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Several Physiological Changes of Cocoa (*Theobroma cacao* L.) in Response to Vascular Streak Dieback Diseases

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ABSTRACT

The physiological response of cocoa plants under VSD infections is still limited because there is no method in artificial inoculation since *Oncobasidium theobromae* was reported as parasitic obligates. The investigation of correlation between cocoa responses and VSD attack becomes important information to figure out the effect of decreased productivity as the consequences of the physiological damages. The objective of this research was to describe the pathogen infection and the symptom of VSD through fungal staining method and to asses the changes of some physiological aspects (chlorophyll and gass exchange character) under VSD pathogen infection. This study was conducted in Kaliwining experimental station, ICCRI, Jember, East Java, Indonesia. The result of the study indicated that late infection stage caused chlorophyll degradation, decreasing transpiration rate, and increasing temperature in both tolerant clone (Scavina 6) and susceptible clone (TSH 858). The rate of photosynthesis, stomatal conductance, and concentration of CO2 in susceptible clone (TSH 858) showed a drastic decrease when infected by VSD pathogen than that of in healthy plants. In resistant clone (Scavina 6), photosynthesis rate, stomatal conductance, and concentration of CO2 showed no significant decrease when infected by VSD pathogen compared to that non-infected condition.

INTRODUCTION

Cocoa (*Theobroma cacao* L.) is an important estate-crop commodity for the food chocolate industry, cosmetics, beverages, and other derivative products (Lima, Almeida, Rob Nout, & Zwietering, 2011). Currently, Indonesia is the third largest producing country of cocoa commodity in the world (ICCO, 2018). The production of cocoa in Indonesia has the potential to increase if the limiting factor can be minimized. One of several limiting factors in cocoa cultivation is the attack of vascular-streak dieback (VSD) pathogen that causes yield loss up to 60 %, it even causes the plants to die out (Sukamto & Junianto, 1986). The spreading of VSD pathogen' attacks have been found in the center of cocoa production area, especially in Sulawesi, and the total area attacked by VSD was around 950 thousand hectares in 10 provinces (Khaerati, Wiyono, & Tondok, 2016). Several efforts to control the VSD pathogen attacks are by planting resistant plant material to VSD and by applying the concept of Good Agricultural Practices (GAP). Under VSD pathogen infection, the phenotypic of plant is controled by genetic factor, environmental factor and both interactions. However, the severity of VSD is mostly influenced by the level of plant resistance. The statement reported by Anita-Sari & Susilo (2014) stated that under the same enviromental condition, the VSD disease severity was genetically controled, that resistant clones is lower than susceptible clones. Santoso, Miftahudin, Sulistyaningsih, & Wiyono (2017) reported that Scavina 6 and Sulawesi 01 clones worked as VSD-resistant clones, while TSH 858, ICS 13, and ICS 60 was VSD-susceptible clones.

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The fundamental research about the resistance mechanism of plant in response to VSD is still limited. It is because the VSD pathogen is difficult to be cultured in the artificial inoculation media (in vitro method). *Oncobasidium theobromae,* the fungus that causes VSD disease, is classified in basidiomycetes group of obligate parasitic (Guest & Keane, 2007). One way to identify the infection of this pathogen in cocoa plant is based on VSD-spesific symptom using scoring method that refers to Nur 'Aini (2015). The spesific symptoms of VSD are 1) the leaves and tig become dry, 2) the second/third leaf from growing point becomes chlorosis with green patches, 3) If the twig and petiole are slashed perpendicularly, there are three yellow-blackish brown spot in the xylem, 4) Moreover, when the twig and petiole are cut longitudinally, there are brown line in xylem tissue, 5) Lenticel in infected twig is more coarse than healthy twig, 6) Necrosis symptom on the leaf midrib similar to potassium deficiency symptom (Nur 'Aini, 2015). To ensure the VSD pathogen infection, histochemical staining (fungal staining) of pathogen hyphae from infected leaves can be used as a tool to detect the existence of the fungus during early until late infection under natural condition (Marques, Soares, & Appezzato-Da-Gloria, 2013). Santoso, Miftahudin, Sulistyaningsih, & Wiyono (2017) used this technique to ensure *O. theobromae* infection.

