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Starch Catabolism Revealed During Secondary Metabolite Released on Vascular Streak Dieback Infected Cocoa (*Theobroma cacao* L.)

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Abstract

Vascular streak dieback (VSD) pathogen may altered some metabolisms in cocoa physiology. This study aimed to investigate the profile of starch content in cocoa leaf and phytoalexin production based on gas chromatography-mass spectrophotometry (GC-MS) analysis at several phases of VSD pathogen infection. Research was conducted at Kaliwining Experimental Station, Indonesian Coffee and Cocoa Research Institute, Jember, East Java. The research was designed by two factors completely randomized block design with three replications. The first factor was clone type based on the level to VSD infection. The resistant and susceptible clones, respectively i.e. Scavina 6 and TSH 858, were used in this study. The second factor was the stage of *Oncobasidium theobromae* infection, i.e. preliminary-, early-, and later-stages. The decrease in the starch content on VSD infected cocoa of Scavina 6 was higher than TSH 858. This indicated a higher starch catabolism rate on the resistant clone. Some secondary metabolites were released during VSD infection, i.e. l-limonene, eugenol and coumaran. Additionally, Scavina 6 clone showed a higher concentration of eugenol and coumaran, on the other hand TSH 858 showed a higher for l-limonene. L-limonene concentration increased in line with the severity of pathogen infection. There were a negative correlation between starch content with l-limonene, eugenol, and coumaran concentrations.

Keywords : cocoa, VSD, secondary metabolite, starch

INTRODUCTION

Vascular streak dieback (VSD) which is caused by *Oncobasidium theobromae* is a main disease in cocoa plantation in Indonesia. VSD disease has spread in more than 20 provinces in Indonesia and potentialy decreasing up to 40% of cocoa production (Halimah & Sukamto, 2007). Like other vascular pathogens, *O. theobromae* colonizes xylem vessels, impairs water transport and causes wilting in some branches or throughout the shoot. *Oncobasidium theobromae* which causes VSD disease is an obligate parasitic fungus where its uses in the research still limited only by artificial inoculation. Recently, the method of VSD inoculation developed was natural inoculation where the healthy plant can be infected immediately from the diseased plant.

The phase of *O. theobromae* attack can be monitored based on the visual symptoms on infected leaves. The common symptoms

of VSD infection was leaf chlorosis with three yellowing spots in leaf petiole. Generally, leaf defoliate after a few days of chlorosis symptoms appeared in the leaves. In a severe phase of infection, *O. theobromae* damages the main stem and causes death especially for susceptible cocoa clones. In addition to visual symptoms, the method of fungal staining had been developed to prove the presence of fungi in the plant tissue and it was also a method to determine the level of *O. theobromae* infection. Detection of *O. theobromae* according to preliminary-, early- and later-stage of infection was conducted by using the fungal staining method refer to the sample preparation method by Santoso *et al.* (2017).

The information regarding to the mechanism of cacao resistance to VSD is limited. The cocoa plant response to pathogen attack is a complex mechanism, involving several defense strategies and different biochemical pathways. The interaction of pathogens directly affects the metabolism of the host plant, which reorganizes its primary metabolism to create defense mechanisms. The mechanism of plant resistance to a pathogen may involve the activation of several pathways of metabolites which are expected to be able to retard the rate of pathogen infection. Primary and secondary metabolite compounds involved in the mechanism of plant resistance to pathogens can be classified as regularly produced compounds and only post pathogen attacks.

The infection of pathogen caused physiological changes in cocoa plants, namely, a decrease in photosynthesis, transpiration, stomatal conductance, increased respiration and CO_2 concentration in the leaves (Santoso & Zakariyya, 2019), which can alter the metabolism of plant. The changes in primary metabolites can be demonstrated through starch content in plants. Starch is a form of carbon and an energy source for plants. As a response of pathogen to infection, the respiration of plants will increase and starch will be degraded as energy for plant. Starch degradation will provide a carbon source for synthesizing sucrose and some volatile compounds (Saraiva *et al.*, 2017). When plant was infected by VSD pathogen, volatile compounds produced as a secondary metabolites and had a role as phytoalexin (Santoso *et al*., 2017).

