

Heterosis Analysis of Leaf Stomatal Characteristics on F₁ Population of Cocoa (*Theobroma cacao* L.) Related to Vascular-Streak Dieback Resistance

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Abstract

Breeding for cocoa resistance to VSD is the main goal in breeding program in Indonesia. Research for selection criteria on cocoa (*Theobroma cacao* L.) resistance to vascular-streak dieback (VSD) has been carried out by exploring leaf stomatal characteristics corresponding to VSD damages. This research had objectives to select best crosses by observing the estimated value of heterosis and heterobeltiosis on F₁ hybrid populations based on leaf stomata characteristics related to VSD resistance. Trial was established at Kaliwining Experimental Station of Indonesian Coffee and Cocoa Research Institute (ICCRI) in a VSD endemic area. Treatments consisted of 5 F₁ hybrid populations generated by biparental crosses of TSH 858(S) x Sulawesi 1 (R), TSH 858 (S) x KEE 2 (R), TSH 858 (S) x Sulawesi 3 (R), Sca 6 (R) x KW 264 (S) and KW 617 (R) x KW 264 (S). Each cross consisted of 100-300 genotypes planted in high planting density of 0.5 m x 0.5 m. The resistance was evaluated by scoring the plant damage in the scale of 0-3 at three years after planting. Stomata characteristics were assessed microscopically based on the variables of density, length, width and diameter of stomata and width of opening stomata. Analysis of correlation indicated that most of stomatal characteristics significantly correlated to VSD damage but the width of opening stomata performed the highest coefficient of correlation ($r = 0.49$) and contributed to VSD damage (0.45). Meanwhile, path analysis indicated higher value of the characteristics performed less resistance of the tested hybrids, KW 617 x KW 264 showed lower value of heterosis and heterobeltiosis for the characteristics which of the best crosses for generating VSD-resistant genotype.

Keyword: Vascular-streak dieback, leaf stomata, heterosis, *Theobroma cacao* L.

INTRODUCTION

Cocoa production in Indonesia tends to decrease since last 5 years due to plant aging, pest and diseases incidence and soil degradation. These factors effect simultaneously to the production reduction that should be addressed in term of integrated approach at the sustaining cocoa production. Vascular-streak dieback (VSD) is the most serious disease

infecting cocoa in Indonesia and other Asia-Pacific region seriously damaging susceptible trees in the main cocoa growing area such as in Sulawesi. Methods for controlling VSD have been successfully applied through integrated management by combining the technical culture approach then sprayed using fungicide to the new-emerged leaves, application in optimum dosage of fertilizer and organic matter, and shade trees management (Febriantomo,

2012). However in term of serious damage the diseases may be effectively controlled using resistant varieties (Guest & Keane, 2007). There are a number of resistant varieties recommended for controlling VSD in Indonesia, such as the clonal materials of Sulawesi 1, Sulawesi 2, ICCRI 03, Sca 6 (Susilo *et al.*, 2012), MCC 01, MCC 02 (Susilo *et al.*, 2015) and hybrids of ICCRI 06 H (Susilo, 2011), ICCRI 08 H (Susilo *et al.*, 2016). Farmer preference to the clonal materials vary among locations depend on the clone adaptability to local environment in which just a few number of clones which mostly preferred for planting in Sulawesi such as Sulawesi 02 and MCC 02 (Susilo *et al.*, 2017). Furthermore breeding programs have to be carried out continuously to generate more resistant genotypes and avail more clones for farming in the anticipating homogeneity of cocoa farm.

Breeding for cocoa resistance to VSD is the main goal in breeding program in Indonesia by which selection aimed to find out resistant hybrids or clonal materials with high yielding (>3 tones/ha/year) and also resistant to other main pests and diseases such as cocoa pod borer (CPB) and phytophthora pod rot (PPR). Breeding strategy was implemented by selecting the resistant genotypes at the germplasm collection then of the selected clones was crossed with other parental clones regard to the potency on yield, bean quality, and cross compatibility (Susilo & Anita-Sari, 2011). The process on selection still take time as the resistance could only be assessed in the field based on field infestation of *Ceratobasidium theobromae* when plant damage could be clearly differentiated between susceptible and resistant genotypes normally occur on peak of dry season (September-October). Furthermore, expressions of the resistance depend on the environmental condition that would affect to selec-

tion effectiveness. Criteria for selection the VSD resistance need to be identified based on stable characteristics which may be expressed at the early stage of cocoa growing due to time limit of evaluation.

