments. The fungi has a mechanism to penetrate, colonize

Table 1. Location of sampling on cacao growing areas with Vascular Streak Dieback (VSD) symptoms in Yogyakarta

Regency	District	Village	Name of location[a]
Gunungkidul	Patuk	Putat	1 Dk. Putat
			2 Dk. Gumawang
			3 Dk. Plumbungan
		Bunder	4 Dk. Gambiran
			5 Dk. Widoro Wetan
		Ngelegi	6 Dk. Trukan
Sleman	Cangkringan	Wukirsari	7 Dk. Salam
			8 Dk. Gondang
			9 Dk. Duwet
	Ngemplak	Bimo Martani	10 Dk. Rindu sari
	Pakem	Hargobinangun	11 Dk. Tanen
Bantul	Dlingo	Terong	12 Dk. Pencit rejo
			13 Dk. Sendang sari
Kulon Progo	Kalibawang	Banjararum	14 Dk. Kanoman
			15 Dk. Semaken
	Samigaluh	Purwoharjo	16 Purwoharjo

Remark: [a]Dk is abbreviation of dukuh (sub-village)

Figure 1. Leaf and stem symptoms of VSD disease on cacao in field; (A) branch defoliation, (B) green spot island, (C) necrotic symptom, (D, E) the browning cambium of infected stem, (F) blackening spot on infected branch

Table 2. Results of in-vitro pathogenicity test of collected isolates after 16 days after inoculation

Group	Respond to treatment		Mycelium growth (day)	First symptom (day)	Number of isolates
	Unwound	Wound			
1	+	+	1−3	4−15	12
2	+	-	1−3	9−14	20
3	-	+	1−3	4−13	14
4	-	-	1−4	-	42
				Total	88

Remark: +: symptom, - : no symptom

and invade the infected plant tissue. In general, phytopathogenic fungi form specialized penetration organs which called appressoria (Knogge, 1996). Those 32 fungal isolates had potential role as the pathogen. The leaf necrotic symptoms were started from the point of inoculation which were then spread into the by tissue on the midrib. Some fungal isolates also caused chlorotic symptom on leaf. The symptom appeared at 4 to 15 days and mycelium of all isolates could show optimum growth on 1 to 4 days after inoculation.

Colony and Conidial Morphology

All isolates showed variation in diversity of colony morphology on PDA after one-week incubation under room temperature (Table 3). Most isolates had circular shape with the round on the edge and mycelia were initially like cottony. There were 8 isolates had irregular edge on their colony.

The Pa2b11, Bu1b2, Kb1d1, Kb1d5, Kb2b1, Kb2b2, Kb2b5, Kb2b6, Kb2d2, Cgn2d3, Pkd31, Ngd3, Dl1b1, Dl1b2 isolates had the similar character of the colony morphology. Their aerial mycelium was abundant. The color on top was grey or grey-dark while on the bottom was black or green-black. Colony shape was circular and the edge was round. Bu2b1f, Kb1b4, Cgn2b1, Cgn2b2, Cgn2b21, Ngds1 isolates had typical form of the surface with smooth and flat. However, the colony color of isolates was different such white, creamy, and pinkish.

Colony features such as the color of surface, shape, edge and mycelia form could be used for morphological identification. The colony features has been used by Amin *et al.* (2014) for observing the macroscopic characterization of endophytic fungi from cacao resistant and susceptible of VSD in South Sulawesi. Some isolates have similar colony

