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Severity and changed symptoms of vascular streak dieback caused by *Ceratobasidium theobromae* in several cocoa (*Theobroma cacao*) clones in Sulawesi, Indonesia

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Abstract

Vascular streak dieback (VSD), caused by the basidiomycete *Ceratobasidium theobromae* (syn. *Oncobasidium theobromae*), is a serious disease of cocoa in Southeast Asia and Melanesia. In the last decade, in many cocoa growing areas in Malaysia and Indonesia, the impact of this disease has increased considerably. In the same period, symptoms of the disease have become dominated by necrotic lesions on infected leaves, in contrast to chlorotic symptoms, characterised by yellowing of the leaf with a typical pattern of remnant green islands, which were formerly ubiquitous. Amplification of basidiomycete ITS and LSU sequences in DNA extracts of VSD-infected plant tissues collected from Sulawesi, Indonesia, and Kerala, India, showing chlorotic symptoms matched previous GenBank accessions (99%). However, neither isolation of an axenic culture nor direct PCR amplification from necrotic leaf tissues was successful. Nevertheless, basidiocarps collected from branches with either necrotic or chlorotic VSD symptom in Sulawesi produced basidiospores and basidia with similar dimensions to those previously documented for *C. theobromae* suggesting that the same pathogen is associated with both symptoms. Incidence of necrotic symptoms was correlated with disease severity in branches. VSD monitored in a range of resistant and susceptible cocoa clones showed rankings for disease incidence and severity that were consistent for three seasons, confirming that disease resistance was maintained despite the prevalence of necrotic leaf symptoms. A lower proportion of symptomatic leaves on branches 5 months after early symptoms were observed in the resistant Malaysian clone PBC123 (32.6%) compared to the more susceptible M04 (46.9%). Clones common to trials in three different districts in Sulawesi showed a consistently high level of resistance to VSD, including PBC123 and two local selections, M05 and GeniJ.

Keywords *Theobroma cacao* · *Ceratobasidium theobromae* · Disease severity · Local genotypes · Resistance · Symptoms

Introduction

Vascular streak dieback (VSD), caused by the unique, wind-borne, xylem-invading basidiomycete, *Ceratobasidium theobromae* (Tulasnellales) (syn. *Thanatephorus theobromae*, *Oncobasidium theobromae*) (Keane and Prior 1991; Talbot and Keane 1971; Samuels et al. 2012; Keane et al. 1972; Roberts and Royal Botanic Gardens 1999), is a serious disease of cocoa in Southeast Asia and the westernmost islands of Melanesia. The fungus has not been reported outside this region and must have evolved on a host or hosts, so far unidentified, indigenous to the region and transferred to introduced cocoa. It has very limited capacity for dispersal and appears to have transferred repeatedly to cocoa introduced to widely separated locations within the region, ranging from New Britain island in Papua New Guinea in the east to

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Kerala State, India, and Hainan Island, China, in the west. Until 2004, the distinctive symptoms of VSD throughout the region were the same as those originally observed in Papua New Guinea in the 1960s (Bridgland et al. 1966a; 1966b; 1967; Keane et al. 1972). Starting on the second or third flush behind the shoot or branch apex, a single leaf becomes chlorotic with many remnant green spots (Keane and Prior 1991). Over time, adjacent leaves become chlorotic with the same pattern. When fully chlorotic, leaves are promptly shed from the branch with a clean break at the abscission zone, and if this occurs during wet weather, yellowish-white fungal hyphae emerge from the three exposed vascular traces in the leaf scar and within a day or two form white corticioid basidiocarps of *C. theobromae* on the scar and surrounding bark (Prior 1979; Keane 1981; Keane and Prior 1991; Keane et al. 1972). Basidiospores are formed and released at night after late afternoon rainfall and there is evidence that they can induce the disease when shed onto young flush leaves of cocoa seedlings (Keane and Prior 1991). Hyphae of the fungus are confined to xylem vessels within leaf veins and stems. As leaves are progressively infected and shed, the shoot or branch apex is eventually killed, giving rise to the ultimate dieback symptoms associated with the disease. In susceptible genotypes of cocoa, the whole tree can be killed. The presence of the disease in branches from which the chlorotic leaves had been shed can be confirmed by scraping the surface off leaf scars and observing three distinct brown-black discoloured vascular traces in the exposed leaf scars.

Seedlings at the pre-jorquette stage of development infected in the main stem are usually killed and so an important control measure has been to raise young plants in covered nurseries rather than in the open beneath or near infected cocoa. Strong partial resistance to VSD was selected in Papua New Guinea and Malaysia, and later in Indonesia, as cocoa growers were forced to propagate from survivors of devastating epidemics. The extinction of some very susceptible clones and the survival of resistant ones observed in clonal trials during the early epidemics allowed identification of useful planting material that became the mainstay of the cocoa plantings in the region (Prior 1979; Keane and Prior 1991; Turner and Keane 1982). The disease caused serious branch dieback and death of trees in Malaysia and Papua New Guinea during the early expansion of cocoa planting in the late 1950s through to the 1970s but since then has become less serious in cocoa genotypes selected for partial resistance to the disease. The resistance is additively inherited and has proved durable for over 50 years (Tan and Tan 1988; Chong and Shepherd 1986; McMahon et al. 2018). A range of local cocoa selections (cloned by top-grafting) were evaluated in four on-farm trials in Sulawesi for VSD incidence and severity, resulting in the identification of resistance in locally selected cocoa genotypes (McMahon et al. 2018).

Studies of pathogen variability and pathogenicity have been limited because the fungus cannot be maintained in culture, sporulates only sporadically on infected branches during prolonged wet weather and produces relatively few spores with a very limited life span (Mainstone et al. 1977; Prior 1977, 1982; Lam et al. 1988; Keane et al. 1972). Recent molecular studies have made possible further studies of pathogen variability and distribution. Evaluation of the regional variation of the fungus based on its ITS and 28S LSU sequences from isolates collected from different parts of the endemic region indicates high similarity between locations, although three regional haplotypes have been identified (Samuels et al. 2012). The *C. theobromae* genome has now been sequenced (Ali et al. 2019) and is available in the public domain.

The disease has been common on the island of Sulawesi, now the most important cocoa growing region in South-east Asia, but since about 2004, it has been observed to be more serious in association with a significant change in some symptoms on diseased leaves (Fig. 1a-c). This change involves a more necrotic reaction and reduced occurrence of the distinctive chlorosis of whole leaves with remnant green spots as originally described (McMahon and Purwantara 2016; Bryceson 2014; Guest and Keane 2007). Diseased leaves remain attached to stems for a prolonged period, allowing sporulation on cracks in the petiole and main vein, never seen previously when chlorotic leaves abscised cleanly at the abscission zone as soon as they became fully chlorotic, allowing sporulation only on and around the leaf scars on stems. A distinct border separates healthy green tissue and necrotic lesions. The prolonged attachment of necrotic leaves gives the trees a more diseased appearance than seen previously when chlorotic leaves abscised abruptly and were commonly found in the leaf litter. Similar changes have been observed in Java, Bali and Papua in Indonesia, and in Malaysia. The present study aimed to establish whether (1) the causal pathogen of VSD, *Ceratobasidium theobromae*, is present in infected leaves displaying necrotic, as well as the original chlorotic, symptoms, (2) symptom type is associated with disease severity and/or host genotype and (3) the durable resistance evident with chlorotic symptoms is still evident in local cocoa genotypes developing necrotic symptoms in the field in Sulawesi.

Materials and methods

Evaluation of VSD in cocoa clone trials

Incidence, severity and symptom type (predominantly necrotic or chlorotic with green spots) of VSD were evaluated on cocoa trees in 2011 and 2012 in two on-farm trials in separate districts in Sulawesi. The trials were established in early

Fig. 1 Chlorotic and necrotic symptoms in vascular streak dieback (VSD)-infected leaves on cocoa farms in Sulawesi. **a** Advanced chlorotic symptoms of VSD with spots of green tissue; **b** both chlorotic and necrotic leaf symptoms of VSD with infected leaves remaining attached to the branch; **c** necrosis at tip of VSD-infected leaf (on upper branch on the left) with a distinct border between necrotic and green tissue



2008 primarily to compare the overall performance of some well-known Malaysian cocoa clones with promising genotypes identified locally by farmers in Sulawesi or obtained from the progeny of hybrid crosses between promising parent clones. Trees were exposed to natural infection in the field.