Identification of the criteria for selection on VSD resistance has being carried out by exploring the characteristics and biochemical compounds of leaf the point where *C. theobromae* entering through the plant to infect xylem bundle sheet. Prawoto *et al.* (2013) reported difference in thickness of cuticle and epidermis layer between susceptible and resistant clones but both characteristics may not represent VSD resistance. Further study analyzed leaf stomata characteristics which demonstrated that lower density and diameter of stomata belong to resistant clone as a guide to VSD resistant characteristics (Anita-Sari & Susilo, 2013). Further, Susilo *et al.* (2016) reported the study on leaf stomata characteristics using more diverse of genetic materials which confirmed that density and width of the opening stomata performed high broad sense heritability (Susilo *et al.*, 2016). Based on that study it could be confirmed that the leaf stomata characteristics are prospective to be developed as criteria for selection. Genetic inheritance of those characteristics should be assessed to confirm their relationship with VSD resistance that breeders are able to construct effective crosses based on leaf stomata characteristics. This research had an objective to analysis the heterosis and heterobeltiosis performance of leaf stomatal characteristics related to VSD resistance on some F_1 hybrid populations that may be identified the best parent in inheriting the resistance. The results were discussed related with consistency of relationship between leaf stomata characteristics with VSD resistance through the high diverse of genetic materials and selecting the best clones for generating VSD resistant genotype regard to the characteristics.

MATERIALS AND METHODS

Trial was established in single plot design with treatments of 5 F₁ hybrid population cross between selected parental clones refer to VSD resistance (Table 1). Each cross consisted of 100-300 progenies planted in high density planting of 0.5 m x 0.5 m at Kaliwining Experimental Station of ICCRI situated with high infection of VSD. The parental clones for crossing and VSD assessment were collected at cocoa germplasm collection at the same location in 45 m asl and C type of climate classification according to Schmidt-Ferguson.

Table 1. The cross combination of parental clones which different background on VSD resistance for generating F₁ hybrid population

Cross combination (♀ x ♂) ^{a)}	Number of progenies
TSH 858 (S) x Sulawesi 1 (R)	300
TSH 858 (S) x KEE 2 (R)	300
TSH 858 (S) x Sulawesi 3 (R)	100
Sca 6 (R) x KW 264 (S)	100
KW 617 (R) x KW 264 (S)	100

Notes: ♀ = female parent, ♂ = male parent, S = susceptible, R = resistant

Three-year old plants were assessed for VSD resistance by scoring the plant damage using 3 scales (Aini, 2015). Evaluation of the resistance were conducted at peak dry season in which time the plant damage could be clearly differentiated among the different level of resistance. Fully-emerged leaves were selected then sent to Plant Breeding Laboratory of ICCRI for stomata characterization using binocular microscope in 40X of magnification (Susilo *et al.*, 2016) by assessing its density per mm², stomata length, width of opening stomata, stomata width and stomata diameter. The samples were prepared according to Haryanti (2010) in which of the full-emerged leaf samples were selected then to determine objected tissue for sampling analysis. Brushing the objected tissue using transparent nail polish, allow the process for about 10-15 minutes till dry then covered the objected tissue using trans-

parent cover-tip, flattened slow down the surface area then uncover the tip carefully. Removing the cover tip containing thin layer of the surface leaf area then place down in the deck glass for stomata analysis under binocular microscope. Stomata measurement was carried out using callibrated micrometer.

The recorded data were analyzed using regression correlation and path analysis between variable of VSD damage score to the stomatal characteristics then be estimated the value of hetetosis and heterobeltiosis.

Heterosis Value

Heterosis value were estimated based on the value of mid parent and the best parent. The formula for calculating the estimated value, namely:

$$\text{Heterosis} = \frac{\mu F_1 - \mu \text{MP}}{\mu \text{MP}} \times 100\%$$

$$\text{Heterobeltiosis} = \frac{\mu F_1 - \text{HP}}{\text{HP}} \times 100\%$$

Note:

- μ F₁ = Mean of F₁
- μ MP = Mean of both parent = (P₁ + P₂) / 2
- HP = Mean of best parent

Correlation

Inter-relationship between the leaf stomatal characteristics was performed by coefficient of correlation based on covariance analysis according to Sigh & Chaudhary (1979):

$$r_{AB} = \frac{\text{cov}(A,B)}{\sqrt{(\sigma_A^2)(\sigma_B^2)}}$$

- Cov (AB) = covariance of phenotypic/genotypic for VSD resistance based on score of plant damage.
- r_{AB} = correlation of phenotypic/genotyoc for VSD resistance based on score of plant damage.
- σ_A² = variance phenotypic/genotypic for resistance to VSD.
- σ_B² = variance phenotypic/genotypic for susceptibility to VSD.