Phytoalexin are compounds that inhibit the development of pathogen which is not activated until there is a contact between host plant and parasitic organisms. Phytoalexin accumulates rapidly as hypersensitive response of plant to pathogens. Generally, volatile phytoalexin compounds produced during an attack are derived from phenols, alkaloids and terpenoids (Sahebi *et al*., 2017). Eugenol is phenolic group that show a good antifungal activities against fungi (Xie *et al.*, 2017). Limonene is monoterpene group that resulted in inducing resistant againts anthracnose in *Arabidopsis thaliana* (Fujioka *et al.*, 2015). Coumaran was reported by Santoso *et al.* (2017) playing a role as phyto-alexin against *O. theobromae*. The release of the secondary metabolites involved in the resistance mechanism required an adequate source of ATP and carbon. This research purpose was to study the profile of starch content in cocoa leaf and phytoalexin production based on GC-MS analysis on phase of VSD pathogen infections.

MATERIALS AND METHODS

Research was conducted at Kaliwining Experimental Station of Indonesian Coffee and Cocoa Research Institute, Jember, East Java. The research was located at an altitude of 45 m above sea level with climate type D based on Schimdt & Ferguson (1951). Secondary metabolites were tested by using gas chromatography-mass spectrophotometry (GC-MS) that was conducted in Integrated Laboratory

of Forestry Development Research Center, Gunung Batu, Bogor. Starch content was tested in the Laboratory of Jember State Polytechnic. Histochemical test for starch detection inside plant tissues was conducted in the Micro-technical Laboratory and Physiology Laboratory, Department of Biology, IPB University.

The research used seven months old of cocoa seedlings derived from top grafting. Cocoa seedlings represented resistant clone to VSD disease, namely Scavina 6 and a susceptible clone, namely TSH 858 clone. The research was designed based on a two factors of completely randomized block design with three replications. The first factor was clone, i.e. the resistant clone (Scavina 6) and susceptible (TSH 858) to VSD infection (Halimah & Sukamto, 2007). The second factor was the stage of *O. theobromae* infection, i.e. preliminary-, early-, and later-stage of infections.

On the histochemical and leaf anatomy observation, the fresh leaf sample were cut transversal section refer to semi-permanent preparation (Sass, 1951). The anatomical characteristic observation includes the petioles of leaf at the end of observation. The measurement of anatomical characteristics was conducted using the image raster software (Macinos, Indonesia). The documentation used an instrument of microscope photo (Optilab Advance–Macinos, Indonesia).

The concentration of eugenol, l-limonene and coumaran were analyzed at three stages of *O. theobromae* infection, namely pre-infection, early-infection and late-infection stages. The secondary metabolite profile was identified using GC-MS Pyrolysis (Shimadzu GCMS-QP2010). The sample of leaf stored at -80ºC using dry ice refer to Chaves & Gianfagna (2007). The results of the GC-MS in form of relative concentrations were were identified by matching them against those from the existing mass spectrum database provided in GC-MS.

The starch content in the stages of pre-infection, early-infection and late-infection of *O. theobromae* was tested refer to Luff Schoorl method (ISI 2002; Apriyantono *et al*. 1989). Three grams of leaf sample was grounded and put into a 500 mL erlenmeyer glass. The Luff Schoorl method began with the hydrolysis of starch (polysaccharides) to reducing sugars (monosaccharides) using acidic solutions. Samples in the erlenmeyer glass were added 200 mL of 3% HCl, then heated for 3 hours and then cooled. The solution was added with 4N NaOH until it was neutral and then added with 1 mL of acetic acid (CH_3COOH). The solution was then added with distilled water until it reached a volume of 500 mL. It was then filtered and 25 mL of filtrate was put into the erlenmeyer glass. The filtrate was added with 25 mL of reagent Luff (CuO). The solution was heated using a hot plate until a red brick precipitation was formed. After cooling, the solution was added 10 mL of 30% KI and 25 mL of 4N H_2SO_4. The excess CuO was reduced with KI, and iodine was released. The released iodine was titrated with Na-thiosulfate solution until the color of solution became clear. The titration stage also required blanks. Blank that used was 25 mL of Luff-Schoorl solution with 25 mL of distilled water. The data was analyzed by ANOVA test and if there was any significant differences ($P < 0.05$), it was then analyzed by using Tukey ($\alpha = 5\%$).