The spreading of VSD diseases is known using basidiocarp organ that only flourishing in petiole of fresh leaf during rainy season (Nur 'Aini, 2015). The fungus spread through airborne and penetrates into young leaves (*flush*) through stomata and penetrated into the xylem (Santoso, Miftahudin, Sulistyaningsih, & Wiyono, 2017). Pathogen, that attack plant through the leaves, can cause physiological changes in the host, which can occur directly through the lytic enzyme from the pathogen and toxin compound or indirectly through the host responses that induced by that pathogen. Physiological changes of leaf plants infected by systemic parasites, such as vascular pathogens like *O. theobromae*, are important factors to be investigated in this context. Among all of those responses, the main physiological process that most related and substantive is the photosynthesis process (Alves, da Silva Guimarães, de Melo Chaves, DaMatta, & Alfenas, 2011; Dallagnol, Rodrigues, Martins, Cavatte, & Damatta, 2011; Zhao, Glynn, Glaz, Comstock, & Sood, 2011). In general, the infection of fungi can affect the net assimilation rate of carbon by the increasing of the leaves respiration, which plant will accelerate the assimilation catabolizing process for the host's metabolic activities (Debona et al., 2014). In VSD disease, the accumulation of pathogenic hyphae in xylem will cause obstruction in vascular tissue to the canopy, so the water and nutrients cannot distribute perfectly to the leaves. From that obstruction, it potentially decreases various physiological process, such as transpiration. This information is important to know how plant reacts or responds during affliction of pathogen infection. The investigation about relation between cocoa responses and VSD pathogen attack also become an important information for researchers and farmers in knowing the effect of decreasing productivity from the consequences of the physiological damages. The objective of this research was to describe the pathogen infection and the symptomp of VSD through fungal staining method and to asses the change of some physiological aspects (chlorophyll and gass exchange character) under VSD pathogen infection.

MATERIALS AND METHODS

Research Site and Experimental Design

The research was conducted in Kaliwining Experimental Station, Indonesian Coffee and Cocoa Research Institute, Jember, Indonesia at an elevation of 48 m above sea level on January – August 2016. The soils were classified as low humic loamy clay with D climate type based on Schmidt & Fergusson classification. The research material used cocoa seedling (Theobroma cacao) from propagation of top grafting aged 7 months that representing resistant group (Scavina 6) and susceptible group (TSH 858) against VSD pathogen attack (Santoso, Miftahudin, Sulistyaningsih, & Wiyono (2017). The experiment using Factorial Randomized Complete Block Design replicated 6 times. First factor is type of clone i.e. resistant clone (Scavina 6) and susceptible clone (TSH 858) against VSD pathogen attack. Second factor is infection treatment i.e. cocoa seedling that naturally infected under the mature cocoa attacked by VSD pathogen was categorized as infected plant while non- infected plant were placed in areas 3000 m away from the mature cocoa plant areas. Cocoa seedling inoculated in rainy season, and the symptoms will show up in dry season.

Fungal Staining Method

Observation of the infection *O. theobroma* using fungal staining method refers to a method developed by Santoso, Miftahudin, Sulistyaningsih, & Wiyono (2017) using triphane blue ($C_{34}H_{28}N_6O_{14}S_4$) with 1g/l concentration in midrib leaves area. One cm of leaf midrib samples (lamina containing midrib) was soaked in a 20 mL 1:1 (v/v) ethanol and glacial acetic acid in dark tube under room temperature for 24 hours. Leaf samples were perpendicularly sectioned, then they were put on glass slide and dropped 0.1 % trypan blue and 85 % lactic acid solution. The existence of fungus was observed by using microscop Olympus CX21 (Olympus, Japan). The fungus was identified by using description key of O. Theobromae according to Samuels et al. (2012). The late infection stage was shown by the hyphae of *O. theobromae* detected on column of cocoa xylem midrib leaves and the leaves had already shown the chlorosis symptom with green patch (Guest & Keane, 2007).