Data of stomata measurement was analyzed using path analysis to study leaf stomata characteristic affecting directly or indirectly on VSD resistance. Calculation of path coefficient used data of intercorrelation. Path coefficient value were calculated according to Singh & Chaudary (1979).

RESULTS AND DISCUSSION

VSD with Leaf Stomatal Characteristics

Information concerning correlation among traits need to be observed for supporting breeding programs as the inter-relation between traits may help breeder to select genotype based on the criteria related to economical traits that would speed up selection process (Astika, 1991). The inter-correlation among traits on plant may be controlled by some linkage-genes or pleiotropic mechanism (Liu, 1998) that multi-trait expressed interdependence. Analysis of correlation among leaf stomata characteristics with the score of VSD damage through F_1 hybrid population indicated significant positive correlation of those characteristics to VSD damage scores (Table 2) that means the higher value of leaf stomata characteristics performing more level of VSD damage scores. This result increasingly strengthen previous report in which the leaf stomata characteristics related to VSD resistance. Generally it could be interpreted that increasing leaf stomata characteristics would

increase the level of plant susceptibility to VSD attack. However it could not yet be determined the range value of those characteristics representing the resistant classification. The closeness of relationship among those variables varied according to the coefficient of correlation in the range of 0.18-0.49 which performing less strength of the interrelation among variables. The width of opening stomata perform highest coefficient of correlation to VSD damage score that would be more important characteristics contributing to the VSD susceptibility.

The density of leaf stomata and width of opening stomata on cocoa were previously reported performing relationship with plant damage due to VSD infection (Susilo *et al.*, 2016). This result is similar with the report, however, coefficient of correlation of these characteristics to VSD damage are small (<0.5) which indicating less strength of the interrelationship. The width of opening stomata performed higher values of the coefficient than of stomata density that would be more important its contribution to VSD susceptibility. The results reported by Susilo *et al.* (2016) represented by 15 clones of genetic materials that would much lower compared to this study using 900 genotypes belong to 5 F_1 hybrid populations which presenting high genetic diversity. Harimurti *et al.* (2004) supported this results by showing that coefficient of correlation among traits may be lower when testing on segregating popu-

Table 2. The coefficient of correlation between the score of VSD damage with the leaf stomatal characteristics through F_1 hybrid populations

Variables	Score of VSD damage	Stomata density	Stomata length	Width of opening stomata	Stomata width	Stomata diameter
Score of VSD damage	1					
Stomata density	0.18 *	1				
Stomata length	0.26 *	-0.23 *	1			
Width of opening stomata	0.49 *	-0.08 ns	0.2 *	1		
Stomata width	0.24 *	-0.22 *	0.44 *	0.32 *	1	
Stomata diameter	0.12 *	-0.3 *	0.41 *	0.12 *	0.5 *	1

Note: the coefficient number between variables which be followed with (*) mark indicate significant and followed with (ns) mark not significant at the significant level of 95%.

Table 3. Path analysis (direct and indirect effects) of the leaf stomatal characteristics contributed to VSD damage

Direct/Indirect	Stomata density	Stomata length	Width of opening stomata	Stomata width	Stomata diameter	Correlation to VSD damage
Stomata density	0.282 *	-0.065	-0.022	-0.062	-0.085	0.178
Stomata length	-0.047	0.201 *	0.039	0.089	0.083	0.262
Width of opening stomata	-0.035	0.089	0.449 *	0.144	0.053	0.487
Stomata width	-0.009	0.019	0.014	0.045 ^{ns}	0.023	0.238
Stomata diameter	-0.013	0.017	0.005	0.021	0.042 ^{ns}	0.116

Note: number written in bold letter indicate direct coefficient of path analysis to VSD damage.

lation (F₁). This study supported the previous report in which the width of opening stomata consistently showed stronger relationship to VSD damage as its coefficient of correlation was stable higher when evaluated at segregating population (F₁).