The trials were established by planting clones, produced by top-grafting seedlings with budwood from the selected clones (McMahon et al. 2015), into the field when approx. 4 months old. The seedlings of each clone were planted in 8-tree plots in a randomised block design with four blocks. Four clones were common to the two trials: PBC123, selected in Malaysia for its resistance to VSD (Chong and Shepherd 1986) and now planted widely in Sulawesi as ‘Sul1’, and three local clones (M01, M05 and GeniJ) selected by Mars Inc. with the cooperation of farmers in South Sulawesi. The trials were established on private farms in Fakkie Village (Tiroang Sub-district, Pinrang District, South Sulawesi) and in Beluak Village (Anreapi Sub-district, Polewali-Mandar, Polman, District, West Sulawesi). The trial sites were managed using normal farm practices, without pesticides. Heavy pruning was conducted in the early wet season and lighter pruning towards the end of the wet season but was avoided in the dry season and on branches evaluated for progress of VSD. While some trees grew poorly or were lost to root rot, for each clone, 6–8 trees per block could be assessed in 2011 and 2012 for disease incidence, severity and type of symptom.

In 2014, eight clones were evaluated for VSD incidence, severity and type of symptom in three districts: the two trials described above (in Polman and Pinrang Districts) and a clone collection in Cendana, East Luwu District. Each of the eight clones was common to at least two sites.

Scoring incidence and severity of VSD

To evaluate VSD incidence and symptoms in the early dry season 2011, the total number of actively growing branchlets (or twigs) with at least 4 expanded leaves was counted for each tree in the Pinrang and Polman trials. The number (and percent) infected by VSD was then determined based on symptoms of slight yellowing or paling, or more advanced chlorosis or necrosis of leaves, and/or evidence of leaf abscission scars that showed discoloured vascular traces when the surface was scraped off. Branchlets with a mixture of symptoms were categorised as having chlorotic symptoms. In 2012 (during the wet season), the total number of branches at least 1 m in length on a tree was counted and assessed in the same way as for branchlets in 2011, calculating the percentage of all infected branches as a total VSD incidence measure. For each infected branch, the symptoms were recorded as necrotic, chlorotic or mixed (both types of symptoms apparent on an infected branch) and the incidence and severity of infection associated with each symptom type were calculated.

Severity of VSD infections in 2011 and 2012 was scored for each infected branch or branchlet as follows: (1) slight infection (1 or 2 leaves with VSD symptoms or fallen with discoloured vascular traces evident); (2) moderate infection (3–5 leaves with VSD symptoms or fallen); (3) severe infection (more than 5 leaves with VSD symptoms or fallen, or most leaves on the branch with symptoms or fallen, or branch with tip or whole branch dead or near-dead). For each tree, the average severity of infections in branches or branchlets was calculated as $(N_1 \times 1) + (N_2 \times 2) + (N_3 \times 3) / \text{total no. infected branches } (N_1 + N_2 + N_3)$, where N_n is the number of branches (or branchlets) with a disease severity score of n . Only infected branches/branchlets were included in the calculation, giving an average severity for infected branches/branchlets only and not for the whole tree. Averages for each plot were then calculated and subjected to statistical tests.

In March and July 2014, the proportion of infections resulting in necrotic, chlorotic or early symptoms (indicated by pale green or slight yellowing of leaves) was determined in individual branches for each clone at three sites. VSD severity was evaluated in the same plots in the Polman and Pinrang clone trials and additionally in a clone collection at Cendana (East Luwu). The number of trees (including all three sites) evaluated per clone ranged from 31 to 64. To score the overall trees for VSD severity, 10 branches were randomly selected at each sampling date in each tree and VSD severity in each branch was scored as follows: 0, healthy (no VSD observed); 1, early signs of VSD; 2, clear signs of VSD (either leaf chlorosis or necrosis) below the branch tip; 3, VSD symptoms in the branch tip (severe infection). The severity scores for the 10 sample branches were summed to give total severity scores for each tree and the mean severity score was calculated for each clone for the March and July assessments.

Development of VSD symptoms in branches

The progressive development of VSD symptoms was observed in the field trial at Pinrang on branches of clones showing susceptibility (MO4) or resistance (PBC123) to VSD over a 5-month period (March–July 2012) during the late wet season. The number of trees in each plot and the number of blocks were as described above. In each block, 10 healthy branches of each clone indicating early signs of VSD infection were labelled and monitored. Attached or fallen infected leaves and their positions on the branches were recorded each month. The numbers of newly formed (new flush or recently expanded) leaves were also counted. The degree of infection was calculated as the proportion of total leaves on

each branch either showing VSD symptoms or having leaf scars of fallen leaves showing evidence of infection (three black vascular traces). The total number of leaves on a branch was determined as the total number of leaf positions (including attached leaves and leaf scars of fallen leaves). Averages for infected branches (reduced to 8 branches in some plots due to loss of branches) in each plot and replicate block were used to determine means and standard errors.

Statistical analyses

Means, standard deviations and standard errors were obtained from the four replicate blocks in the two trials or, in 2014, from replicated measurements for individual trees in 2–3 sites. Data were subjected to ANOVA and means separated by Tukey's test or the Games-Howell test in cases when the Levene's test indicated non-homogeneity of variances. For the 2014 severity evaluations, the Kruskal–Wallis test was applied when Levene's test indicated non-homogeneity of variances or in the case of homogeneity, ANOVA was used and homogenous subsets of clones with similar resistance ratings were separated. Bivariate relation between variables was determined using Pearson's correlation test. IBM SPSS Ver 19 was used for statistical analyses.

Rainfall data

Data on the total rainfall per month from January 2011 to October 2012 in Polewali-Mandar were provided by the District Department of Agriculture. Rainfall data for 2011 and 2012 at Tiroang, Pinrang and for 2014 (January–August) at Pinrang and North Luwu (nearby the clone collection at Cendana) were provided by Mars Indonesia (Mars Inc.).

Observation of basidiocarps and spores

White, corticioid basidiocarps on leaf scars on branches (occasionally) or on leaf laminae associated with cracking of veins (most commonly) were collected periodically from the two trial sites and at Tarengge, East Luwu, South Sulawesi, especially after periods of wet weather when they had a thick, velvet-like appearance indicating that they may have been fertile (Keane et al. 1972). The leaf or branch section with a basidiocarp was placed in a plastic bag and kept moist until examined in the early morning on the day after collection from the field. Small pieces of basidiocarp removed with fine forceps were initially mounted in water on a microscope slide and covered with a coverslip for examination under a high-power microscope (Olympus CH or Nikon E100). If interesting structures were found, the specimen was stained with lacto-cotton-blue stain. Structures were

measured with an eyepiece graticule scale and photos were taken with an eyepiece camera.

Fungal isolation

Putative *C. theobromae* in tissues of infected cocoa with VSD symptoms were isolated onto artificial media (Varghese et al. 1981; Keane et al. 1972) as follows. Branches or branchlets showing symptoms of VSD-infection in the leaves (necrotic or chlorotic) were collected in Pinrang, Polman, North Luwu and other districts in Sulawesi, placed into slightly moistened plastic bags and within 24 h used for isolation of *C. theobromae*. Healthy branches were collected and subjected to the same isolation method as for infected branches. Infection was confirmed by the presence of vascular streaking in the petiole and/or stem. To isolate fungi, infected petioles and leaf lamina including the mid-rib (approximately 2 cm × 1 cm) were surface sterilised in the following steps: 70% ethanol (2 min), 3–10% sodium hypochlorite (3 min), 70% ethanol (1 min) and then rinsed in sterile distilled water and blotted dry on sterile filter paper. The pieces were trimmed with a flame-sterilised scalpel or scissors to expose fresh xylem tissue then placed onto 1.7–2% water agar in 5- or 10-cm-diameter Petri dishes. At room temperature, mycelium generally grew from the cut petiole or mid-rib ends within a day or two. Three to four days after plating on water agar, the hyphae at the edge of the colony were removed under sterile conditions to 10-cm-diameter Petri dishes containing modified liquid Corticium culture medium (CCM) (Kotila 1929) with the following composition: 1.25 g KH_2PO_4 , 2.36 g CaNO_3 , 0.59 g MgSO_4 , 0.25 g maltose, 6.25 g malt extract made up to 1 L. The liquid sub-cultures were maintained in a cabinet in an air-conditioned room (at approx. 26 °C) for up to 3 weeks.

Infected petioles and leaves were also collected for direct extraction as discussed below. Petioles or leaves were surface-sterilised as described above and stored in 2% cetyltrimethylammonium bromide (CTAB) in small plastic screw-top tubes. Samples were collected in this way from Polman, Pinrang and North Luwu in Sulawesi. Samples were also collected in Kerala, southern India, where VSD has been a significant disease of cocoa (Peter and Chandramohan 2011). The samples from Kerala were collected from VSD-infected trees displaying the classic chlorotic symptoms, while those in Sulawesi displayed either necrotic or chlorotic symptoms. The samples were stored in the freezer at –20 °C and used within 3 months for DNA extraction.