Physiological Aspect (Gas Exchange and Chlorophyll Analysis)

The observation of photosynthesis rate, stomatal conductance, CO_2 concentration (Ci), leaf temperature, and transpiration rate were observed at 09.00 am – 12.00 pm with portable open-system infrared gas analyzer (LI-6400, LI-COR Inc., Lincoln, NE), with external CO_2 concentration in 400 µmol/mol). Leaf chlorophyll was observed using SPAD meter (Minolta 502) and then converted to the equation $10^{M^{0,265}}$ .The equipments were tagged on the fully expanded leaf, which are the most physiologically active leaf.

Data Analysis

The obtained data of pathogen infection and symptomp of VSD was analyzed by using descriptive method. The data of physiological aspect (chlorophyll content and gas exchange character) was analyzed by using analysis of variance (ANOVA) with α = 5 %. If the result of variance analysis obtained that F value > F table, it means there is significant different between each factor, and then it is followed by Tukey Test (Gomez and Gomez, 1995).

RESULTS AND DISCUSSION

Pathogen Infection and The Symptom of VSD

VSD symptoms in late infection stage began to show up after 2 weeks since the first-time hyphae of *O. theobromae* was discovered (Fig. 1). The fungi formed basidiospore that became the source of infection of this fungus, and spread through airborne in the night when the moisture was high (Ardianti, Umrah, & Asrul, 2017). Fig. 1E and Fig. 1F showed the spesific symptom of VSD disease that was three brownish-yellow spots in the xylem of leaf petiole. The first infection only occured in young leaf, after that, the infection would spread following the xylem. The formation of the fungi colony in xylem column will become main characteristic that differentiate *O. theobromae* with the other fungi (Samuels et al., 2012).

To confirm whether the fungus that attacked cocoa seedlings was VSD, besides using specific symptoms in plant, using fungal staining in xylem is also possible. This observation used fungal staining in midrib tissue of cocoa leaves (white arrow in Fig. 2a). In healthy leaves, there was no colonization of hyphae that makes occlusion in xylem vessel was not found (Fig. 2b). Hyphae occlusions were found in xylem vessel (marked by arrow and x in Fig. 2c). Furthermore, the specific of VSD fungal could be seen morphologically, with the type of specific perpendicular branch fungus to the hyphae bodies derived from basidiomycetes group (Fig. 2d). In paradermal section, there was found fungal hyphae that identified as *O. theobromae* based on the characteristic described by Talbot & Keane (1971). The 90^o of the branch of fungal hyphae inside of the xylem vessels is the (marked by ring and x in Fig. 2d.). Hyphae colonizing on the xylem vessel is also an identifier of *O. theobromae* (Talbot & Keane, 1971) and the character distinguish from others fungi attack on cacao (Samuels et al., 2012). Ardianti, Umrah, & Asrul (2017) explained that the hypae of *O. theobromae* has irregular septate and branched micelium. Moreover, Trisno, Refflin, & Martinius (2016) stated that the hyphae of the pathogen is characterized with perpendicular branch and dolipore septate. The pathogen is wind-borne disease and xylem-infecting fungus which has been discovered mostly associated only with cocoa and may be related to other endemic plant species and has it transferred to cocoa (McMahon & Purwantara, 2016).

Physiological (Chlorophyll Content and Gas Exchange) Character

The accumulation of the colony in this xylem vessel caused disruption in physiological process in plant and then it would affect on the growth and development of plants. The main physiological process that was disrupted from this disease infection is photosynthetic apparatus, especially chlorophyll. VSD pathogen attack consistently showed the necrotic and chlorotic symptoms was that the infected leaves remain attached to the branch. Santoso, Miftahudin, Sulistyaningsih, & Wiyono (2017) stated that water and nutrient translocation via xylem vessel would be disrupted and it led plant to be wilting, chlorosis, and necrosis. Zakariyya & Prawoto (2015) reported that chlorophyll is an important physiological component in cocoa plant that related with the yield.