The analysis of correlation just show relatedness of the stomata characteristics to VSD damage, however it could not be identified of which characteristic directly contribute to VSD susceptibility. Furthermore, to meet the purpose path analysis was used to overview the contribution of each characteristic to VSD susceptibility. By using path analysis it could be observed the causal interaction between independence variables (predictor) and dependence variables (response variable) as the coefficient of correlation among variables could be separated into direct and indirect effects (Dewey & Lu *cit.* Samonte *et al.*, 1998). Data presented in Table 3 indicate that the width of opening stomata had positive direct effect VSD damage scores, similar with density and length of stomata. It was also shown that the coefficient of correlation of the width of opening stomata as higher as the path coefficient of the direct effect to of VSD damage scores that means the width of opening stomata reconfirmed of which characteristics had higher contribution to VSD susceptibility. Furthermore, the width of opening stomata could be developed as criteria for selection the VSD resistance regard to the less value of the characteristics. Li *et al.* (2015) also reported the leaf stomata

characteristics significantly different between the resistant variety of transgenic cotton and traditional variety regard to their resistance to *Fusarium oxysporum*. The report informed that the resistant variety of transgenic cotton performed significant higher size of stomata size and less stomata density compared to the traditional variety (non-trangenic). Maan *et al.* (2017) also supported the results which reported the stomata density of potato leaves were negatively correlated to their resistance to apical leaf-curl disease. Refer to these reports it could be concluded that the leaf stomata characteristics play specific role to the resistant mechanism regard to the plant species. The leaf stomata function on VSD resistance may play physically on facilitating the entering of *C. theobromae* fungus through the leaf tissue to infect the xylem bundle sheets by which the larger size of those stomata characteristics are related with the higher VSD susceptibility.

Heterosis and Heterobeltiosis

There are two genetic parameters to show hybrid vigor, namely heterosis representing hybrid vigor of F₁ hybrid compared to the mean of both parent and heterobeltiosis representing hybrid vigor of F₁ hybrid compared to the best parent. The value of heterosis and heterobeltiosis depend on the genetic distance between parental crosses (Daryanto *et al.*, 2010). Heterobeltiosis is more important parameter as the value be compared

to the best parent that means vigorosity of the hybrid better quality than the best parent. In this research, the term of hybrid vigor was determined with the lower score of VSD damage and lower value of the stomata characteristics due to the resistance related to the lower value of those parameters. The analysis showed that the heterosis and heterobeltiosis values of the tested hybrids ranged between -50.42–27.87% and -33.58–1,763% respectively. The wide range of the values indicates differences on the effectiveness of resistant inheritance through F_1 hybrid between the resistant clones. Based on the data, it showed that the hybrid of KW 617 x KW 264 had lower value of heterosis and heterobeltiosis for the VSD damage scores by which could be selected as best crosses to generate VSD resistant genotypes. Singh *et al.* (2017) selected the best crosses for early blight resistance based on the lower heterosis values for the damage score and higher values of heterosis for yield.

Selecting crosses for generating more vigorous hybrids, beside refer to the estimated values of heterosis and heterobeltiosis, also many refer to the mean of F_1 and the combining ability (Arif *et al.*, 2012). This tested hybrids were not crossed in accordance with crossing design for identifying the combining ability of parental clones that the parents could not be estimated of their combining abilities, both general or specific combining abilities. Based on the mean value of F_1 hybrid it showed that the F_1 hybrid of KW 617 x KW 264 crosses also performed lower value of the mean that supporting the conclusion they were better crosses to generate VSD resistant genotypes. Other than the crosses, the cross combination of TSH 858 x Sulawesi 3 also performed lower value of the estimated heterosis but having higher mean value of F_1 hybrids compared to the crosses of TSH 858 x Sulawesi 1, TSH 858 x KEE 2 and Sca 6 x KW 164

which of their F_1 hybrid performed higher value of the estimated heterosis. Furthermore refer to the parameters of heterosis, heterobeltiosis and mean value of F_1 hybrid that it could be selected in order the best crosses to generate VSD resistant genotypes, namely KW 617 x KW 264, TSH 858 x Sulawesi 1 and TSH 858 x KEE 2.