ITS and LSU sequences of *C. theobromae*

DNA extraction

DNA was extracted from either fungal mycelium sub-cultured into modified CCM or directly from infected petioles or leaves. The mycelium of samples growing in liquid sub-culture was rinsed three times in sterile pure water, blotted dry on sterile filter paper and weighed. To extract DNA directly from infected petioles (either freshly obtained or stored in buffer), after rinsing in sterile water and blotting dry, the epidermal layer of the petiole was removed with a razor blade, or by rubbing with sterile filter paper, and the petiole sample was weighed. Similarly, leaf sections that included the mid-rib were rinsed, blotted dry and weighed.

To obtain genomic DNA, either mycelium or leaf/petiole tissue was extracted in 750 µl Carlson lysis buffer (pH 9.5) pre-heated in a water bath (65 °C). The buffer was prepared using a protocol modified from Carlson et al. (1991) with the following composition: 100 mM Tris-HCl, 2% CTAB, 1% PEG, 1.4 M NaCl, 20 mM EDTA. Prior to use for extraction, 2 µl mercaptoethanol per ml was added. The tissue samples (approx. 100 mg, but ranging from 70–120 mg) were placed in a small mortar and ground quickly with a small amount of sand with a pestle, 500 µl lysis buffer added and the samples homogenised further. The ground samples were transferred to 1.5 ml microcentrifuge tubes (Eppendorf™). To remove the remainder of the tissue from the mortar and pestle, another 250 µl buffer was added (total extraction volume, 750 µl). Samples were incubated for 30–45 min at 65 °C (inverting the tubes every 5–8 min). The tubes were centrifuged at 14,000 rpm for 15 min. The supernatant was removed into new Eppendorf tubes and the volume measured. An equal volume of chloroform/isoamyl alcohol (v/v 24:1) was added; the tubes inverted gently to mix and then centrifuged at 14,000 rpm for 15 min. The supernatant was pipetted into a new tube. An equal volume of cold isopropanol was added to precipitate the DNA. The tubes were centrifuged at 12,000 rpm (4 °C) for 10 min. Precipitated DNA was washed by adding 200–220 µl cold 70% ethanol and centrifuging at 10,000 rpm (4 °C) for 1 min; the supernatant was discarded. This procedure was repeated three times. The DNA precipitate was air-dried at room temperature for 60 min and resuspended in TE buffer (30–50 µl). The DNA extracts were stored at 4 °C for the short term and –20 °C for long term use.

ITS and LSU amplification

Portions of the ITS and 28S nuclear LSU rDNA regions in genomic DNA extracted from fungi, petioles and leaf pieces were amplified by PCR. Reaction mixtures

included 5 µl of the DNA extract, 7 µl Go Taq Green Master Mix (Promega), 1.5 µl forward and reverse primers (10 mM per primer final concentration) and 2 µl deionised water (total volume, 17 µl). Forward/reverse primer pairs included ITS5/ITS4, ITS1F/ITS4B, ITS1/ITS2 and LROR/LR7. To amplify the sequences, the following PCR cycle was used: pre-cycle temperature 95 °C for 15 min (denaturation) followed by 94 °C, 30 s; 55 °C, 30 s (annealing); 72 °C, 1 min (extension). This cycle was repeated 35 times followed by a final extension step at 72 °C, 10 min and then 4 °C for 30 min. PCR products were separated by electrophoresis on 1% agarose in TBE buffer, 80 V, 40–45 min. PCR products were purified and

sequenced by PT Genetika Science, Jakarta, Indonesia and 1st Base Pty Ltd, Singapore.

Results

Incidence and severity of VSD in branches and trees of cocoa clones

VSD incidence for each year (2011 and 2012) for the 12 cocoa clones in the Polman and Pinrang trials is shown in Tables 1, 2, 3 and 4, while disease severity scores are shown in Table 5. In 2011, since not all branchlets were infected even on heavily infected branches, the incidence

Table 1 Mean incidence (% of branchlets on tree infected) of vascular streak dieback infections with necrotic, chlorotic or mixed symptoms, in branches of 12 cocoa clones in a field trial at Beluak, Polman, West Sulawesi, in May/June 2011. The clones are ranked from lowest to highest total incidence. Standard errors are shown in parentheses. Within a column, means followed by the same letter are not significantly different ($P < 0.05$, ANOVA); no significant differences detected between means of the incidence of chlorotic/mixed symptoms

Clone	Incidence of all infections %	Incidence necrotic symptoms %	Incidence chlorotic (and mixed) symptoms %	Ratio Necrotic:chlorotic
PBC123	4.1 (0.6) ^a	1.1 (0.5) ^a	3.1 (0.5)	1:2.8
M05	3.8 (1.3) ^a	2.1 (1.1) ^{abc}	1.7 (0.3)	1:0.8
Geni J	3.8 (1.3) ^a	1.4 (0.6) ^a	2.2 (0.6)	1:1.6
KW617	7.2 (1.4) ^{ab}	0.9 (0.3) ^a	6.3 (1.2)	1:7
KW523	9.4 (2.8) ^{abc}	2.2 (0.2) ^{abc}	7.0 (3.0)	1:3.2
Br25	9.4 (1.1) ^{abc}	0.5 (0.3) ^a	8.6 (1.2)	1:17.2
Muhtar	10.1 (2.8) ^{abc}	3.0 (1.1) ^{abc}	6.9 (2.0)	1:2.3
Ilham	10.8 (3.6) ^{abcd}	2.6 (0.9) ^{abc}	7.6 (2.5)	1:2.9
KW516	10.9 (1.7) ^{abcd}	2.5 (0.6) ^{abc}	8.2 (1.5)	1:3.3
M01	12.9 (5.9) ^{bcd}	1.9 (0.5) ^{ab}	11.0 (5.7)	1:5.8
Nasir R	16.4 (1.7) ^{cd}	6.8 (0.2) ^{bc}	9.4 (1.4)	1:1.4
Husbitori	18.3 (2.2) ^d	7.3 (4.2) ^c	10.3 (4.6)	1:1.4
Average	9.8	2.7	6.9	1:2.6

Table 2 Mean incidence (% of branches > 1 m on tree infected) of vascular streak dieback (VSD) infections with necrotic, chlorotic or mixed symptoms in branches of 12 cocoa clones in a field trial at Beluak, Polman, Sulawesi, in February 2012. The clones are ranked from lowest to highest total incidence (2011 rankings shown in the final column). Within a column, means followed by the same letter are not significantly different ($P < 0.05$, ANOVA)

Clone	VSD incidence (%)				2011 ranking	Ratio Necrotic:chlorotic plus mixed
	Total	Necrosis	Chlorosis	Mixed		
PB123	37.1 ^a	32.5 ^a	3.0 ^a	1.6 ^a	1	7.1:1
MO5	40.7 ^a	34.5 ^{ab}	3.3 ^a	2.5 ^a	2	6:1
Geni J	48.3 ^{ab}	34.7 ^{ab}	6.0 ^a	1.4 ^a	3	4.7:1
KW523	60.5 ^{abc}	57.1 ^{bcde}	3.2 ^a	2.6 ^a	5	9.8:1
KW516	60.6 ^{abc}	49.7 ^{abc}	7.4 ^a	2.0 ^a	9	5.3:1
KW617	74.8 ^{bcd}	68.7 ^{defg}	3.6 ^a	2.1 ^a	4	12:1
MO1	74.8 ^{bcd}	64.1 ^{cdef}	7.3 ^a	3.1 ^a	10	6.2:1
BR25	76.5 ^{cd}	44.8 ^{abc}	22.3 ^b	8.9 ^b	6	1.4:1
Muhtar	80.3 ^{cd}	73.7 ^{efg}	1.1 ^a	2.5 ^a	7	11.2:1
Nasir R	86.4 ^{cd}	79.6 ^{fg}	3.6 ^a	2.7 ^a	11	12.6:1
Ilham	86.8 ^{cd}	82.0 ^{fg}	2.7 ^a	1.6 ^a	8	19.1:1
Husbitori	94.2 ^d	88.7 ^g	2.9 ^a	1.7 ^a	12	19.3:1
Average	68.4	59.2	5.8	2.7		7:1

Table 3 Mean incidence (% branchlets infected) of vascular streak dieback infections that were either predominantly necrotic or chlorotic (includes branchlets with mainly chlorotic symptoms but also those that had a mixture of the two symptoms) in branchlets of 12 cocoa clones in a field trial in Tiroang, Pinrang, South Sulawesi, in