Fig. 1. Character of cocoa plant seedling (A) Clone Scavina 6 (Resistant) Healthy/Not infected; (B) Clone TSH 858 (Susceptible) Healthy/Not infected; (C) Clone Scavina 6 infected; (D) Clone TSH 858 infected by *O. theobromae;* (E and F) Three Brownish-Yellow color spots as specific symptoms of VSD

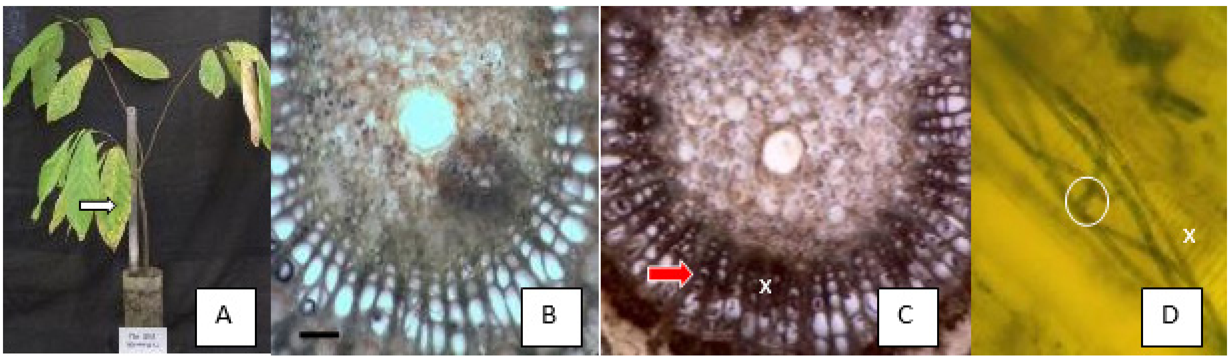

Fig. 2. Histocemical detection of *O. theobromae* infection with triphane blue in leaf midrib (A). Midrib across incision in healthy plant (B) and late infection stage (C). Hyphae of fungi in longitudinal midrib incision (D). The circle mark showed type of specific perpendicular hyphae branch of *O. theobromae*. Bar scale= 30 µm. The arrows showed hyphae of *O. theobromae*. Bar scale = 50 µm; x = xylem

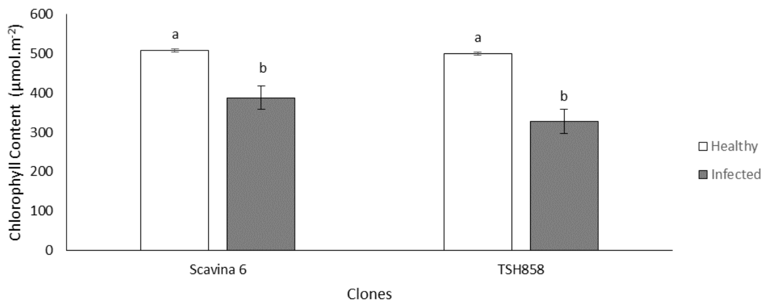

Fig. 3. Leaf Chlorophyll content of cocoa seedling leaf that infected VSD disease (showing late symptoms stage) and healthy/not infected in resistant (Sca 6) and susceptible (TSH 858) clones

Fig. 3. showed that both of VSD resistant and susceptible clone significantly decreased in chlorophyll content under late VSD pathogen infection. In average, the decreasing of chlorophyll content in both clones when infected by pathogen was up to 29.07 % than healthy condition. However, the decreasing of chlorophyll content in susceptible clone is more drastic compared to resistant clone, where chlorophyll content in the susceptible clone (TSH 858) was 499.97 μmol/m^2 in health condition, but in infected condition it decreased become 327.87 μmol/m^2

The degradation of chloropyhll and leaf abscission may be associated with the increasing ethylene levels and/or toxic compund caused by pathogens (Broekaert, Delauré, De Bolle, & Cammue, 2006; Scarpari et al., 2005). In some diseases that caused by fungi, the photosynthesis apparatus was decreased because of toxic that they produced, such as tentoxin and tabtoxin. Those toxins could inhibit the enzyme which involved directly or indirectly in chlorophyll synthesis (Yunasfi, 2008). Moreover, fungi infection intensifies generate of reactive oxygen species (ROS) that cause the degradation of chlorophyll (Dehgahi et al., 2015).