RESULTS AND DISCUSSION

Oncobasidium theobromae infects cocoa plants through leaves and branches, through stomata and lenticell. The inocula as basidiospores are released in the wet season during high humidity and infect young leaves. Fungi formed colonies in xylem vessels after infection and damaged the leaf vessel tissue.

In the advanced stage of infection, the fungal hyphae has formed a colony which results in a blockage in the xylem column. Blockage the xylem column by hypha *O. theobromae* results in the appearance of chlorosis symptoms in the leaves. Fungal colonization is important stage to be passed by a pathogen in its life cycle before entering the pathogen dissemination stage. The formation of fungal colonies in the xylem column is the main marker character that distinguishes *O. theobromae* from other fungi that attack cocoa (Samuel *et al.*, 2012).

The results of analysis of variance were presented in Table 1. It was demonstrated that there were interaction between clones and infection stage. Meanwhile, the single factor of clones affected on l-limonene, eugenol and coumaran content. Infection stage of pathogen affected on l-limonene. Primary metabolites, especially starch content, were significantly altered due to pathogen infection in leaf tissue (Trapero *et al*., 2018). In addition, Prawoto *et al*. (2013) stated that secondary metabolites, polyphenol and terpenoid compound played important role in cocoa resistance to vascular streak dieback disease.

Starch is the primary metabolism compound that had benefit to plant as an energy. Plant store most abundant carbohydrates in form of starch. Its metabolism and function depends upon the cell-type from which it is derived. In the leaves, starch typically accumulates gradually during the day using a fraction of the carbon assimilated through photosynthesis. The mobilization of starch in the leaf of cocoa was also investigated through histochemical analyses at different pathogen infection stages (Figure 2.). All images were from transversal sections through leaf midrib. There were remarkable differences during infection stages with regard to the accumulation of starch granule location and rate of degradation. The type of starch on this research was amylose. According to Jackson (2003), the blue color staining indicated that there was reaction between amylose and iodine.

In pre-infection, the starch granule from resistant and succeptible clones embedded in a densely stained in the pith of leaf midrib. The starch granule form of Scavina 6 clone (resistant clone) was more dense than TSH 858 (succeptible clone). In early pathogen infection stage, starch was more degraded in line with the fungus infection stage. Starch granule was more limited in pith cell and more accumulated in cells near the xylem of leaf. At the advanced infection, starch granule was found lesser both in the pith area and cells near to xylem. Sinaga (2003) stated that soluble starch and nutrition was accumulated around infection area. This was proved that there was translocation of nutrient and starch from host to pathogen. The condition was also needed by pathogen to acquire nutrient from plant host. In the infected area, the metabolism was increased, included the increasing of respiration during the infection stages.

A quantitative Luff Schoorl test showed that starch content decreased in line with the infection stages of pathogen (Figure 2.). There was an interaction between treatment of clones and phase of *O. theobromae* infection.

Table 1. Analysis of variance of starch content, l-limonene, eugenol, and coumaran concentration as affected by clones and infection stage of *O. theobromae* and their interaction

Variabel	Clones	Infection stage	Interaction
Starch content	69.07 **	402.37 **	93.28 **
l-limonene	7.39 *	17.72 **	2.18 ns
Eugenol	5.48 *	3.04 ns	2.05 ns
Coumaran	5.53 *	2.47 ns	1.83 ns

Notes: *,** = Significant at the 0.05 or 0.01 levels of probability negatively; ns = non significant.