July 2011. The clones are ranked from lowest to highest total incidence. Standard errors are shown in parentheses. Within a column, means followed by the same letter are not significantly different ($P < 0.05$, ANOVA); no significant differences detected between means of the incidence of chlorotic/mixed symptoms

Clone	Incidence of all infections %	Incidence necrotic symptoms %	Incidence chlorotic and mixed symptoms %	Ratio Necrotic:chlorotic
Geni J	4.3 (0.8) ^a	2.1 (0.8) ^a	2.2 (0.5)	1:1
M05	4.9 (0.7) ^a	3.1 (0.9) ^{ab}	1.8 (0.6)	1:0.5
PBC123	8.0 (0.5) ^{ab}	3.0 (0.4) ^{ab}	4.9 (1.0)	1:1.6
DRC15	13.8 (2.8) ^{bc}	10.8 (1.6) ^c	3.0 (1.2)	1:0.3
PCK	14.0 (1.8) ^{bc}	4.4 (1.6) ^{ab}	9.6 (1.7)	1:2.2
M01	14.5 (3.1) ^{bc}	6.0 (1.4) ^{abc}	8.5 (2.3)	1:1.4
M06	14.7 (1.9) ^{bc}	8.2 (0.7) ^{bc}	6.5 (1.9)	1:0.8
ICCRI04	15.6 (1.3) ^c	7.5 (2.0) ^{abc}	8.1 (1.2)	1:1.1
RB	18.0 (2.5) ^c	5.0 (0.4) ^{abc}	13.0 (2.4)	1:2.6
TR01	18.4 (3.5) ^c	10.5 (2.7) ^c	7.9 (0.8)	1:0.8
Pan R	19.4 (2.4) ^c	6.2 (1.4) ^{abc}	13.1 (2.8)	1:2.1
M04	20.3 (3.7) ^c	16.1 (3.9) ^d	4.2 (1.5)	1:0.3
Average	13.8	6.9	6.9	1:1

Table 4 Mean incidence (% of branches on tree infected) of vascular streak dieback (VSD) infections with necrotic, chlorotic or mixed symptoms in branches of 12 cocoa clones in a field trial at Tiroang, Pinrang, Sulawesi, in February 2012. The clones are ranked from lowest to highest total incidence (2011 rankings shown in the first column). Within a column, means followed by the same letter are not significantly different ($P < 0.05$, ANOVA); no significant differences in incidence of chlorotic or mixed symptoms were detected

Clone	VSD incidence (%)				2011 ranking	Ratio Necrotic:chlorotic plus mixed
	Total	Necrosis	Chlorosis	Mixed		
Geni J	39.2 ^a	30.4 ^a	6.7	2.0	1	3.5:1
PBC123	39.9 ^a	35.4 ^{ab}	2.9	1.5	3	8:1
M05	44.8 ^{ab}	38.4 ^{abc}	4.7	1.5	2	6.2:1
PCK	57.3 ^{abc}	45.7 ^{abcd}	7.5	3.4	5	4.2:1
M01	61.7 ^{abc}	49.1 ^{abcd}	10.2	2.3	6	4:1
RB	64.2 ^{abc}	53.8 ^{abcd}	7.3	3.1	9	5.2:1
M06	70.0 ^{abc}	59.5 ^{bcd}	7.3	3.3	7	5.6:1
ICCRI04	74.7 ^{bc}	62.3 ^{cd}	7.5	4.2	8	5.3:1
TR	75.2 ^{bc}	64.7 ^d	7.3	2.8	10	6.4:1
DRC15	76.3 ^{bc}	61.7 ^{cd}	10.3	4.3	4	4.2:1
M04	79.0 ^c	65.3 ^d	9.7	4.7	12	4.5:1
Pan R	80.9 ^c	65.9 ^d	8.7	3.5	11	5.4:1
Average	63.6	52.7	7.5	3.0		5:1

estimated by this method was lower (Tables 1 and 3) than in 2012 (Tables 2 and 4) when only infected branches longer than 1 m were counted to evaluate incidence.

Significant differences in total VSD incidence occurred between the most susceptible and most resistant clones. Cocoa clones PBC123, Geni J and M05 consistently had the lowest VSD incidence at both sites in both 2011 and 2012 (Tables 1, 2, 3 and 4) and relatively low severity scores (Table 5), while Husbitori, M04 and Panimbu Red had the highest disease incidence in both years. M01, a productive local farmer selection, indicated moderate

VSD incidence at both locations. In Polman, KW clones selected from the progeny of crosses between VSD-resistant and high yielding parents in East Java also had moderate disease incidence (Tables 1 and 2).

Most of the cocoa clones developed both leaf symptoms (necrotic or chlorotic) (Fig. 1), especially at early stages of infection. In many cases, leaves showed early signs of chlorosis and then became necrotic, but in some clones, such as BR25, infected leaves often developed only symptoms of chlorosis (yellowing with remnant green areas) before abscission. A longer period of attachment of infected leaves

Table 5 Vascular streak dieback (VSD) severity, scored from 1 (low) to 3 (high), in 2011 and 2012 in cocoa clones at Beluak, Polman and Tiroang, Pinrang, Sulawesi. In 2012, severity was evaluated in branches displaying either necrotic (N), chlorotic (Ch) or mixed (M) VSD symptoms. Within a column, means followed by the same letter are not significantly different ($P < 0.05$, ANOVA); in the evaluation of the Pinrang trial in 2012, no significant difference between clones occurred in total severity or severity of individual symptoms

Clone	Polman					Pinrang				
	2011	2012				2011	2012			
	Total	Total	N	Ch	M	Total	Total	N	Ch	M
PBC123	2.6 ^a	2.4 ^a	2.5 ^{ab}	0.8 ^a	0.5 ^a	2.1 ^{abc}	3.0	3.0	1.3	0.8
MO5	2.5 ^a	2.7 ^a	2.8 ^{ab}	0.8 ^a	0.8 ^{ab}	2.0 ^{ab}	3.0	3.0	1.8	0.9
Geni J	2.2 ^a	2.8 ^a	2.6 ^{ab}	1.1 ^a	0.3 ^a	1.8 ^a	3.0	3.0	2.0	0.8
KW523	2.4 ^a	2.9 ^a	2.8 ^{ab}	0.8 ^a	0.4 ^a	-	-	-	-	-
KW516	2.5 ^a	2.9 ^a	2.8 ^{ab}	0.9 ^a	0.4 ^a	-	-	-	-	-
KW617	2.5 ^a	2.8 ^a	2.9 ^{ab}	1.1 ^{ab}	0.8 ^{ab}	-	-	-	-	-
MO1	2.8 ^a	2.9 ^a	2.8 ^{ab}	1.4 ^{ab}	1.3 ^{ab}	2.1 ^{bcd}	3.0	3.0	2.3	1.1
BR25	2.6 ^a	2.9 ^a	3.0 ^{ab}	2.6 ^b	1.7 ^b	-	-	-	-	-
Muhtar	2.8 ^a	3.0 ^a	3.0 ^{ab}	1.1 ^a	0.6 ^a	-	-	-	-	-
Nasir R	2.3 ^a	3.0 ^a	2.7 ^a	1.1 ^{ab}	0.8 ^{ab}	-	-	-	-	-
Ilham	2.9 ^a	2.8 ^a	2.9 ^{ab}	0.6 ^a	0.3 ^a	-	-	-	-	-
Husbitori	2.9 ^a	3.0 ^a	3.0 ^b	0.7 ^a	0.4 ^a	-	-	-	-	-
PCK	-	-	-	-	-	2.2 ^{bcd}	3.0	3.0	2.4	1.4
RB	-	-	-	-	-	2.3 ^{bcd}	3.0	3.0	2.0	1.2
M06	-	-	-	-	-	2.2 ^{bcd}	3.0	3.0	2.0	1.5
ICCRI04	-	-	-	-	-	2.4 ^{cd}	3.0	3.0	2.1	1.4
TR	-	-	-	-	-	2.4 ^{cd}	3.0	3.0	2.1	1.1
DRC15	-	-	-	-	-	1.9 ^{ab}	3.0	3.0	2.5	1.9
M04	-	-	-	-	-	2.4 ^{cd}	3.0	3.0	2.5	2.0
Pan R	-	-	-	-	-	2.5 ^d	2.9	3.0	2.4	1.7
Average		2.8	2.8	1.1	0.7	2.2	3.0	3.0	2.1	1.3

occurred with leaves with necrotic symptoms than with those that developed chlorotic symptoms. Some necrotic leaves remained attached until fully dried and brittle, but this was never observed with chlorotic leaves.

