Analysis of Secondary Metabolites as Potential Phytoalexins, Their Secretion Sites and Proposed Resistance Markers to Vascular Streak Dieback in *Theobroma cacao* L.

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Abstract

Study on resistance mechanism to vascular-streak dieback (VSD) disease in cacao (*Theobroma cacao* L.) is limited due to the lack of fungal spores for artificial inoculation. This research was conducted to study the production of secondary metabolites that appear to be evidence of defense signaling in resistant clone of Sca 6 and susceptible clone of TSH 858 to *Ceratobasidium theobromae* natural infection. A fungal staining method was employed to detect *C. theobromae* hyphae at early infection stages, before VSD symptoms appear. Metabolite profiling was analyzed using pyrolysis gas chromatography mass spectrometry (Py-GCMS) at pre-, early and late stages of *C. theobromae* infection. Histochemical and anatomical characteristics of both healthy and infected leaves were also observed to identify the accumulation sites of secondary metabolites on and in cocoa leaf tissues. The results confirmed that fungal staining using trypan blue can detect early stages of *C. theobromae* infection; at the 14th week (on susceptible seedlings) and the 18th week (on resistant clones), following placement of the seedlings under infected cacao plants. Phenylpropanoid biosynthesis, terpenoid biosynthesis, environmental information processing signal transduction pathways, and aromatic biodegradation were detected as important metabolite pathways during defense mechanism. I-limonene (terpenoid), p-ethylguaiacol (phenols) and 2.3 dihidrobenzofuran (heterocyclic compounds) were proposed as an active defense produced by the host after infected by pathogen mainly on late infection of *C. theobromae*. Terpenoid and phenol compounds were accumulated on the glandular trichomes, idioblast of upper and bottom epidermis, phloem vessel and cortex idioblast of cacao leaves. Epidermis thickness of resistant clone was significantly greater than that of susceptible clone on both surfaces. Leaf epidermis tissue and the accumulated compounds in epidermis idioblast may act as the physical and biochemical markers of cocoa resistance to VSD.

Keywords: *Theobroma cacao*, fungal staining, histochemical, Py-GCMS, resistances, VSD

INTRODUCTION

Vascular streak dieback (VSD) is a major constraint of cacao (*Theobroma cacao* L.) production in Southeast Asia and Melanesia (Samuel *et al*., 2012). In Southeast Asian countries, which include Indonesia as the world third largest cocoa producer (ICCO, 2014), VSD caused 40-60% losses of the national cacao production. The occurrence

of VSD had been firstly reported in Papua New Guinea (PNG), and the disease had expanded northwestward to Kerala State in India, and Hainan Island, the southernmost of China (Keane & Prior, 1991). Wind-dispersed basidiospores of *C. theobromae* are released during periods of continously heavy rainfall and high humidity. After penetrating cacao leaf, the fungal hyphae colonize xylem vessels, causing browning of the veins of lamina; which spreads to leaf midrib and petioles, and eventually reaches the branch. Colonization by *C. theobromae* is found only in the xylem vessels of cacao (Talbot & Keane, 1971); as a characteristic that distinguishes it from other diseases caused by fungi in cacao (Samuel *et al*., 2012). The recognizable symptoms of VSD include chlorosis of the leaves, with scattered spots on the lamina remaining green (Guest & Keane, 2007).

VSD was caused by the basidiomycete fungus of *C. theobromae*, which is a member of the genus Ceratobasidium (Talbot & Keane, 1971). This fungus is an obligate parasite and cannot be grown in pure culture. The only experiment that successfully induced the sexual stage of the fungus was conducted in Malaysia using solid Corticium culture media (CCM) and liquid coconut water media, incubated at $25^{\circ}C$ under 10/14 hours of light/ dark (Lam *et al*., 1988), but this experiment could not be repeated in PNG (Dennis, 1991). Therefore, the challenge is to study the plant response to the fungus without the option of artificial inoculation.

Cacao resistance mechanism in response to *C. theobromae* infection is still unclear. Resistance evaluation and selection of plants for evaluation can only be done in a VSD epidemic area due to the lack of spores that could be used for artificial inoculation (Guest & Keane, 2007). The resistance response to VSD varies among cacao clones.

Halimah & Sri-Sukamto (2007) classified some cacao clones of Sulawesi 1, Sulawesi 2, and Sca 6 as VSD-resistant, based on a VSD-symptom-scoring method; whereas other cacao clones of ICS 60 and TSH 858, were classified as VSD-susceptible (Susilo & Anita-Sari, 2009). Other potentially VSD-resistant clones of DRC 15, KA 2-10 and VSD2Ldg were also found in Sulawesi (McMahon *et al*., 2010).