In other case, the spesific chlorosis and necrosis symptoms of VSD-infected cocoa leaf were similar to potassium deficiency. Abdoellah (2009) reported that K concentration in VSD-infected leaves was 20 % lower than healthy leaves. Furthermore, McMahon & Purwantara (2016) also stated that in some clones that has necrotic and chlorotic tissues were substantially lower about 40 % in K concentration than in healthy leaves. It indicated that there were disruption of uptake of K element occur in response to the infection of VSD pathogen. Zhao, Glynn, Glaz, Comstock, & Sood (2011) explained that potassium is a macro-essential elements that is needed in large quantities and associated with chlorophyll synthesis. However, the spesific mechanism of chlorophyll degradation caused by VSD disease is still unclear.

Stomatal conductance is the ability of stomata in conducting gases for diffusion, for either exiting or entering the gases into the leaf. The exchange gas can be CO_2 (entering) or H_2O (exiting or vaporization as the effect of transpiration). Based on this observation (Fig. 4), there were interactions between resistant and susceptible to VSD clones in healthy condition and late infection stage. The infection of VSD pathogen in both clones decreased in stomatal conductance up to 36.63 %, approximately. Stomatal conductance in VSD-resistant clone when infected were not significantly different compared to the healthy condition, meanwhile, in susceptible clone infected by VSD pathogen, it significantly declined by 48.98 % than its healthy condition (Fig. 5). This result was similar to Dehgahi et al. (2015) research that fungal toxin caused the decreasing in stomatal conductance. This phenomena might be indicated that there were strong correlation between stomatal conductance and stomatal closure. The possible reason causing stomata closed due to the pathogen was the inhibiton of water transportation via xylem which causes the limitation of water in leaf tissue (Wang, Zheng, Shen, & Guo, 2013). In the other hand, the limitation of K+ uptake in guard cells was one of the factors that affect stomatal apperture (Misra, Acharya, Granot, Assmann, & Chen, 2015). Moreover, some pathogens also activated abscisic acid (ABA) signaling as a defence mechanism to the pathogen, meanwhile, ABA also played a role in stomatal mechanism, such inhibiting the stomatal opening and promoting stomatal closure (Cao, Yoshioka, & Desveaux, 2011; Mine et al., 2017).

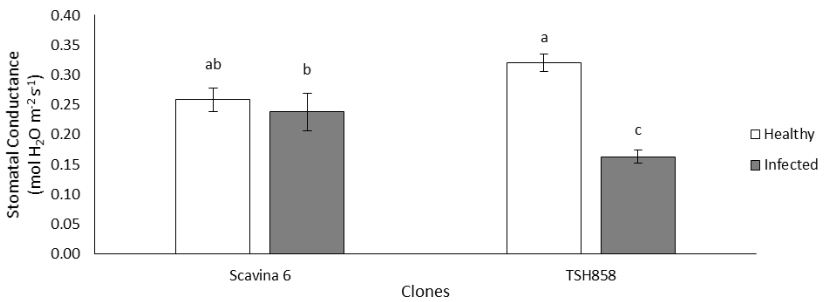

Fig. 4. Stomatal conductance of cocoa seedling leaf that infected VSD disease (showing late symptoms stage) and healthy/not infected in resistant (Scavina 6) and susceptible (TSH 858) clones