In 2011, the average ratio of incidence of necrotic to chlorotic symptoms was 1:1 at Pinrang and 1:2.6 at Polman, with the outlier being BR25 where the ratio was 1:17.2 (Table 1). Monthly rainfall totals were relatively high when the 2012 evaluation was conducted, while the 2011 evaluation was conducted in a drier period (Supplementary Figs. 1 and 2). In 2012, there was a much higher incidence of necrotic than chlorotic (including mixed) symptoms, with the average ratio of incidence of necrotic to chlorotic symptoms being 5:1 at Pinrang and 7:1 at Polman (Table 2). Outliers at Polman were BR25 that, as in 2011, had a relatively higher incidence of chlorotic symptoms (ratio of necrotic to chlorotic symptoms 1.4:1) than all other clones, while the very susceptible clones, Ilham and Husbitori, had a very high incidence of necrotic symptoms (ratio of necrotic to chlorotic symptoms more than 19:1).

Disease severity scores in 2011 ranged from a low of 1.8 and 2.2 for Geni J at Pinrang and Polman, respectively, to a high of 2.9 for Ilham and Husbitori at Polman, while the highest severity scores were recorded

in 2012 in Pinrang (Table 5). Significant correlations between total incidence and severity occurred in Polman in 2012 and in Pinrang in 2011 (Pearson's $r = 0.76^{**}$ and $r = 0.83^{**}$, respectively). In Pinrang, incidence and severity of chlorotic and mixed symptoms were significantly correlated ($r = 0.91^{**}$; $r = 0.94^{**}$, respectively). In Polman in 2012, incidence of infected branches that had predominantly necrotic leaf symptoms (Table 2) was significantly correlated with overall disease severity in branches (Table 5) ($r = 0.652^*$) but no significant correlation occurred between disease severity and the incidence of either chlorotic or mixed symptoms. No significant correlation between disease severity and incidence of either chlorotic or mixed symptoms occurred at this site in 2012.

Re-evaluation in 2014

In the first clone evaluation in 2014 (March), the incidence of VSD-infected branches ranged from 12.1% (M05) to 54.1% (Panambu Red) but had increased by July 2014, to 28% and 66%, respectively (Table 6). Necrotic symptoms were predominant in all clones in March, but by July branches with chlorotic symptoms were more common in

Table 6 Mean branch incidence and severity of vascular streak dieback (VSD) observed in eight clones of cocoa in South and West Sulawesi, March and July 2014. The results were obtained from a ran-dom sample of 10 branches in each tree at each sampling time and the severity determinations for each branch were summed to give a severity score for each tree^a. Standard errors are shown in parentheses

Clone	No. of trees observed	Evaluation (2014)	Mean of sum of severity scores (SE)	Incidence of VSD (SE)			
				% branches infected	% branches with necrotic symptoms	% branches with chlorotic symptoms	% branches with early symptoms ^b
M05	58	March	2.5 (0.3)	12.1	5.8 (0.3)	3.9 (0.2)	2.4 (0.1)
		July	5.4 (0.5)	28.0	18.6 (0.3)	7.2 (0.1)	2.2 (0)
Geni J	54	March	2.9 (0.3)	13.9	6.5 (0.4)	4.8 (0.4)	2.6 (0.2)
		July	5.9 (0.5)	32.0	12.8 (0.2)	18.0 (0.3)	1.2 (0)
KW617	31	March	4.4 (0.6)	26.0	15.2 (0.6)	5.7 (0.2)	5.1 (0.2)
		July	6.1 (0.7)	30.9	9.7 (0.3)	15.4 (0.4)	5.8 (0.2)
PBC123	76	March	4.9 (0.4)	28.9	14.4 (0.6)	7.7 (0.3)	6.8 (0.3)
		July	6.7 (0.4)	33.0	20.1 (0.8)	10.8 (0.4)	2.1 (0.1)
M01	64	March	7 (0.5)	38.0	17.5 (0.6)	9.0 (0.3)	11.5 (0.4)
		July	8.1 (0.5)	41.9	23.4 (0.7)	13.3 (0.4)	5.3 (0.2)
M06	39	March	7.6 (0.6)	37.1	25.0 (0.9)	4.6 (0.1)	7.5 (0.2)
		July	9.8 (0.6)	43.0	36.2 (0.9)	5.3 (0.1)	1.5 (0)
KW516	40	March	10.2 (0.7)	52.1	24.4 (1.0)	10.6 (0.4)	17.1 (0.7)
		July	8.7 (0.6)	35.0	15.1 (0.6)	18.5 (0.7)	1.4 (0.1)
Panimbu Red	36	March	11.1 (0.7)	54.1	28.0 (1.0)	7.8 (0.5)	18.3 (0.7)
		July	13.3 (0.6)	66.0	35.6 (0.3)	24.6 (0.2)	5.8 (0.1)

^aHomogenous subsets of cocoa clones ranging from low to high severity of vascular streak dieback for the March evaluation that were significantly different from each other included: M05 and GeniJ; KW617 and PBC123; M01 and M06; KW516 and Panimbu Red; and for the July evaluation: M05, Geni J, KW617 and PBC123; PBC123 and M01; M01 and M06; M06 and KW516; Panimbu Red. ^bSlight paling green or slight yellowing of leaves

the KW clones and Geni J. Rainfall data collected in 2014 indicates average rainfall was lower in July than March in Pinrang, but was higher in Luwu (Supplementary Fig. 3). With the exception of the KW clones, the incidence of both necrotic and chlorotic symptoms increased in the evaluated clones. Paired differences (not shown) indicated

increases in necrotic symptoms between the two evaluations were significant ($p = 0.05$) for M01 and in chlorotic symptoms for KW617 and KW516.

Average severity for all of the trees (for the eight clones combined) in the sample increased from 6 to 7.7% between the two evaluations. In both March and July, clones showed

Table 7 The progress of vascular streak dieback (VSD) infection in the branches of two clones, M04 (susceptible) and PBC123 (resistant), during a 5-month period in the 2012 late wet season at the trial site in Tiroang, Pinrang, South Sulawesi. For each clone, the average proportion (%) of the total leaves on the VSD-infected branches occurring as flush leaves, healthy expanded (mature) leaves or infected leaves was determined each month. The data shown are the mean (and SE) of the four replicates in the clone trial

Clone	Category of leaves	Proportion (%) of total leaves ^a on branch ^b				
		March	April	May	June	July
M04	Healthy mature	56.2 (5.0)	74.3 (5.1)	74.6 (2.8)	58.9 (2.9)	52.0 (3.5)
	Flush	28.9 (5.5)	9.2 (3.9)	3.9 (1.5)	1.2	1.0
	Total healthy	85.1 (10.5)	83.5 (9.0)	78.5 (4.3)	60.1 (2.9)	52.0 (3.5)
	Infected, attached	8.6 (2.5)	6.0 (1.0)	8.9 (1.5)	22.6 (3.1)	23.0 (0.5)
	Fallen with VSD	3.7 (0.9)	10.5 (2.8)	12.6 (4.0)	17.3 (3.0)	23.9 (3.8)
	Total infected	12.3 (3.0)	16.5 (2.5)	21.5 (4.2)	39.9 (3.1)	46.9 (4.0)
PBC123	Healthy mature	56.5 (6.6)	77.4 (2.2)	73.7 (4.2)	69.2 (6.2)	62.3 (6.0)
	Flush	30.0 (6.0)	4.0 (1.6)	0.0	1.7 (1.0)	5.1 (2.7)
	Total healthy	86.5 (12.6)	81.4 (3.8)	73.7 (4.2)	71.9 (7.2)	67.3 (8.7)
	Infected, attached	3.1 (1.8)	5.8 (0.6)	10.2 (3.2)	9.8 (4.0)	11.2 (3.0)
	Fallen with VSD	10.3 (2.9)	12.7 (2.0)	16.1 (1.8)	19.2 (2.1)	21.4 (2.6)
	Total infected	13.4 (3.0)	18.5 (2.2)	26.3 (4.2)	29.0 (6.1)	32.6 (4.3)

^aTotal number of leaf positions (including attached and fallen leaves); ^b8–10 branches per clone in each block evaluated

significant differences in VSD severity (Table 6). The lowest severity scores occurred in Geni J, M05, KW617 and PBC123, and the highest in M06, KW516 and Panimbu Red.