The epidemiology of VSD is controlled by a strong association between the fungus survival and environmental factors, and therefore it is necessary to study the host response to *C. theobromae* infection under natural conditions. However, it is difficult to determine early infection by *C. theobromae* based on obvious VSD symptoms. Histochemical staining of *C. theobromae* hyphae from infected leaves can be used to detect the presence of the fungus during early natural infection. The presence of *C. theobromae* hyphae may also be an indication of secondary metabolite production; which acting as phytoalexins, in response to defense signaling as part of cocoa resistance mechanism response to the pathogen.

The objectives of the research were to analyze profiles of secondary metabolites produced by *C. theobromae*-infected cacao leaves, to identify secondary metabolite production sites in the infected leaf tissues, and to identify any leaf anatomical differences as characteristics of VSD-resistant and VSD-susceptible cacao clones. This paper reported the secondary metabolites produced during *C. theobromae* infection may be as phytoalexins, histochemical analysis of secondary metabolite accumulation in leaf tissues, and leaf anatomical data. Information gathered from this study can be used for pathogenesis studies and selection tools to support breeding and development of VSD-resistant cacao.

MATERIALS AND METHODS

Two cacao clones, i.e. Sca 6 (resistant) and TSH-858 (susceptible) were used in the experiment. Seven-month-old seedlings propagated through top grafting, were grown in and outside VSD epidemic area.

The study was conducted in a VSD epidemic area, Kaliwining Experimental Station of Indonesian Coffee and Cacao Research Institute (ICCRI), Jember, Indonesia. Histo-chemical and anatomical analyses were carried out in Microtechnique Laboratory, Department of Biology, Bogor Agricultural University, Bogor, Indonesia. Secondary metabolite analysis using pyrolysis gas chromatography mass spectrometry (Py-GCMS) was carried out at the Chemical and Proximate Integrated Laboratory, Forestry Research and Development Center, Gunung Batu, Bogor, Indonesia.

The experiment used a randomized complete block design in factorial with three replications. The first factor was two cacao clones, i.e. VSD-resistant (Sca 6) and VSD-susceptible (TSH 858). The second factor was two VSD infection treatments, i.e. naturally VSD-infected cacao seedlings, placed under infected cacao trees, and non-infected cacao seedlings, placed outside the area of cacao plants at a distance of approximately 3000 m. Observation for the presence of *C. theobromae* infection and VSD symptoms was performed every two weeks using three plant samples per experimental unit. Metabolite profiling was carried out at pre-, early and late infection stages using three biological replications with two technical replications per biological replication. Phenolic, terpenoid and alkaloid histochemical staining was done using three biological replications with five observation views per replication.

Pre-infection is the pre-exposure condition, one day before seedlings are placed under infected plants, and when neither *C. theobromae*

nor VSD symptoms have yet been detected. Early infection is defined as the first time *C. theobromae* hyphae have been detected in the xylem vessel of the infected leaf, but VSD symptoms have not yet appeared. Late infection is the condition when both *C. theobromae* and VSD symptoms have been detected.

The presence of *C. theobromae* hyphae was checked every two weeks using a fungal staining method based on Liberato *et al.* (2005). Leaf midrib samples (lamina containing midrib) of 1 cm^2 were soaked in a 20 mL 1:1 (v/v) solution of glacial acetic acid and absolute ethanol in a closed container at room temperature for 24 hours. Leaf samples were cross-sectioned using a razor blade, then mounted on glass slide and given a drop of 85% lactic acid and 0.1% trypan blue. Any presence of the fungus was observed under light microscope Olympus CX21 (Olympus, Japan), and identified using species description of *C. theobromae* as done by Junianto & Sri-Sukamto (1986). The hyphae of *C. theobromae* are typically characterized by right-angle branching, and colonize only xylem vessels of cacao.

Secondary metabolite profiles were identified using a Shimadzu QP2010 (Shimadzu, Japan) Pyrolysis GCMS, and the sample preparation method was based on Chaves & Gianfagna (2007). Leaf samples were homogenized using a pestle and mortar, and a 0.002 g homogenized sample was then put into the GCMS instrument. The chemical components of the sample were identified under the following settings/conditions: pyrolysis temperature at 400°C; GC oven temperature at 50°C; injector temperature at 280°C; and interface temperature ion source at 280°C. Helium gas was used as the carrier.

The mass spectrograms were identified by matching them against those from the existing mass spectrum database provided

in the GCMS instrument, which cross-refers to the National Institute of Standard and Technology (NIST) Library, Maryland, USA. Analysis of peak area chromatogram data was performed using the One-Way Analisis of variance (Anova) and Tukey test at 5% of confidencial level. The types of metabolite were identified using the PubChem (http://pubchem.ncbi.nlm. nih.gov/) database. Metabolite pathways were developed using the pathway maps of the KEGG database (http://www.genome.jp/kegg/pathway).