Table 3. Colony morphology of some selected isolates

No.	Isolate	Colony colour		Surface	Pattern	Edge	Mycelia
		Top	Bottom				
1.	Pa1b1	purplish	purplish	rough	circular	round	cottony
2.	Pa1d1	white-grey	white-green	rough	circular as ring	irregular	cottony
3.	Pa1d2	yellowish	brown	rough	circular as ring	irregular	cottony
4.	Pa2d2	brown	light brown	smooth, thick	branched	irregular	cottony
5.	Pa2b1	white-black	white-green	round, thick	circular	round	cottony
6.	Pa2b11	grey dark	green-blackish	aerial, spread over	circular	round	cottony
7.	Bu1b2	grey dark	black	aerial, spread over	circular	round	cottony
8.	Bu2b1f	creamy	creamy	smooth, flat	circular	round	cottony
9.	Kb1b4	white	creamy	smooth, flat	circular	round	cottony
10.	Kb1d1	grey	green-blackish	aerial, spread over	circular	round	cottony
11.	Kb1d2a	grey dark	black	aerial, spread over	circular	irregular	cottony
12.	Kb1d3a	white	white-green	bumpy	branched	irregular	cottony
13.	Kb1d5	grey	green-blackish	aerial, spread over	circular	round	cottony
14.	Kb2b1	grey	green-blackish	aerial, spread over	circular	round	cottony
15.	Kb2b2	grey	green-blackish	aerial, spread over	circular	round	cottony
16.	Kb2b5	grey	green-blackish	aerial, spread over	circular	round	cottony
17.	Kb2b6	grey	green	aerial, spread over	circular	round	cottony
18.	Kb2d2	grey	green-blackish	aerial, spread over	circular	round	cottony
19.	Cgn2b1	pinkish	pinkish	smooth, flat	circular	round	cottony
20.	Cgn2b2	pinkish	pinkish	smooth, flat	circular	round	cottony
21.	Cgn2b21	white	yellowish	smooth, flat	circular	round	cottony
22.	Cgn2d3	grey	green-blackish	aerial, spread over	circular	round	cottony
23.	Cgn3d1	white	white-brown	bumpy	circular	irregular	cottony
24.	Cgn3d2	grey-brown	brownish	smooth, thick	circular	irregular	cottony
25.	Pkd3	white-green	white-green	rough	circular as ring	round	cottony
26.	Pkd31	grey dark	green-blackish	aerial, spread over	circular	round	cottony
27.	Ngd3	grey dark	black	aerial, spread over	circular	irregular	cottony
28.	Ngds1	white	yellowish	smooth, flat	circular	round	cottony
29.	Ngdf	white	creamy	rough, convex	circular	round	cottony
30.	Dl1b1	grey dark	green-blackish	aerial, spread over	circular	round	cottony
31.	Dl1b2	grey dark	green-blackish	aerial, spread over	circular	round	cottony
32.	Dl1d2	white	creamy	rough, convex	circular	round	cottony

morphology character which indicates that those were possibly the same isolates.

There were 24 isolates which produced conidia and some of them could produced pycnidia (Table 4). All isolates which had conidia could be identified. Meanwhile, there were 8 isolates could not produce conidia and some of them such Pa1d1, Pa1d2 and Kb1d3a isolates were produced sclerotia on PDA. Microscopically, some isolates could be observed from conidia form either macroconidia or microconidia. It was also observed immature and mature conidia on certain isolates.

Microscopic observations demonstrated that morphological characters of conidia were different to each other, identified as genus *Fusarium, Colletotrichum, Pestalotiopsis, Lasiodiplodia*. Conidia were oval to cylindrical, hyaline, typically thin walled, sickle-shaped and septate (Figure 2.A). We assumed that the isolates belong to the genus *Fusarium*. Conidia of *Fusarium* sp. had two type i.e. macro and micro conidia. Microconidia were hyaline, 1-2 cells, ovoid shape or form and arch. Meanwhile, macroconidia hyphae were hyaline as well, forms up to 2 cells, appear curve or canoe and

the edge of peak appears hooked (Amin *et al.* 2014). The morphological characters of the most ubiquitous of *Fusarium* spp. were micro and macro-conidium as well as monophyalid or polyphyalid (Sutejo *et al*., 2008).

Conidial characters of *Colletotrichum* sp. were showed in Figure 2.B. Conidia were hyaline, guttulate, one-celled and cylindrical (Mo *et al.* 2018). Microscopically, conidia of *Colletotrichum s*p., was appeared oblong and consist of single cell sterigmata. Conidiophore was short and non septa (Amin *et al.* 2014). Figure 2.C showed the conidia of *Pestalotiopsis* sp. which only has been produced by Pa2b1 isolate. Genus *Pestalotiopsis* is one of genera *Pestalotia* based on the conidial forms with 5-celled conidia (Maharachchikumbura *et al*., 2014). Conidia character of *Pestalotiopsis* sp. has been described by Liu *et al.* (2007) that was fusiform, erect or slight curving, slight constricted at septa, intermediate colored cells subcylindrical, thick-walled, smooth, concolorous, 13−15 µm long; second cell from the base pale brown, third cell olivaceous and fourth cell pale brown to olivaceous. Exterior hyaline cells was small, trigonal, bearing 1 setula, rarely 2 or 3 setulae, short with 1−10 µm long and basal appendage was absent. Conidiogenous cells were integrated, lageniform to ampulliform or subcylindrical, colorless, smooth-walled.