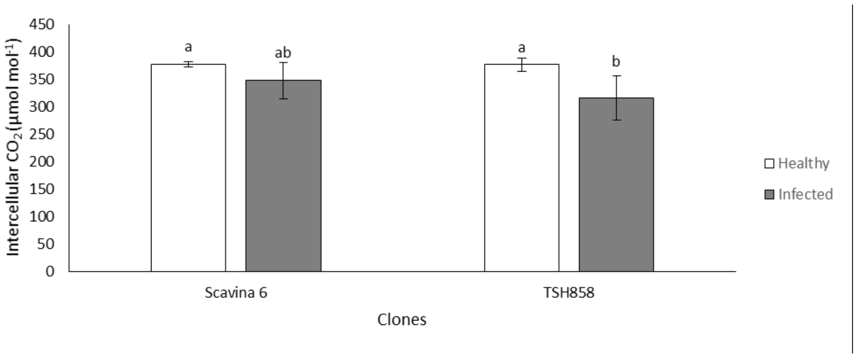

Fig. 5. Intercellular CO_2 (Ci) of cocoa seedling leaf that infected VSD disease (showing late symptoms stage) and healthy/not infected in resistant (Sca 6) and susceptible (TSH 858) clones

The decreasing of stomatal conductance implicated on intercelullar CO_2 concentration in leaf tissue. Intercelullar CO_2 concentration of Scavina 6 (resistant clone) was not significantly different both in healthy condition and under the pathogen infection. Meanwhile, in TSH 858 (succeptible clone), intercelullar CO_2 concentration was 16.06 % lower than under the pathogen infection. This result was similar to the research of Pinkard & Mohammed (2006) which proved that the more severe damage of Eucalyptus globulus' leaf caused by Mycosphaerella leaf disease, the lower CO_2 intercelullar concentration became.

The decreasing of stomatal conductance also affected on transpiration rate. Under the pathogen infection, the transpiration rate of both clones (susceptible clone) decreased significantly compared to in healthy condition (Fig. 6). In average, the transpiration rate of both clones under the pathogen infection decreased by 19.57 % than in healthy condition. Lobato et al. (2010) reported similar lower transpiration rate when common bean plant were infected by anthracnose. Cavalcanti, Resende, Lima, Silveira, & Oliveira (2006) also found that *Xanthomonas vesicatoria* infection in tomato decreased transpiration. Lobato et al. (2010) stated that the decreasing transpiration rate in infected plant by pathogen, indicated that there were inhibition in water transportation from the roots to the leaves by the pathogen infection.

Leaf temperature in both resistant (Scavina 6) and susceptible (TSH 858) clones were significantly increased under *O. Theobromae* infection. However, the increasing of temperature in Scavina 6 (resistant clone) is relatively smaller than TSH 858 (susceptible clone). Leaf temperature of TSH 858 clone under the late infection stage was 30,55^{o}C and was significantly different with its healthy condition where the temperature was 27.76^{o}C (Fig. 7). In other hand, the pathogen infection could be decreasing the values of both clones (resistant and susceptible clone). Leaf temperature became higher because the decreasing of leaf transpiration rate. Kerbauy (2008) study reported that transpiration rate play a role to be a cooling effect in leaf surface or leaf thermoregulation.

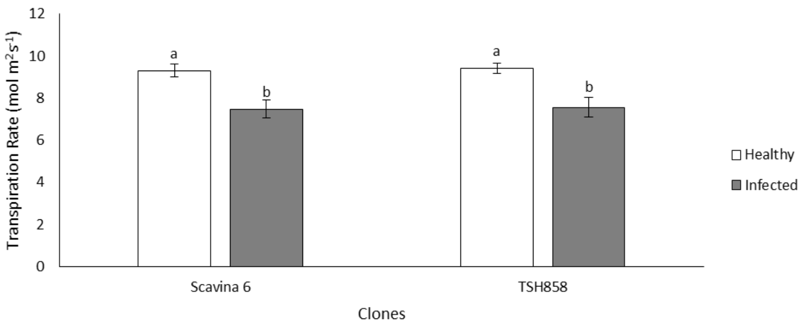

Fig. 6. Transpiration Rate of of cocoa seedling leaf that infected VSD disease (showing late symptoms stage) and healthy/not infected in resistant (Sca 6) and susceptible (TSH 858) clones