Metabolite accumulation sites on and in leaf tissues were identified by histochemical test. The leaf used for sampling in the analysis was the sixth leaf back from the tip in each case. Fresh samples were cross-sectioned using a freezing microtome: a Yamato RV-240 (Yamato Kohki Ind Co, Japan) with 30 μm razor-blade thickness. Terpenoids were identified using 5% copper acetate, following a procedure suggested by Harborne (1987); phenolic compounds were identified using a 5% ferric trichloride procedure suggested by Johansen (1940). Anatomical characteristics of the cacao leaf were also investigated, based on both transversal and paradermal sections. Samples were fixed in 70% alcohol, and then cross-sectioned using a freezing microtome: a Yamato RV-240 (Yamato Kohki Ind Co, Japan) in 20 μm thickness. Paradermal sections of each leaf were prepared as semi-permanent samples using a whole-mount method first suggested by SASS (1951). The alcohol-fixed leaves were rinsed with distilled water and soaked in 50% nitric acid solution, before being rinsed again with distilled water three times. Adaxial and abaxial surface layers of leaves were peeled using a razor blade. Samples were observed under an Olympus CX-21 light microscope (Olympus, Japan), and photographed using an Optilab Advance camera (Miconos, Indonesia). Measurement of anatomical characteristics, i.e. epidermis thickness, stomatal size, stomatal density,

stomatal index, palisade thickness, glandular trichome density, glandular trichome length and glandular trichome width, was performed using Image Raster Software (Miconos, Indonesia). Stomatal index was measured based on number of epidermis divided by number of stomata.

RESULTS AND DISCUSSION

Plant disease resistance mechanisms involve activation of a series of defense pathways as part of the defense mechanism against pathogen attack. Plants have a regulatory mechanism to activate particular defense responses according to the specific stimuli received (Chaves & Gianfagna, 2007). Phytoalexin is a chemically diverse group of secondary metabolites with strong antimicrobial actions, and these metabolites accumulate around the infection site. They are generally undetectable in the plant before infection, but are synthesized very rapidly after pathogen attack (Taiz & Zeiger, 2010).

Fungal staining successfully detected the early infection stage of *C. theobromae* in VSD-susceptible and VSD-resistant plants, at the 14th and 18th weeks, respectively, after cacao seedlings had been placed under VSD-infected cacao plants. Susceptible clone (TSH 858) was infected faster than resistant clones (Sca 6). Although the hyphae had been detected in the xylem vessels, the leaves appeared normal and healthy, exhibiting no visible symptoms of VSD (Figure 1 & 2). The VSD symptoms of leaves used in this study were interpreted according to observed leaf chlorosis with green spots, as described by Guest & Keane (2007).

During onset of *C. theobromae* infection, cacao leaves produced 20 different metabolites. Chromatogram areas from Py-GCMS analysis of both VSD-resistant (Sca 6) and VSD-susceptible clones (TSH 858) were not statistically different at the pre-infection
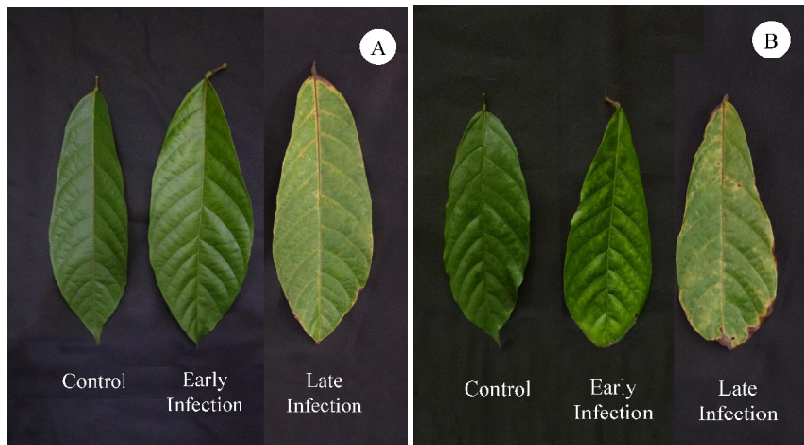
Figure 1.   Morphological symptom on VSD-susceptible clone of TSH 858 (A) and VSD-resistant clone of Sca 6 (B) at the three infection stages of *C. theobromae*
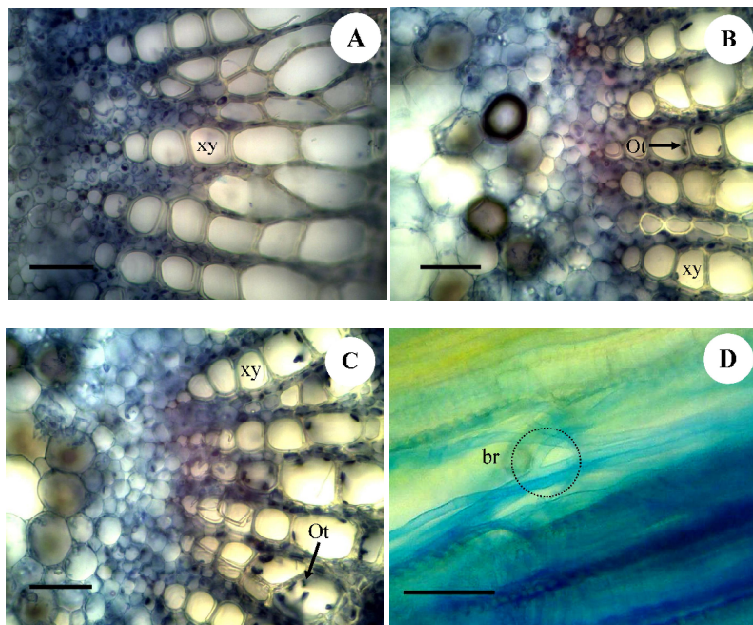