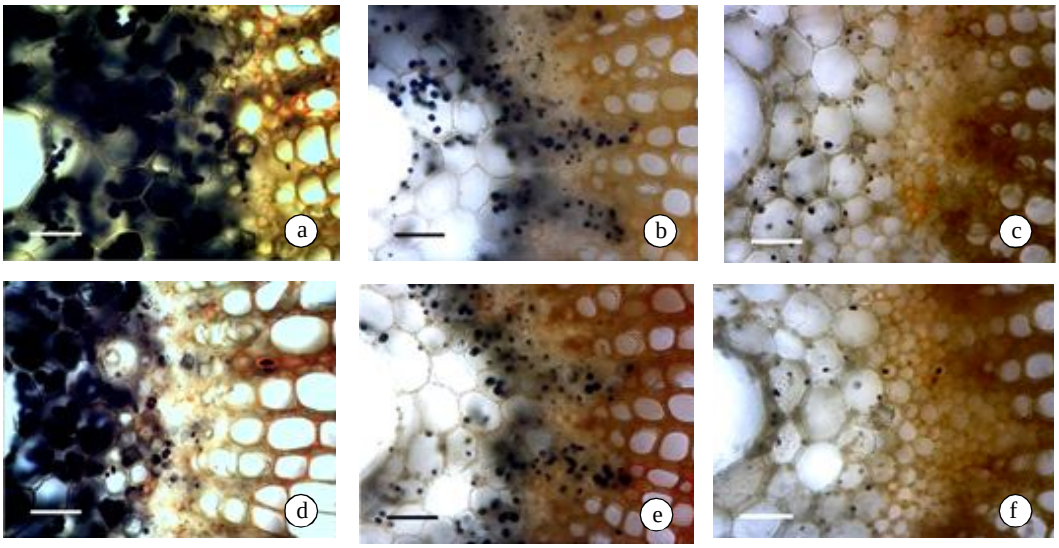

Figure 1. Starch content in pith of leaf midrib of Scavina 6 clone (resistant clone) at (a) healthy condition, (b) early infection stage, (c) late infection stage; and TSH 858 at (d) healthy condition, (e) early stage of infection, (f) advanced stage of infection. (xy = xylem, p = pith, ot = hyphae of *O. theobromae*); Bar scale = 50 µm (a-f)

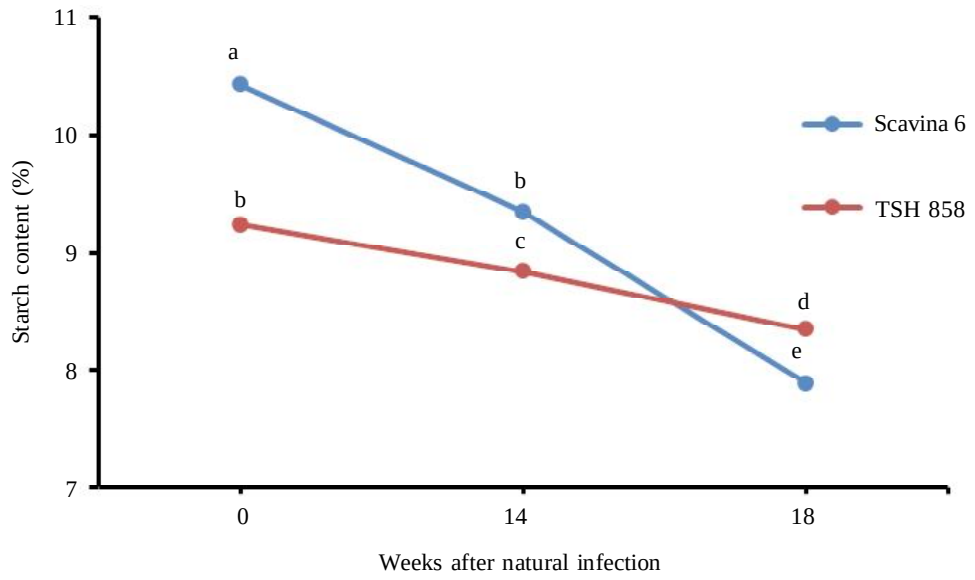

Figure 2. Total starch content of Scavina 6 and TSH 858 clones in preliminary-, early-, and advanced-stage of *O. theobromae* infections