Development of VSD symptoms in branches

The development of VSD symptoms in a susceptible (M04) and resistant (PBC123) clone was monitored for 5 months (March–July 2012) in Pinrang on 32–40 branches (8–10 branches per block for each clone) that initially showed the earliest signs of VSD infection (Table 7). By the end of the evaluation period, the proportion of infected leaves on branches on the susceptible clone (M04, 46.9%) was almost 1.5-fold higher than on the resistant clone (PBC123, 32.6%). The proportion of leaves showing symptoms increased more rapidly in M04 than PBC123 over the last 2 months of assessment. The changed symptoms of VSD observed since 2004 and throughout this study were evident as dark necrotic blotches or margins on leaves that tended to remain attached to branches, rather than as a developing chlorosis with green spots of whole leaves that abscised as soon as they were fully chlorotic, without any sign of leaf necrosis. Over the 5-month period, necrotic leaves in both clones remained attached to the branch for an extended period (30% of the infected leaves in PBC123 and 50% in M04) (Table 7). Sporulation, confirmed as *C. theobromae*, was commonly observed on cracks in the petiole and on the adjacent leaf lamina.

Sporocarp observations and spore shape and size

In the 2011–2012 wet season, basidiocarp numbers were greatest at Polman in the early wet season (in November and December, pers. comm., farmer). Nevertheless, by February 2012, basidiocarps were still detected, the greatest number being produced on the moderately VSD-susceptible

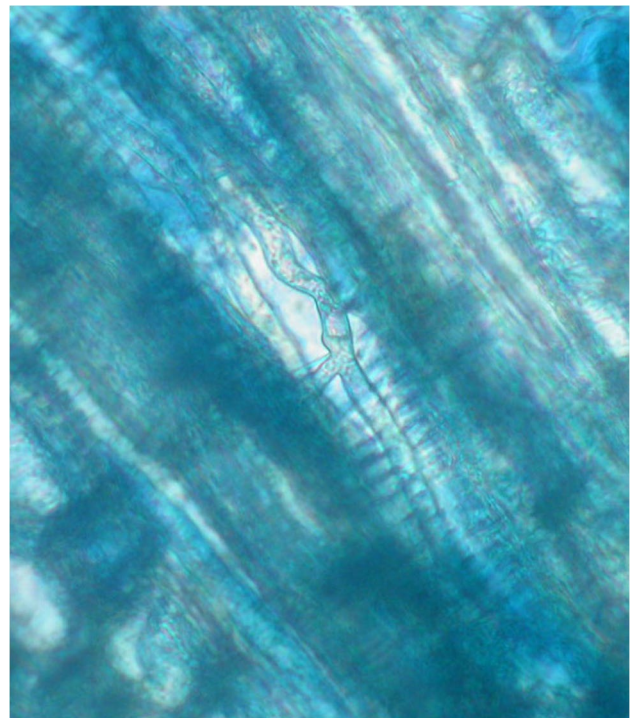


Fig. 3 *Rhizoctonia*-like hyphae in xylem vessel of vascular streak dieback (VSD)-infected cocoa

clones BR25, Ilham and M01. They were most commonly formed from hyphae emerging from cracks in the leaf mid-rib (Fig. 2b) or leaf petioles, but basidiocarps on leaf scars were also found (Fig. 2a). The hyphae emerging from leaves or leaf scars and beginning to form basidiocarps were generally typical of *C. theobromae* with right angle branching and thick hyphae. *Rhizoctonia*-like hyphae were also observed in xylem vessels in longitudinal sections of stem cut with a razor blade and examined under a HP microscope (Fig. 3). Hyphal width was approx. 5 μm in the vegetative hyphae with dolipore septa although hyphae became much wider (up



Fig. 2 **a** A new position of sporulation (through a crack in a petiole) for the basidiomycete, *Ceratobasidium theobromae* (the causal pathogen of vascular streak dieback) because infected leaves tend to remain

attached to branches. Both stem pieces collected from the same infection in Polman, West Sulawesi. **b** Sporulation on a crack in the leaf mid-rib as a result of the leaf remaining attached to the branch

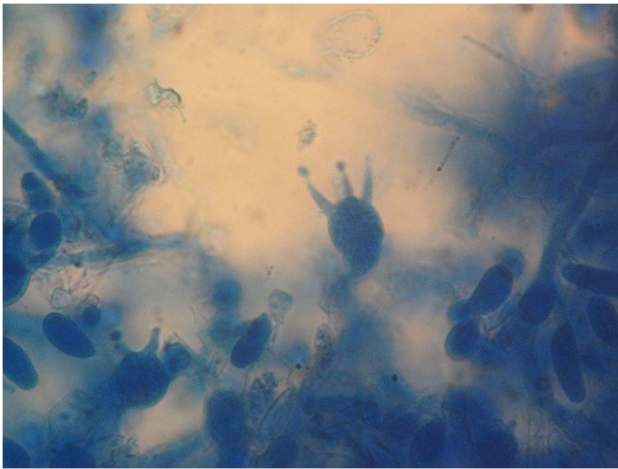


Fig. 4 Loose *Ceratobasidium theobromae* spores shown under a HP microscope ($\times 400$ magnification) in basidiocarps collected from vascular streak dieback (VSD)-infected cocoa in East Luwu, Sulawesi, recognisable by their asymmetric shape and a basidium (centre), with spores forming on the sterigmata. Dimensions of reproductive structures were consistent with those published by Talbot and Keane (1971)

to 10 μm) in sporophores that were developing basidia. However, a number of basidiocarps contained hyphae of other fungi. *Fusarium* sp. micro- and macroconidia were sometimes found in association with mycelia characterised by narrow hyphae. When basidiocarps still attached to leaves or stems were stored for 2 or more days in plastic bags, macro- and micro-conidia of *Fusarium* sp. and putative *Fusarium* sp. mycelia were found more frequently. However, in fresh basidiocarps, the mycelium was usually dominated by *C. theobromae*-like hyphae.

Basidiospores were rare or absent in the basidiocarps collected. However, in March 2012 following a period of heavy rainfall, hyphae in the advanced stages of basidiocarp development prior to sporulation, as described by Talbot and Keane (1971), were evident and some basidiocarps also had formed basidia and basidiospores (Fig. 4). Spore dimensions were within the range described by Talbot and Keane (1971) as typical of *C. theobromae*, ranging from 15 to 20 μm in length and 7.5 to 8 μm in width. Many had a slightly asymmetric shape as described by Talbot and Keane (Fig. 4). Sterigmata observed were approximately half the length of the spores (consistent with the descriptions of Talbot and Keane). The spore dimensions and shape and their association with basidiocarps which consisted of *Rhizoctonia*-like hyphae support their identification as *C. theobromae* spores. Whether other *Ceratobasidium* spp. particularly *C. ramicola*, were present also (as reported by Samuels et al. (2012)) is uncertain as the hyphae of *C. ramicola* and *C. theobromae* are very similar, but small basidiospores with dimensions of approx. $6 \times 4 \mu\text{m}$, typical of *C. ramicola* (Kotrianta and

Saarenoksa 2005) were not found. Also, the frequent anastomosis reported in *C. ramicola* (Samuels et al. 2012) was not observed, either in the mycelia collected from sporophores or in isolates from cut petioles and leaf midribs.

Isolation of the causal pathogen, *C. theobromae* and rDNA sequences

Fungi isolated onto water agar from petioles and leaves with symptoms of VSD infection were consistently *Rhizoctonia*-like with hyphae commonly 5 mm in diameter as described by Keane et al. (1972). Some isolates obtained from infected cocoa with chlorotic leaf symptoms could be grown as axenic cultures in CCM nutrient medium. These grew slowly in culture as reported previously (Keane et al. 1972; Samuels et al. 2012). Other species of fungi were frequently isolated from VSD-infected cocoa leaves and petioles (both chlorotic and necrotic), even after surface sterilisation. The morphology of hyphae emerged from the cut ends of petioles and leaf mid-ribs in the early stages of isolation and, additionally, in sporocarps, in samples collected from cocoa with necrotic symptoms confirmed the association of *C. theobromae*, as indicated by morphological characteristics, with necrotic, as well as chlorotic, symptoms.