Figure 2.   Fungal staining by trypan blue for detection of *C. theobromae* presence. Midrib cross-sections at pre-infection stage (A), early infection stage (B), and late infection stage (C). Midrib longitudinal section: typical branching of *C. theobromae* hyphae at right angles (D) (xy = xylem, Ot = *C. theobromae* hyphae, br = hyphae typical branching). Scale bars = 50 µm (A-C); 30 µm (D)

stage (Table 1). There was then an increase in production of I-limonene (a terpenoid compound) and p-ethylguaiacol (a phenolic compound) in both clones at the early stage of *C. theobromae* infection, and again, there was no significant difference between the VSD-resistant and VSD-susceptible clones (Table 1). However, at the late infection stage, production of I-limonene, p-ethylguaiacol, and 2,3 dihidrobenzofuran (terpenoids), were significantly higher in resistant clones than in susceptible clones (Table 1).

Early infection defined as the first time *C. theobormae* hyphae detected in the xylem vessel of infected leaf, but VSD symptoms not yet appear. Early infection occurred in week 14th (TSH 858) and week 18 th (Sca 6) following placement of cacao seedlings under VSD-infected cacao plants. Control was cacao seedlings of the same age, placed outside area of infected cacao plants.

Late infection is defined as when both *C. theobormae* and VSD symptom have been detected. Late infection in TSH 858 and Sca 6 occurred at 15th and 19th weeks respectively, after cacao seedlings placed under VSD infected cacao plants. Control was cacao seedlings of the same age placed outside area of infected cacao plants.

In the early stage of *C. theobromae* infection there was increasing production of I-limonene and p-ethylguaiacol in both VSD-resistant and VSD-susceptible clones, with no significant difference between the two types of clone at this stage (Table 1). This might be due to similar basic responses of both VSD-resistant and VSD-susceptible cells to the infection, and similar rates of phytoalexin formation (Kuc, 1992). Increased production of I-limonene and p-ethylguaiacol, as well as 2,3 dihidrobenzofuran, occured more significantly in the VSD-resistant clones at the late infection stage (Table 1). It is suggested that I-limonene, p-ethylguaiacol

and 2,3 dihidrobenzofuran could be considered as indicators of a triggered defense signaling mechanism, and are produced by host plants induced by *C. theobromae,* mainly in late infection stage. I-limonene, p-ethylguaiacol, and 2,3 dihydrobenzofuran were synthesized during *C. theobromae* infection stages through different metabolite pathways. I-limonene is a monoterpenoid that is synthesized through methyl erythritol phosphate (MEP) in the terpenoid backbone metabolism.

Limonene has been reported as a monoterpenoid that exhibits an antifungal action that is similar to the commercial fungicide, carbendazim. Limonene and carbendazim have been tested against *Rhizoctonia solani*, *Fusarium oxysporum*, *Penicillium digitatum* and *Aspergilus niger* (Marei *et al.*, 2012). Fungi produce a cellulase enzyme that can degrade cell walls during pathogenesis (Milling & Richardson, 1995), and it was reported that limonene has a strong inhibitory effect, similar to that of commercial fungicides, on cellulase activity (Marei *et al.*, 2012).

Monoterpenoid is a type of metabolite product synthesized in large quantities through a signal transduction pathway, as a result of jasmonic acid activity (KEGG, 2015). Jasmonic acid is a signal molecule that induces a signal transduction pathway, which leads to the production of phytoalexins as part of the resistance mechanism of *Glycine max,* in response to *Rhizoctonia solani* infection (Aliferis *et al.,* 2014).