Table 4. Microscopic features of isolates selected

No.	Isolate	Conidia	Fruiting body	Sclerotia	Genus
1	Pa1b1	√	-	-	*Fusarium* sp.
2	Pa1d1	-	-	√	Unidentified
3	Pa1d2	-	-	√	Unidentified
4	Pa2d2	-	-	-	Unidentified
5	Pa2b1	√	-	-	*Pestalotiopsis* sp.
6	Pa2b11	√	√	-	*Lasiodiplodia* sp.
7	Bu1b2	-	-	-	Unidentified
8	Bu2b1f	√	-	-	*Fusarium* sp.
9	Kb1b4	√	-	-	*Fusarium* sp.
10	Kb1d1	√	√	-	*Lasiodiplodia* sp.
11	Kb1d2a	-	-	-	Unidentified
12	Kb1d3a	-	-	√	Unidentified
13	Kb1d5	√	√	-	*Lasiodiplodia* sp.
14	Kb2b1	√	√	-	*Lasiodiplodia* sp.
15	Kb2b2	√	√	-	*Lasiodiplodia* sp.
16	Kb2b5	√	√	-	*Lasiodiplodia* sp.
17	Kb2b6	√	√	-	*Lasiodiplodia* sp.
18	Kb2d2	√	√	-	*Lasiodiplodia* sp.
19	Cgn2b1	√	-	-	*Fusarium* sp.
20	Cgn2b2	√	-	-	*Fusarium* sp.
21	Cgn2b21	√	-	-	*Fusarium* sp.
22	Cgn2d3	√	√	-	*Lasiodiplodia* sp.
23	Cgn3d1	√	√	-	*Colletotrichum* sp.
24	Cgn3d2	-	-	-	Unidentified
25	Pkd3	√	-	-	*Colletotrichum* sp.
26	Pkd31	√	√	-	*Lasiodiplodia* sp.
27	Ngd3	-	-	-	Unidentified
28	Ngds1	√	-	-	*Fusarium* sp.
29	Ngdf	√	-	-	*Colletotrichum* sp.
30	Dl1b1	√	√	-	*Lasiodiplodia* sp.
31	Dl1b2	√	√	-	*Lasiodiplodia* sp.
32	Dl1d2	√	-	-	*Colletotrichum* sp.

Remark: √ = formed, - = not formed

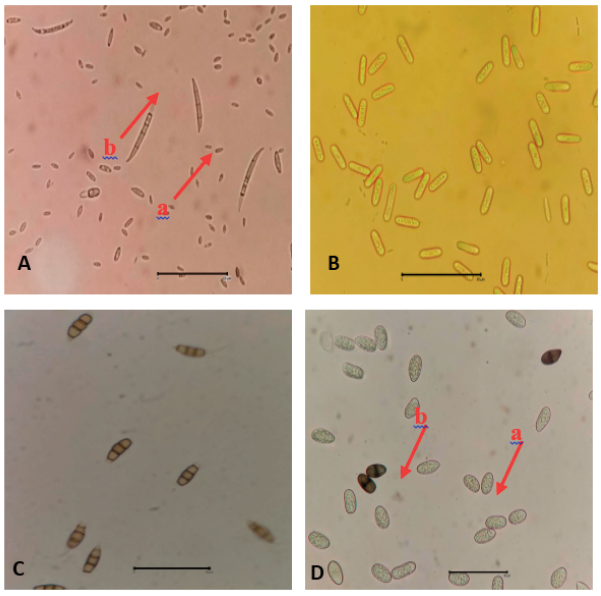

Figure 2. Conidia characterization of isolates selected; (A) *Fusarium* sp. (a. macroconidia, b. microconidia), (B) *Colletotrichum* sp., (C) *Pestalotiopsis* sp., (D) *Laiodiplodia* sp. (a. immature, b. mature)

Another form of conidia was identified as of genus *Lasiodiplodia* (Figure 2.D). Genus *Lasiodiplodia* had two types conidia i.e. micro-conidia (immature) and macro-conidia (mature). Immature conidia were initially unicellular, ellipsoidal, hyaline, thick-walled with granular content, and 25–27 × 11–13 µm. Mature conidia were one-septate, dark brown and with longitudinal striations. Some isolates were referred to as Lasiodiplodia could grow rapidly on PDA medium with a white to light or dark grey aerial mycelia and later produces abundant black pigmentation which can be clearly viewed from the reserve side of the PDA culture (Zhang, 2014; Alvindia & Gallema, 2017).

In this study, we still could not able to identify the species on the fungal isolates because isolates which had the same morphological characters could be from different species, although they were in the same genera. On the other hand, isolates which had different characters morphologies could be from the same species. Therefore, further identification the fungal isolates, such as by molecular techniques is necessary.

CONCLUSION

The fungi which associate with VSD on cacao have a variation in colony morphology. Thirty-two fungal isolates produced necrotic symptoms indicating that they were pathogenic. Some isolates produced conidia, pycnidia and sclerotia. However, some isolates did not produce those features on PDA so they could not be identified. Based on conidial morphology, the genera of fungi associated with VSD disease were identified as *Lasiodiplodia*, *Fusarium*, *Colletotrichum*, and *Pestalotiopsis*.

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