Starch was degraded significantly on both clones in line with phase of pathogen infection. In healthy condition (no infection), until early-stage of infection of pathogen, Scavina 6 (resistant clone) had starch content higher than TSH 858 (susceptible clone). On the other hand, at the later-stage of infection, starch content in resistant clone was lower than susceptible clone. Starch content in Scavina 6 in advanced-stage of infection was 24.33% lower than healthy condition (no infection), however, starch content in TSH 858 in later stage of infection was 9.63% lower than healthy condition (no infection). This indicated that starch degradation rate on resistant clone was higher than susceptible clone.

The degradation of starch during pathogen infection was also reported by Gamm *et al.* (2011). The degradation of starch under pathogen infection was caused by respiration activity in plant tissue to produce other compund that play an important role in defense mechanisms of plant against infection (Agrios, 2005). The response in degrading starch on resistant clone was faster than susceptible clone. It indicated that the metabolism activity, especially respiration in resistant clone was higher than susceptible clone. Gortrari *et al.* (2018) reported that respiration rate of *Populus deltoides* Bartr. ex Marsh that infected by pathogen was increased up to 80%. Further information was demonstrated by da Silva *et al.* (2017) that respiration in resistant and susceptible clone of Eucalypt plant increased under *Ceratocystis ûmbriata* infection, however, the increment of respiration rate in resistant clone was higher than susceptible clone.

Respiration is a source of various biochemical activities in plants including the activation of secondary metabolite compounds (Major *et al*., 2010). The results of carbon metabolism from respiration are intermediate compounds in the formation of phenol group compounds through the shikimic acid pathway and malonic acid pathways as well as the formation of terpenoid group compounds through the mevalonic acid pathway and the methyl erythtritol phosphate pathway (Taiz & Zeiger 2010).

Secondary metabolites status that have represented a group of secondary metabolites had been analyzed at the preliminary-, early-, and advanced-stage of *O. theobroma* infection by GC-MS. Commonly, metabolites are grouped according to the type of chemical compound, its function and biosynthetic pathway. Single factor clone was affected by limonene, eugenol, and coumaran (Table 2). In this research, phenolic compound investigated were eugenol, meanwhile, terpenoid compound was l-limonene. Scavina 6 had higher concentration of eugenol and coumaran than TSH 858. However, for limonene compound, TSH 858 had higher concentration than Scavina 6. Limonene concentration was released higher in susceptible clone than resistant clones. This shows that limonene is a response to pathogen attack, whereas eugenol and coumaran are plant mechanisms against pathogens. In the infection stages, limonene concentration was higher in line with the severity of pathogen infection.

The eugenol, l-limonene and coumaran compounds were synthesized at different stages of *O. theobroma* infection through different metabolite pathways. Eugenol was a phenolic compound that had been reported to play an important role in inhibiting the growth of *Botrytis cinerea* fungus (Wang *et al*., 2010). Silva *et al.* (2019) reported that limonene were generally released as a chemical compound related to the resistance. Coumaran, also known as 2,3-dihydrobenzofuran, was heterocyclic compound that act as a precursor in the pathway of degradation of aromatic compounds. Degradation of the aromatic compound 2,3-D will produce salicylate which has long been known in signal transduction of plant resistance to pathogens (Aliferis *et al*., 2014).

There were a negative correlation between starch content with concentration of l-limonene (r = - 0.74), eugenol (r = - 0.44), and coumaran (r = - 0.32) (Figure 3). This showed that starch degradation potetially increased the production of secondary metabolites. Starch, specifically amylose was converted from amylase to maltose. Furthermore, maltose was converted by maltase to glucose (Sinaga, 2003). Glucose was the main source of energy at the stage of glycolysis in respiration. In glycolysis, plant produce ATP, reduce NADH and produce intermediate products to support the biosynthesis of other materials (Taiz & Zeiger, 2010). In the formation of limonene, pyruvate was converted through monoterpenoids synthesized in plastids via the methylerythritol phosphate (MEP) pathway in terpenoid metabolism. IPP (isopentenyl diphosphate) through the enzyme IPP isomerase was converted to geranile diphosphate as a monoterpene substrate (Taiz & Zeiger, 2010). Eugenol compound was phenol group that was synthesized through the cyclic acid pathway to produce phenylalanine. Furthermore, phenylalanine was converted into cinamate and then coumaryl alcohol. Coumaryl alcohol was converted to coniferyl alcohol and then converted to eugenol. Coumaran was a heterocyclic compound that act as a precursor in the path of degradation of aromatic compounds. The degradation of coumaran would produce salicylate which have long been known as signals for transduction of