Phenolic compound p-ethylguaiacol is synthesized through phenylpropanoid pathways. Secretion of the compound from plants is induced by wounds and is involved in the communication and defense systems during Agrobacterium and Rhizobium infection (Bhattacharya *et al.*, 2010). Accumulation of phenolic compounds associated with a hypersensitive response by a plant to pathogen infection has been found in coffee plant

Table 1. Cacao metabolite profiles based on chromatogram peak area in pre-infection, the early stage of infection, and the late stage of infection by *C. theobromae*

RT	Metabolites	% from total ion chromatogram			% from total ion chromatogram					% from total ion chromatogram				
		Resistant clone Sca 6	Susceptible clone TSH 858	P Value	Resistant clone Sca 6		Susceptible clone TSH 858		P Value	Resistant clone Sca 6		P Value	Susceptible clone TSH 858	
					Control	Early Infections	Control	Early Infections		Control	Late infections		Control	Late infections
3.608	Methyl acetate*	3.06	3.39	0.608	1.37 b	1.03 b	2.39 a	2.00 ab	0.047	1.44 a	1.52 a	0.155	2.17 a	2.28 a
4.082	Diacetyl	1.10	1.60	0.480	2.00 a	1.08 a	1.82 a	1.54 a	0.426	1.06 a	1.37 a	0.231	2.16 a	1.03 a
4.275	2-Methylfuran*	-	-	-	0.83 a	1.12 a	0.71 a	0.93 a	0.227	1.07 a	0.92 a	0.502	0.70 a	0.91 a
4.802	Ethylic acid	8.68	8.35	0.863	20.37 a	20.20 a	15.71 ab	12.70 b	0.032	16.66 a	15.55 a	0.01	8.99 ab	5.68 b
5.342	Acetol	3.83	3.90	0.893	3.13 a	5.24 a	4.93 a	4.29 a	0.548	2.23 a	2.25 a	0.005	2.06 a	1.04 b
12.543	I-limonene	-	-	-	0.73 b	2.14 ab	0.89 b	5.60 a	0.001	0.00 c	4.25 ab	0.009	2.02 b	7.60 a
13.066	Corylon	1.34	1.10	0.193	1.88 a	2.20 a	1.63 a	1.65 a	0.722	1.53 a	1.40 a	0.124	1.31 a	1.10 a
13.658	p-Methoxyphenol	6.25	6.80	0.634	5.69 a	5.08 a	5.34 a	5.60 a	0.852	5.06 ab	3.98 b	0.022	7.00 a	4.96 ab
14.144	2-N-Propyl- Oxetan	2.99	3.14	0.544	-	-	-	-	-	3.04 a	2.94 a	0.857	2.81 a	2.48 a
14.863	2-Methoxy-4-methylphenol	2.68	2.91	0.435	2.49 a	2.38 a	2.46 a	2.84 a	0.687	2.10 a	1.97 a	0.199	2.73 a	2.50 a
15.508	2,3 dihydrobenzofuran	0.91	0.89	0.932	2.35 a	2.09 a	2.20 a	1.13 a	0.596	0.70 b	1.62 a	0	1.37 a	0.00 c
15.712	p-ethylguaiacol	3.37	3.34	0.881	0.00 b	2.50 a	2.78 a	3.17 a	0.006	0.70 b	1.80 a		1.94 a	1.99 a
15.664	2,5 Dimethoxytoluene	-	-	-	1.79 a	1.77 a	0.71 b	0.71 b	0.000	3.73 a	0.99 a	0.103	3.98 a	1.71 a
16.128	2-Methoxy acetophenone	4.97	4.97	0.979	6.31 a	5.14 ab	1.89 b	2.86 ab	0.107	1.23 b	2.34 a	0.06	2.43 a	2.01 ab
16.492	2,6-Dimethoxyphenol	4.60	4.36	0.719	5.46 a	5.79 a	4.92 a	5.34 a	0.394	4.82 a	4.99 a	0.323	5.21 a	3.85 a
17.344	Eugenol	4.06	4.37	0.727	2.61 ab	2.79 a	2.46 ab	2.24 b	0.020	6.38 a	6.97 a	0.417	4.99 a	4.86 a
17.925	Toluene, 3,4,5-trimethoxy	1.37	0.89	0.088	1.91 a	2.16 a	1.63 a	2.04 a	0.627	2.61 a	1.60 a	0.144	1.90 a	1.64 a
19.352	4-Allyl-2,6-dimethoxyphenol	2.12	1.98	0.493	2.86 a	3.71 a	2.70 a	2.21 a	0.130	3.26 a	2.26 ab	0.042	2.20 ab	1.29 b
19.889	Phytol	1.15	0.70	0.638	2.51 a	1.32 a	1.98 a	1.62 a	0.544	1.08 a	1.08 a	0.909	1.08 a	0.88 a
20.888	Palmitic acid	2.69	2.41	0.644	1.35 b	1.19 b	1.77 ab	2.18 a	0.060	1.25 a	1.27 a	0.756	1.61 a	1.36 a

Note: Figures in the same line followed by the same letter are not significantly different, based on Tukey test $\alpha = 5\%$; *Data were transformed by $(\sqrt{\bar{x}+0.5})$, where x = chromatogram peak area of x metabolite, RT = retention time (minutes); pre infections = one day before cacao seedlings placed under cacao trees infected by VSD.

defense mechanism against *Colletotrichum kahawae* (Loureiro *et al.,* 2012).