ITS and LSU sequences

DNA extracts of *Rhizoctonia*-like fungi isolated from VSD-infected cocoa with clear symptoms of leaf chlorosis, or direct DNA extracts of infected chlorotic leaves or petioles, were subjected to PCR using universal and basidiomycete-specific primers and the PCR products sequenced, enabling the identification of *C. theobromae* (syn. *Oncobasidium theobromae*, *Thanatephorus theobromae*) by matches with previous ITS sequences submitted to GenBank (Table 8) (Samuels et al. 2012). In addition, sequences of the 28S nuclear LSU region had 100% identity with *C. theobromae* LSU sequences previously deposited in GenBank (Table 8). While *C. theobromae* isolated from petioles and mid-ribs of leaves with VSD infections showing typical chlorotic symptoms could be cultured or detected with molecular methods *in planta*, this proved to be challenging for VSD-infected leaves with necrotic symptoms, which were common on clones such as Husbitori and Panimbu Red. Even though in the early stages of isolation *Rhizoctonia*-like hyphae were observed to emerge from cut petioles or leaf midribs (see above), these hyphae were subsequently overgrown by faster growing species. DNA extracts of these faster-growing fungi were subjected to amplification using ITS5/ITS4 or ITS1F/ITS4B primer pairs. ITS sequences of the PCR products provided close matches (97–100%) to Basidiomycetes wood-rotting species including (with GenBank

Table 8 *C. theobromae* partial sequences of the internal transcribed spacer (ITS) or large subunit (LSU) (28S) rDNA regions obtained either from DNA extracts of fungal isolates (with *Rhizoctonia*-like characteristics) grown in nutrient media or directly from vascular streak dieback (VSD)-infected leaves. The leaves, all of which indicated classic VSD symptoms of chlorosis, were collected from hybrid cocoa in Sulawesi, Indonesia, and Kerala, India, or from the

local cocoa clone, M06, in Pinrang, Sulawesi. Identity (99–100%) was found with sequences previously deposited in NCBI's GenBank (see Samuels et al., 2012), including ITS and LSU sequences of VSD-infected cocoa in Sulawesi (HQ424246.1, HQ424242.1) and ITS sequences in Malaysia (HQ424250.1, FJ763574.1), East Java (FJ763573) and Vietnam (FJ763576.1)

Province, country	District	Source of DNA	Sequence (bp)	GenBank accession
South Sulawesi, Indonesia	North Luwu	Isolate	ITS (615) LSU (812)	KM523668 KU319575
		Isolate	ITS (651)	KU255725
	East Luwu	Petiole	ITS (789) LSU (812)	KU255724 KU319576
		Isolate	ITS (776)	KU255726
	Pinrang	Petiole	ITS (638) LSU (812)	KU319574 KU319577
		Leaf mid-rib	ITS (778)	KU319572
		Leaf mid-rib	ITS (750) LSU (735)	KU319573 KU319578
		Petiole	ITS (706)	KU310940
		Petiole	ITS (844)	KU310941
Kerala, India	Monipali	Petiole	ITS (706)	KU310940
	Vempey	Petiole	ITS (844)	KU310941

Accession numbers) *Ceriporia lacerata* (KP297476.1), *Meripilus giganteus* (KU366500) and *Lentinus squarrosulus* (KU366501).

Discussion

Observations of a higher frequency of necrotic, compared to chlorotic symptoms, in VSD-infected cocoa in recent years were confirmed by the evaluations of on-farm trials in three districts in Sulawesi. The predominant leaf symptom in all of the evaluated clones was necrotic blotches eventually developing into necrosis of the whole lamina. The prolonged period of attachment of VSD-infected leaves with necrotic symptoms was also evident. For two cocoa clones, each of which showed predominantly necrotic leaf symptoms, 30–50% of infected leaves remained attached 5 months after early VSD symptoms were recorded (Table 7). This contrasts with the rapid abscission of leaves soon after they become fully chlorotic recorded for VSD infections in Papua New Guinea from the 1970s through to the present time (Keane and Prior 1991). In addition to a higher incidence of necrosis and a prolonged period of leaf attachment, our observations confirmed changes in sites of sporulation of the pathogen (Fig. 2a, b).

The leaf symptoms, whether necrotic or chlorotic, were always associated with other distinctive symptoms of VSD as previously described. They always began on the second or third growth flush behind the branch tip and were always associated with the discolouration of the three vascular traces in leaf scars (observed by scraping off the dry surface

layer of tissue) and discolouration of xylem in veins and stems, except that the discolouration was generally much darker than that associated with chlorotic leaves in the early descriptions of the disease. The presence of *Rhizoctonia*-like hyphae typical of *C. theobromae* was always associated with the disease as originally described (Talbot and Keane 1971; Keane et al. 1972).

Observations of basidiocarps on VSD-infected cocoa with either necrotic or chlorotic symptoms in two of the trial locations (Pinrang and Polman), as well as other cocoa-growing locations in Sulawesi (Parawansa 2012; Parawansa et al. 2015), confirmed the consistent presence of a fungus with hyphae, basidia and basidiospores characteristic of *C. theobromae* (formerly *Oncobasidium theobromae*) as described in earlier studies (Talbot and Keane 1971; Keane et al. 1972; Lam et al. 1988). Although the pattern of sporulation has changed with the increased frequency of necrotic symptoms and the prolonged attachment of infected leaves (so that basidiocarps are now frequently found on cracks in leaf mid-ribs and petioles as well as leaf scars), collections of basidiocarps in Polman in March 2012 from trees showing predominantly necrotic symptoms revealed asymmetric basidiospores (Fig. 4) flattened on one side and with dimensions within the range first described by Talbot and Keane (1971). Furthermore, in collections of basidiocarps from East Luwu, the length of sterigmata compared to spore length was similar as first shown in the illustrations by Talbot and Keane (1971), indicating it is likely that the species of pathogen present on cocoa in Sulawesi today is the same as in cocoa affected by VSD some five decades ago (Keane et al. 1972). Furthermore,

microscopic examination of the xylem tissue of infected stems and petioles consistently revealed the presence of hyphae in xylem vessels (see Fig. 3) with the characteristics of *C. theobromae*, 5 mm in width, with right-angle branching and dolipore septa (McMahon 2012).

The consistent occurrence of *C. theobromae* in VSD-infected cocoa in three districts in South Sulawesi was confirmed by ITS and LSU sequences obtained from DNA extracts of fungal isolates (Table 8), which had typical *Rhizoctonia*-like hyphae as previously described for fungi regularly isolated from VSD-infected cocoa (Keane et al. 1972). In addition, *C. theobromae* was identified from ITS and LSU sequences amplified directly from DNA extracts of VSD-infected host tissues (Table 8). All the DNA sequences of *C. theobromae* in this study were obtained from infected cocoa with chlorotic leaf symptoms. *Rhizoctonia*-like mycelium also grew from cut petioles or leaf mid-ribs of infected cocoa with necrotic symptoms but subculturing these isolates proved difficult since, even following surface sterilisation, other saprotrophic fungi associated with the necrotic VSD-infected cocoa tissues overgrew the slower-growing *C. theobromae* mycelia. Additionally, direct amplification of *C. theobromae* DNA from necrotic leaves was not successful.

Although the incidence of necrotic symptoms has strongly increased since VSD was originally described in Papua New Guinea, Malaysia and Indonesia (Keane and Prior 1991; Turner and Keane 1982), this study demonstrated variation between clones in the relative proportion of the two symptoms (necrotic or chlorotic), indicating a degree of clone dependence. Clones with a relatively high proportion of chlorotic symptoms included KW617 from East Java and BR25, a Malaysian clone.

The positive correlation between incidence of necrotic symptoms in branches and overall disease severity, with the exception of the clones evaluated in 2012 in Pinrang, which demonstrated a very high proportion of necrotic symptoms compared to chlorotic (or mixed) symptoms, suggests that increased VSD severity is associated with a greater preponderance of necrotic symptoms. It should be noted that the formation of basidiocarps on cracks on the lamina and petiole means that sporulation is likely to be more common than previously because the lamina can be wet by overnight dew, while the leaf scars on branches require heavy rain for wetting. A link between increased disease severity and necrotic symptoms is also supported by data on VSD intensity in cocoa grown at relatively high and low altitudes, with a much lower average severity and an absence of necrotic symptoms recorded in VSD-infected cocoa at higher altitudes, compared to higher disease severity and a preponderance of necrotic symptoms at lower altitudes (Bryceson 2014).

The study in 2014 demonstrated that in July (the dry season in Pinrang and Polewali-Mandar), a higher proportion

of branches in some clones showed chlorotic symptoms than in March (the late wet season). This is consistent with the greater proportion of chlorotic symptoms found in the 2011 evaluations (conducted during the dry season, Supplementary Figs. 1 and 2), compared to 2012 when clones evaluated in the late wet season showed a higher proportion of necrotic symptoms. Possibly, drier conditions in July 2014 in these locations (although rainfall totals were high at this time of year in Luwu, Supplementary Fig. 3) favoured the development of chlorotic symptoms (and leaf loss), while an adequate water supply supported a necrotic response, in which green tissue of still attached, partly necrotic leaves could continue to photosynthesise. This heightened necrotic response supports the hypothesis that an increased incidence of necrotic symptoms is related to a general enhanced resistance response in the cocoa (Bryceson 2014). The more rapid and extensive development of a dark necrosis of infected leaf tissue, including the invaded xylem tissue resulting in darker vascular discolouration than originally reported, is symptomatic of a hypersensitive necrotic response of a type not reported in the original descriptions from Papua New Guinea, Malaysia or Indonesia. As a 'new-encounter' disease caused by a fungus that can only have transferred to cocoa since the introduction of the crop to Southeast Asia, the host–pathogen relation is likely to be unstable and susceptible to disturbance or rapid change, for example in response to environmental changes. A strong necrotic response was noted in the local clone TR01 at Pinrang in 2011 (Table 3); yet, this clone continued to grow well and proved to be productive despite the infection (authors' observation). While necrotic symptoms have also been observed to reach leaves at the branch tip in some clones resulting in branch dieback, in a number of other clones, the branches were able to outgrow the disease (McMahon et al. 2018).