Table 2. Concentration of l-limonene, eugenol, and coumaran between two cocoa clones at different infection phases of *O. Theobromae*

Clone	Limonene*) (%)	Eugenol (%)	Coumaran (%)
TSH 858 (Sus)	4.40 a	4,59 b	0.49 b
Sca 6 (Res)	2.19 b	6,11 a	1.56 a
Infection stages			
Pre	0.10 m	4,21 m	0.38 m
Early	3.87 n	5.92 m	1.61 m
Advanced	5.93 o	5.92 m	1.09 m

Notes: Number in the same row followed by the same letter are not significantly different, based on Tukey-test at α = 5%; *)Data was transformed by using sqrt + 0.5.

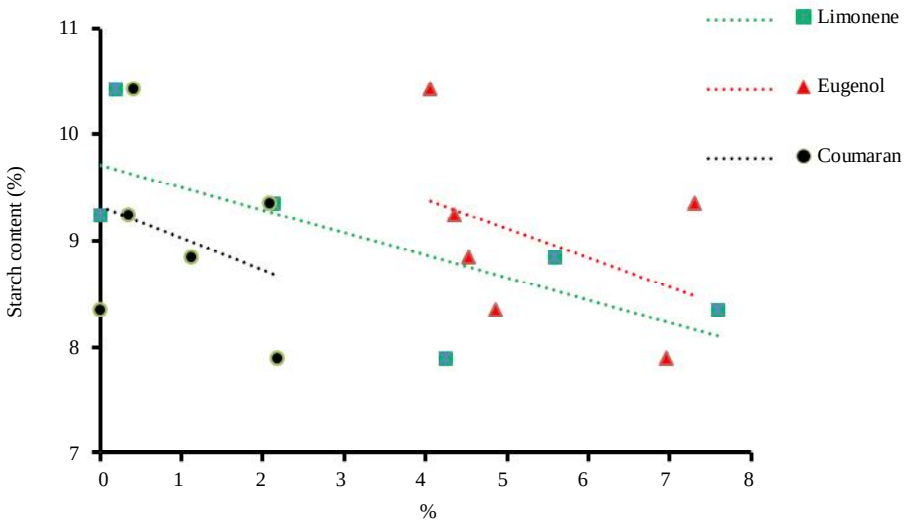

Figure 3. Relationship between l-limonene, coumaran, eugenol and starch content

plant resistance signals to pathogens (Aliferis *et al.*, 2014). Normally, coumaran was a precursor of acetaldehyde in the biosynthesis of acetic acid in the tricarboxylic acid cycle. In pathogen-infected conditions, coumaran also a precursor of salicylate of a signal transduction in the release of proteins related to the defense mechanism.

CONCLUSIONS

Starch, l-limonene, eugenol, and coumaran were the compounds that played role in VSD in cocoa. Starch content in Scavina 6 (resistant clone) in the advanced-stage of infection was 24.33% lower than healthy condition (no infection), however, starch content in TSH 858 (succeptible clone) in later stage of infection decreased only 9.63% than healthy condition (no infection). Starch degradation rate on resistant clone was higher than susceptible clone. Scavina 6 had higher concentration of eugenol and coumaran than TSH 858. However, the concentration of limonene compound of TSH 858 was higher than Scavina 6. L-limonene concentration increased in line with the severity of pathogen infection. There were negative correlation between starch content and concentration of l-limonene, eugenol, and coumaran.

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