2,3-dihydrobenzofuran is a heterocyclic compound that is synthesized through degradation of certain aromatic compounds (KEGG, 2015). Asperfuran is an example of an antifungal 2,3 dihydrobenzofuran produced by a strain of *Aspergillus oryzae.* Asperfuran inhibits chitin synthase produced by *Coprinus cinereus* (Pfefferle *et al*., 1990). Chitin is a major component of fungal cell walls, which play a vital role in hyphal tip growth and fungal morphogenesis. Chitin is synthesized by chitin synthase and it is well known that the latter is inhibited by commercial fungicides (Kong *et al*., 2012). The 2,3 dihydrobenzofuran compound has also been reported to be produced by *Bauhenia purpurea* and shows antifungal activity against *Candida albicans* (Abyaneh & Rai, 2013).

Role of terpenoid and phenolic compounds in cacao resistance mechanism to pathogens have been reported as phytoalexins in respect of *Verticillium dahlia* infection. Production of triterpenoid arjunolic acid and the phenolic compounds 3,4 dihydroxyacetophenone and 4-hydroxyaceto-phenone, significantly increases in cacao infected by *Verticillium dahlia* (Resende *et al*., 1996). In addition to these terpenoid and phenolic phytoalexins, elemental sulphur is produced as an antifungal substance, in cacao during *Verticillium dahlia* infection (Cooper & Williams, 2004; Resende *et al.*, 1996).

Metabolite Accumulation Sites

Metabolite accumulation sites were found in healthy leaves of both VSD-resistant and VSD-susceptible clones at pre-infection stage. Both clones had leaf laminae consisting of single-layered epidermis tissue with idioblast cells containing phenolic and terpenoid compounds in the upper and lower epidermises (Figures 3B & 3D). Phenolic compounds were also detected in palisade tissue (Figure 3A). Glandular trichomes containing phenolic and terpenoid compounds were distributed across both cacao leaf surfaces, although mainly on the leaf midrib (Figures 3C & 3E). Phenolic compounds also appeared in numerous idioblast cells in the cortex of the midrib (Figures 4A, 4B, & 4C).

Similar accumulation sites were found in the leaves of VSD-resistant and VSD-susceptible clones during the infection stages of *O. theobromae*. Histochemical staining showed that phenolic and terpenoid content of the phloem in VSD-resistant clones was higher than that of susceptible clones. This was indicated by increasing intensity of dark brown color of the phloem at the time of infection (Figure 5, Table 4).

The secretion of terpenoid and phenolic compounds during *C. theobromae* infection involves phloem tissues. The role of phloem in the secretion of phenolic compounds has been reported in the case of *Picea abies* infected by *Ophiostoma polonicum* (Brignolas *et al.,* 2007). It is understandable that phenolic and terpenoid contents in the phloem of VSD-resistant clones were higher than in susceptible clones. This might be related to the secretion of the compounds during the infection stage. Cacao leaves have glandular and non-glandular trichomes (Nakayama *et al*., 1996). In some plants, glandular trichomes have a role as physical and chemical barriers to spores (Martin & Clover, 2007; Chattopadyay *et al*., 2011). However, there is lack of study findings regarding the metabolite content of cacao glandular trichomes. This research showed that glandular trichomes of cacao, containing phenolic and terpenoid compounds, were distributed on both surfaces of cacao leaves, mainly on the leaf midribs. However, the trichomes were generally more abundant on the abaxial than on the adaxial

leaf surface. Cacao glandular trichomes are of the multiseriate capitate type: composed of a basal cell, two-cell uniseriates and eight cells making up the secretory head. The distribution of glandular trichomes on both VSD-resistant (Sca 6) and VSD-susceptible clones (TSH 858) did not differ statistically, suggesting that the glandular trichome is not closely related to cacao resistance mechanisms to VSD.
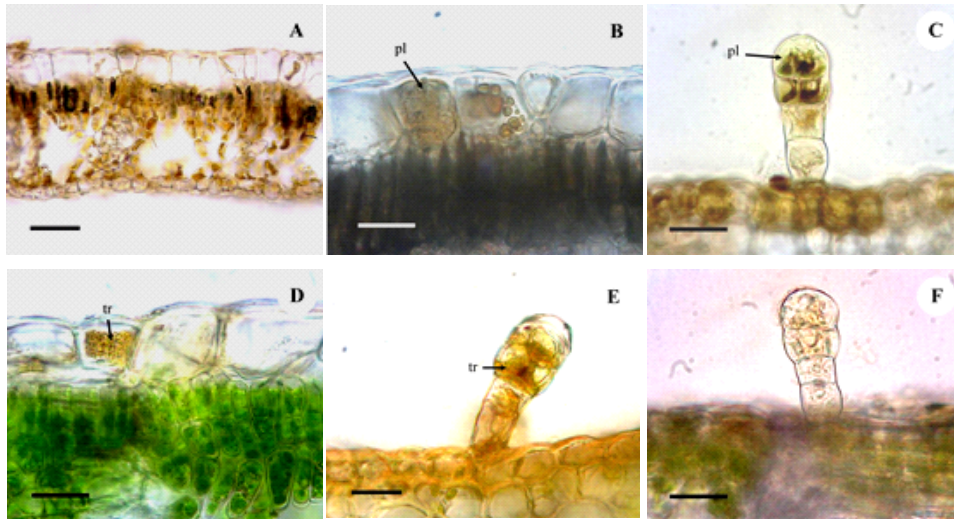