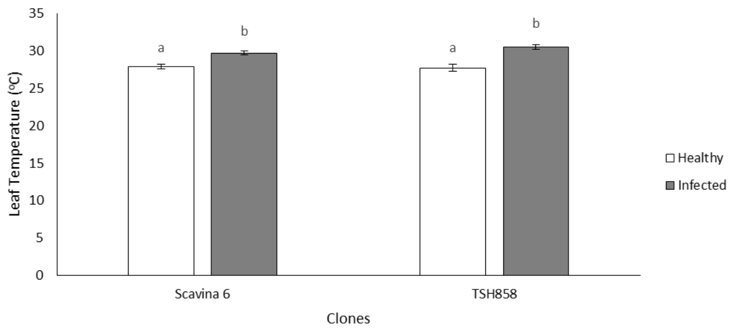

Fig. 7. Leaf Temperature of cocoa seedling leaf that infected VSD disease (showing late symptoms stage) and healthy/not infected in resistant (Sca 6) and susceptible (TSH 858) clones

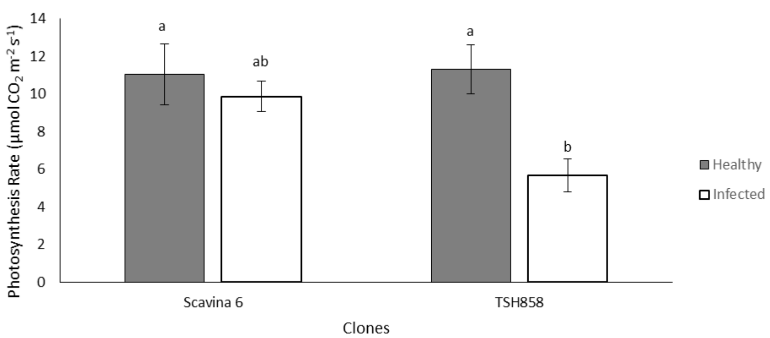

Fig. 8. Photosynthesis rate of cocoa seedling leaf that infected VSD disease (showing late symptoms stage) and healthy/not infected in resistant (Sca 6) and susceptible (TSH 858) clones

Fig. 8. demonstrated photosynthesis rate on resistant and susceptible cocoa clones in healthy plant and infected by pathogen. Photosynthesis rate of both clones (resistant and susceptible) significantly decreased under the pathogen infection. Photosynthesis rate in all clones decreased approximately 49.89% than their healthy condition. This is indicated from susceptible clone (TSH 858), which in their healthy condition, their photosynthesis rate is can be 11.32 µmol CO_2 /m^2 s^1, whereas in the late infection stage symptoms has decreasing become 5.65 µmol CO_2 / m^2 s^1.

Photosynthesis consists of two processes namely light and dark reactions. In light reaction, chlorophyll captures light energy and then generates energy from electron chain in the form of ATP and NADPH. The decreasing of photosynthesis rate in plant infected by fungi may be affected by the degradating of chloropyll content. This statement was also explained by Lobato et al. (2010) and Debona et al. (2014). Fig. 9 demonstrated the highly positive correlation between chlorophyll content and photosynthesis process. It proved that the lower chlorophyll content, the lower photosynthetic rate would be. The reduction of chlorophyll content consequently reduced the capacity of light absorbtion in leaf tissue. In dark reaction, the limitation of photosynthesis process is caused by CO_2 fixation by Rubisco enzyme. The reduction of stomatal conductance contributed on the reducing of CO_2 diffusion in to the leaf. Fig. 10. presented a positive linear relationship between intercelullar CO_2 concentration and photosynthesis rate. It meant that the lower intercelullar CO_2, the lower photosinthesis rate. The light and dark reactions in photosynthesis process were signficantly affected by pathogen infection. Weintraub & Jones (2009) stated that pathogen attack resulted in declining the photosynthetic activity was caused by the decreasing of some thylakoid membrane proteins and soluble protein such as RUBP.