Resistance ratings in local clones were maintained over the three seasons covered in this study. Both symptoms were observed regardless of the degree of resistance of the clones (Tables 1, 2, 3, 4, 5 and 6). Clones indicating resistance included the well-known Malaysian clone, PBC123 (known locally as Sulawesi 1), and two local selections, M05 and Geni J. In all locations, these three clones had the lowest disease incidence in the shoots in 2011, 2012 and 2014, indicating stable resistance. Resistance in these clones has been identified in trials located in other districts of Sulawesi, while PBC123 has maintained a high degree of resistance since the 1980s in Malaysia and Sulawesi (Chong and Shepherd 1986; McMahon et al. 2010; 2018). In the current study, PBC123 had a relatively high proportion of healthy leaves on the branch 5 months after early stages of infection (Table 7). On the other hand, some clones have indicated consistent susceptibility in different locations in Sulawesi and East Java (McMahon et al. 2018; Susilo and Anita-Sari 2011), including the highly productive farmer

selection M04 in this study (Tables 1, 2, 3, 4, 5 and 7). The hypothesis that VSD-resistance is heritable and horizontal (van der Plank 1963), i.e. quantitative and durable (Tan and Tan 1988; Prior 1979; Keane 2012), is supported by the consistent response of the clones in these trials in Sulawesi (McMahon et al. 2018). Additionally, VSD resistance was recorded in KW617, a progeny of a cross between the VSD-resistant PBC123 and the high yielding clone TSH858 (Tables 1, 2, 5 and 6).

In the 5-month longitudinal study comparing two contrasting clones (VSD resistant and susceptible), disease severity was determined by the number of infected or fallen leaves (Table 7): in the resistant clones even following successful infection, a lower proportion of leaves were infected compared to susceptible clones, suggesting that the fungus or its effect (for example blockage of transport in the xylem) did not readily spread within the branch to other leaves. The distribution of infections within branches is more limited in the resistant PBC123 than in susceptible clones (McMahon et al. 2018), suggesting it is more able to restrict the spread of the initial infection to other leaves. Resistance characteristics identified in local clones are most useful in breeding programmes that combine resistance and quality characteristics: Geni J, for example, is highly susceptible to cocoa pod borer, and so would not be useful for uptake by farmers.

As reported previously, the incidence and severity of VSD are highly dependent on rainfall (Keane 1981; McMahon et al. 2018). In the current study, the increased VSD incidence between March and July 2014 (Table 6) could be explained by infections initiated in the peak wet season (November–February) and the 3-month incubation period prior to symptom development (Keane and Prior 1991). Infections initiated in the later wet period during March/April (Supplementary Fig. 3) would have appeared as symptoms in July adding to the total incidence assessed in July. Furthermore, disease progression results in increased severity as more leaves develop symptoms (Table 7) and infections approach the branch tip (McMahon et al. 2018). This is supported by the higher proportion of early symptoms (pale green leaves) in March (3 months after likely infection) compared to July when most infections had already developed into either leaf chlorosis or necrosis. Nevertheless, resistance rankings were maintained despite the increase in disease incidence and severity (Table 6).

It remains uncertain whether the mechanism(s) of resistance to VSD infection at the later stage of infection (when the fungal hyphae grow through the xylem vessels) is the same as, or linked to, mechanism(s) at the early stage of infection (germination of the spores and penetration of the young leaves) (Prior 1979). However, the positive correlation found between branch infection incidence and severity in clones in each of the trials suggests that resistance to initial infection of young leaves and to the growth of the

fungus via the xylem has a common mechanism or is otherwise linked.

Necrotic symptoms emerged in different locations in the region of VSD endemicity around the same time in the early 2000s. The cause of this widespread change remains unknown. If it is a resistance response (see above), then a more gradual spread of the new symptomology (as new cocoa genotypes are introduced to farms) would be expected. Other potential explanations are that necrotic symptoms have emerged due to (1) a host response modified by other fungi: weak pathogens, secondary invaders or endophytes; (2) changes in plant nutrition, for example inadequate potassium due to prolonged cropping, although Bryceson (2014) found similar potassium and phosphorus levels in infected leaves showing either symptom; (3) smoke haze and emissions resulting from widespread peat and forest fires that have impacted Indonesia in recent years (Bryceson 2014), resulting in a gradient of increasing carbon dioxide concentrations from high to low altitudes; (4) the emergence of a new strain of *C. theobromae*, although molecular sequence data for collections from Kerala to Papua New Guinea do not support this (see below); or (5) a response to climate change (Parawansa et al. 2015) such as higher temperatures (impacting lower lying areas) or increases in carbon dioxide concentration. It is interesting to note that recent experience from 2014 to 2020 by one of the authors (PJK) in three widespread provinces of Papua New Guinea (East New Britain, Madang and East Sepik) known to have VSD showed that the originally described symptoms are still overwhelmingly predominant there. Over the 6-year period, only one clear example of the necrotic symptoms now very common in Indonesia and Malaysia was found in Papua New Guinea. Also, the original symptoms still prevail in Kerala, India. This may indicate that if some environmental change is responsible for the change in symptoms, it could be linked to factors unique to the Indonesian-Malaysian region such as greatly elevated carbon dioxide concentrations in the atmosphere due to extensive forest burning and prolonged burning of peat beds.

Since leaves with necrotic symptoms remain attached for much longer periods before abscission than those with chlorotic symptoms, it is possible that the necrosis is itself a consequence of the longer period during which infected leaves remain attached. If this is the case, the question remains: what factor(s) delays leaf abscission? One possible explanation for the change in symptomology of VSD-infected cocoa in the 2000s (see above) is that a new strain of *C. theobromae* has emerged. However, as dispersal of *C. theobromae* spores is limited to within 100 m (Keane 1981), the practically simultaneous emergence of the new symptoms throughout the Southeast Asian region in the early 2000s is unlikely to be solely the result of a new strain of the pathogen, which could be expected to occur by chance in one

location and spread slowly. Furthermore, regional variation of *C. theobromae* is low: for example, ITS sequences of *C. theobromae* from Kerala, India, had 99% identity with previously submitted sequences from Malaysia, East Java, Vietnam and Sulawesi (Table 8). Nevertheless, three regional haplotypes of *C. theobromae* were identified by Samuels et al. (2012), indicating the occurrence of some regional variability. Data on variability of the pathogen remains limited and, to date, molecular investigations of *C. theobromae* populations have been restricted to ITS and LSU sequences. In a regional collaboration involving scientists from Indonesia and Malaysia, sequencing of the *C. theobromae* genome has been completed (Ali et al. 2019) and this information will be invaluable in the future detection of genetic variants. Nonetheless, even if another virulent strain of the pathogen has emerged, a region-wide environmental change is more likely to have triggered the widespread, synchronous change in VSD symptomatology.

This study provides evidence that the pathogen (*C. theobromae*) causing the original described chlorotic leaf symptoms of VSD of cocoa is also the cause of the necrotic leaf symptoms that have become common in Indonesia and Malaysia since about 2004. Although the proportion of leaf symptoms (necrotic or chlorotic) varies between cocoa genotypes, disease resistance rankings have remained consistent. Therefore, resistance selected prior to 2004 in a clone such as PBC123 remains effective. The cause of the change of symptoms (from predominantly chlorotic to necrotic leaf symptoms) remains unknown, but is possibly due to a region-wide environmental change that has triggered a change in host response to *C. theobromae*.

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Author contribution The authors contributed as follows: field evaluations and statistical analyses in 2012, pathogen collections, culture preparation and data analysis (AKP), culture preparation and PCR analyses (APF), field evaluations and analyses in 2014 (SB), field evaluations and analyses in 2011 (EV), development of culture techniques, plant and fungal DNA extraction and PCR protocols for the study (AP), study design and supervision, field evaluations, development of direct DNA extraction method for infected plant tissues, submission of sequences, microscopic observations (PJM and PJK).

Data availability Original data and data sets analysed in this study are available by request from the corresponding author.

Declarations

Conflict of interest The authors declare no competing interests.

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