Figure 3.   Histochemical staining of cacao leaves. Phenolic compounds detected in palisade tissue (A), idioblast epidermis (B) and glandular trichomes (C). Terpenoids compounds detected in idioblast epidermis (D) and glandular trichomes (E). Glandular trichomes in water as control (F). (pl = phenolic compounds, tr = terpenoids). Scale bar: 50 μm (A), 20 μm (B - F)

Tabel 2.   Secondary metabolite production sites during *O. theobromae* infection, based on histochemical staining

Cell/tissue	Pre infection				Early infection				Late infection			
	Sca 6		TSH 858		Sca 6		TSH 858		Sca 6		TSH 858	
	P	T	P	T	P	T	P	T	P	T	P	T
Glandular trichomes	+	+	+	+	+	+	+	+	(+)	(+)	(+)	(+)
Idioblast of upper epidermis	+	+	+	+	+	+	+	+	+	+	+	+
Idioblast of lower epidermis	+	+	+	+	+	+	+	+	+	+	+	+
Palisade tissue	+	-	+	-	+	-	+	-	+	-	+	-
Phloem vessels	++	+	++	+	+++	+++	+	++	+++	+++	++	+++
Idioblast of cortex	+	+	+	+	+	+	+	+	+	+	+	+

Note: P = phenolic compounds; T = terpenoids; - = not detected, + = fairly detected, ++ = moderately detected, +++ = strongly detected; (+) = lysis.
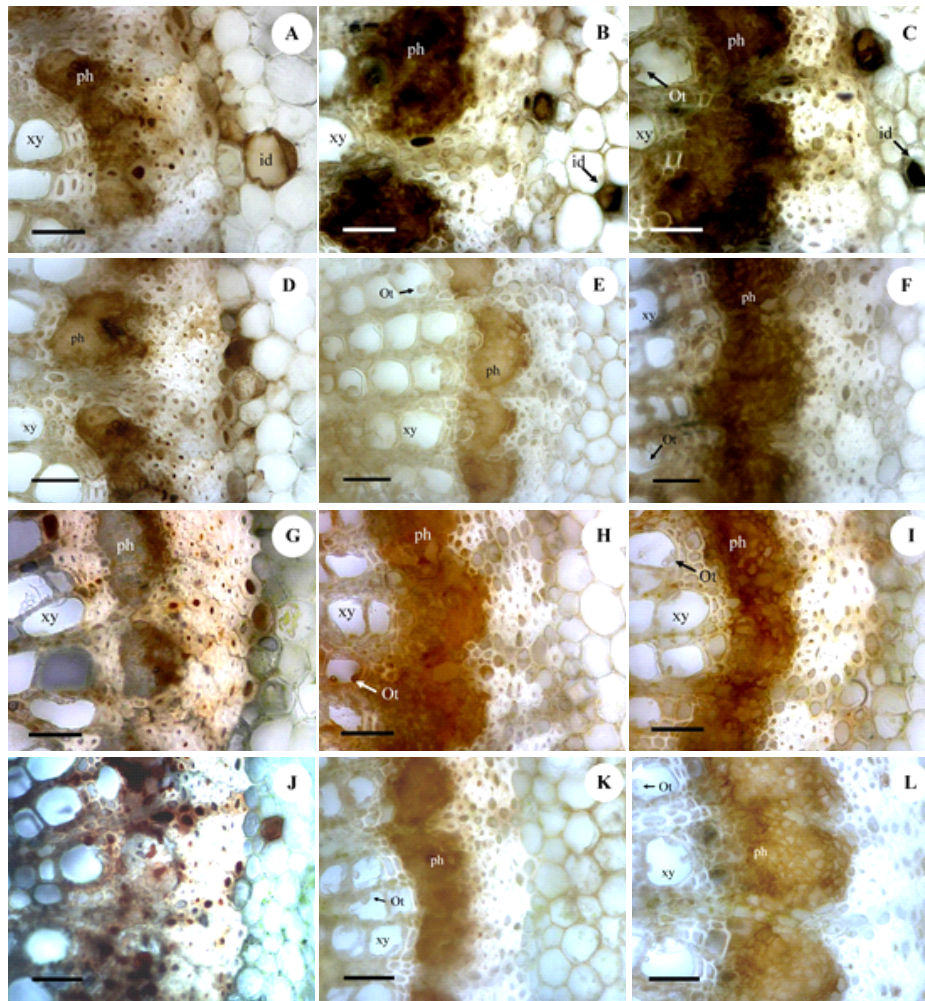
Figure 4.   Histochemical staining of phenolic compounds (A-F) and terpenoids (G-L) in cacao leaf midrib of Sca 6 (A-C and G-I) and TSH 858 (D-F and J-L) showing positive results in phloem during pre-infection (A, D, G, J), early infection (B, E, H, K) and late infection (C, F, I, L). (xy = xylem; ph = phloem; id = idioblast; Ot = *O. theobromae* hyphae). Scale bar: 50 μm (A-L)