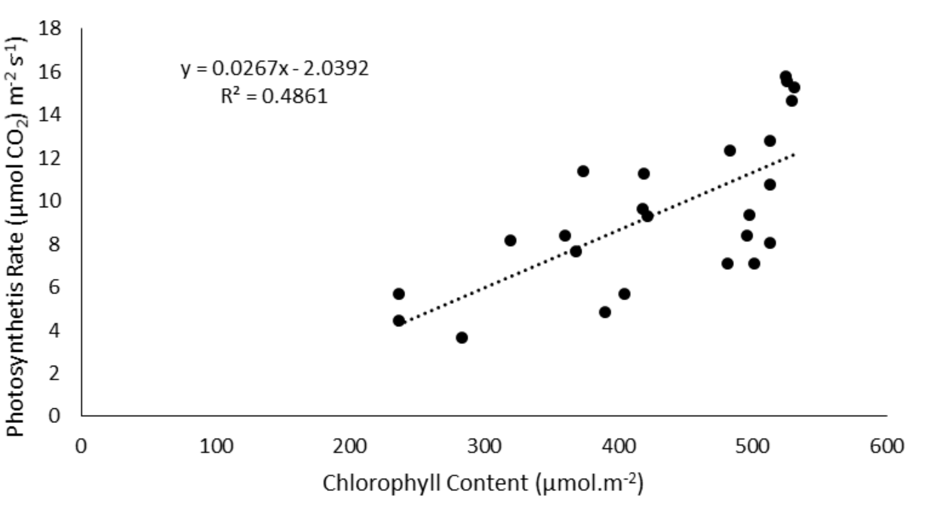

Fig. 9. Relationship between Chlorophyll content and Photosynthesis rate of cocoa seedling

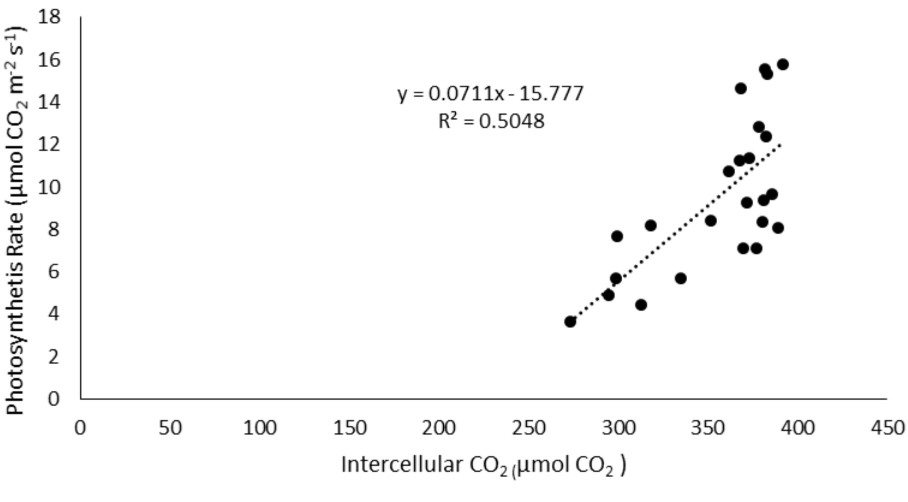

Fig. 10. Relationship between Intercelullar CO_2 and Photosynthesis rate of cocoa seedling

CONCLUSION AND SUGGESTION

Late infection stage caused chlorophyll degradation, decreased transpiration rate and increased temperature both in resistant clone (Scavina 6) and susceptible clone (TSH 858). The photosynthesis rate, stomatal conductance, and CO_2 concentration in susceptible clone (TSH 858) under the VSD pathogen infection drastically decreased compared to its healthy condition. In resistant clone (Scavina 6), photosynthesis rate, stomatal conductance, and CO_2 concentration parameter when infected by VSD pathogen, decreased though not significantly compared to its healthy condition.

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