Epidermis as a Barrier

The leaf epidermis tissue of VSD-resistant clones was significantly thicker than that of susceptible clones, for both leaf surfaces (Table 3). However, size and density of stomata, as well as the distribution of glandular trichomes, were not significantly different between VSD-resistant and VSD-susceptible clones. Interestingly, stomatal pores on the VSD-susceptible clones were found to be wider than those of resistant clones.

Pathogenesis caused by infectious agents begins when the pathogen contacts host plants. Certain anatomical characteristics of leaves can act as physical barriers to pathogens penetrating a host plant. Most pathogens gain access to the plant interior through natural openings such as stomata (Agrios, 1988).

The VSD-resistant and VSD-susceptible clones were not significantly different in terms of stomatal density, length and width. The study suggested that these stomatal characteristics were not directly related to cacao resistance mechanisms to VSD. This finding differs from a previous study that reported a positive association between stomatal density and cacao resistance to VSD (Susilo & Anita-Sari, 2009). However, our research found that the stomatal pores of susceptible clones were wider than those of resistant clones. We suspect that pathogens have more opportunities to penetrate susceptible than resistant plants through the former wider stomatal openings.

A previous study explained that hyphae penetrated young leaves by growing directly through cuticle layers into mesophyll tissues (Prior, 1979). This study found that cacao leaves have a very thin cuticle layer, even on the leaves of resistant clones. Our result offers the possible explanation that physical and biochemical resistance mechanisms to VSD infection may involve leaf epidermis tissue characteristics. The leaf epidermal tissue, on upper and lower surfaces, of resistant clones was significantly thicker than that of susceptible clones on both surfaces. In addition, our results also confirmed that phenolic and terpenoid compounds accumulated on both surfaces of the leaf epidermis. Phenolic compounds that accumulated in the idioblast of epidermis tissue may be involved in biological interaction between cacao and pathogens. Epidermis tissue plays a role in the penetration by pathogens of the host plant through cell wall degradation (Agrios, 1988). Therefore, we propose that epidermis characteristics and the compounds accumulated in the epidermis, be considered as anatomical and biochemical markers of cacao resistance to VSD.

Table 3. Anatomical characters of the leaves of *Theobroma cacao* clones Sca 6 and TSH 858

Anatomical characteristics	P Value	Cocoa clone	
		Sca 6	TSH 858
Upper epidermis thickness, μm	0.000	22.616 a	16.953 b
Lower epidermis thickness, μm	0.000	10.448 a	8.679 b
Palisade tissue thickness, μm	0.000	22.335 b	27.018 a
Density of adaxial glandular trichomes, mm^{-2}	0.271	1.897 a	1.743 a
Density of abaxial glandular trichomes, mm^{-2}	0.278	5.282 a	4.974 a
Length of glandular trichomes head, μm	0.314	28.705 a	32.688 a
Width of glandular trichomes head, μm	0.308	26.700 a	29.057 a
Stomatal density, mm^{-2}	0.184	1138.5 a	1076.9 a
Stomatal index	0.007	14.842 a	13.380 b
Length of stomata, μm	0.063	12.410 a	12.730 a
Width of stomata, μm	0.083	13.764 a	14.120 a
Length of stomatal pore, μm	0.000	4.950 b	5.972 a

Note: [*)]Number in the same row followed by the same letter are not significantly different, based on T-test $\alpha = 5\%$.

CONCLUSIONS

I-limonene, p-ethylguaiacol and 2,3 dihidrobenzofuran may act as potential phytoalexins produced by the host after infected by pathogen mainly on the late infection of *C. theobromae*. Terpenoid and phenol compounds were accumulated on idioblast of upper and bottom epidermis of cacao leaves. Epidermis thickness of resistant clone was significantly greater than that of susceptible clone on both surfaces. Leaf epidermis tissue and the accumulated compounds in epidermis idioblast are proposed as a physical and biochemical markers of cocoa resistances against VSD disease.

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