This result confirmed the differences on resistant inheritance among some VSD resistant clones such as Sulawesi 1, KEE 2 and Sca 6. It was reported that the differences on the pattern of VSD resistant inheritance between KEE 2 and KW 162 (Sulawesi 1) in which KEE 2 performed monogenic pattern of inheritance (1:1) and Sulawesi 1 performed digenic pattern of inheritance (3:1) (Susilo, 2015). KEE 2 was previously reported to show specific combining ability for VSD resistance that it should be crossed with specific clones which had better combining ability to KEE 2. However, in contrary the parental clone of Sulawesi 1 performed good general combining ability for VSD resistance that could be crossed to many other cross-compatible clones. Furthermore, it was proposed to construct best crosses for generating VSD-resistant genotypes by combining the crosses of Sulawesi 1 x KEE 2 as both of which are cross-compatible genotype. By referring to the studies therefore it could be overviewed the differences on inheritance effectiveness of the resistant clones by ordered from the highest to the lowest are KW 617, Sulawesi 1, KEE 2, Sulawesi 3 and Sca 6.

The estimated values of heterosis and heterobeltiosis for leaf stomata characteristics were identified in similar pattern of their inheritance with the scores of VSD damage. The best crosses of KW 617 x KW 264 performed lower values of heterosis and heterobeltiosis for most of the stomata characteristics such as the length, width

Table 4. Performance of parental clones (P1, P2), F₁, mid parent (MP) value, heterosis (H) and heterobeltiosis (HB) of the cross for VSD scores and the stomatal characteristics

Variables	Crosses	P1	P2	F ₁	MP	H	HB
Score of VSD damage	TSH 858 x Sul 1	2.52	1.45	1.39	1.98	-29.97	-4.14
	TSH 858 x KEE 2	2.52	1.06	1.48	1.78	-17.32	39.62
	TSH 858 x Sul 3	2.52	2.09	1.5	2.30	-34.92	-28.23
	Sca 6 x KW264	0.08	2.25	1.49	1.16	27.897	1762.50
	KW 617 x KW 264	1.34	2.25	0.89	1.79	-50.42	-33.58
Stomata density	TSH 858 x Sul 1	92	104	100	98	2.04	8.70
	TSH 858 x KEE 2	92	65	88	78.5	12.10	35.38
	TSH 858 x Sul 3	92	63	95	77.5	22.58	50.79
	Sca 6 x KW264	78	114	106	96	10.42	35.90
	KW 617 x KW 264	50	114	86	82	4.88	72.00
Stomata length	TSH 858 x Sul 1	18	19.6	19.85	18.8	5.59	10.28
	TSH 858 x KEE 2	18	21.4	21.7	19.7	10.15	20.56
	TSH 858 x Sul 3	18	16	19.12	17.0	12.47	19.50
	Sca 6 x KW264	18.8	27.8	18.89	23.3	-18.93	0.48
	KW 617 x KW 264	20.6	27.8	16.78	24.2	-30.66	-18.54
Width of the opening stomata	TSH 858 x Sul 1	6.7	4.9	5.81	5.8	0.17	18.57
	TSH 858 x KEE 2	6.7	5.4	5.02	6.05	-17.02	-7.04
	TSH 858 x Sul 3	6.7	5.1	4.92	5.9	-16.61	-3.53
	Sca 6 x KW264	4.7	7.1	4.65	5.9	-21.19	-1.06
	KW 617 x KW 264	4.9	7.1	4.47	6.0	-25.50	-8.78
Stomata width	TSH 858 x Sul 1	20.6	22.5	20.11	21.55	-6.68	-2.38
	TSH 858 x KEE 2	20.6	25.7	20.98	23.15	-9.37	1.84
	TSH 858 x Sul 3	20.6	22.9	21.4	21.75	-1.60	3.88
	Sca 6 x KW264	18.3	23.9	22.14	21.1	4.93	20.98
	KW 617 x KW 264	23.1	23.9	20.15	23.5	-14.25	-12.77
Diameter of stomata	TSH 858 x Sul 1	59.6	56.7	55.56	58.15	-4.45	-2.01
	TSH 858 x KEE 2	59.6	54.7	58.41	57.15	2.21	6.78
	TSH 858 x Sul 3	59.6	56.7	57.92	58.15	-0.39	2.15
	Sca 6 x KW264	56.2	66.2	57.39	61.2	-6.23	2.12
	KW 617 x KW 264	51.7	66.2	58.99	58.95	0.068	14.10

Notes : P1 = mean of female parent, P2 = mean of male parent, F1 = mean of F1 population, MP = mean of both parent, H = heterosis, HB = heterobeltiosis.

of opening stomata and stomata width. However, the crosses of TSH 858 x Sulawesi 1 had lower values of the heterosis and heterobeltiosis for stomata density. This results show a consistency inter-correlation between leaf stomata characteristics to VSD damage scores in term of inheritance pattern that the characteristics would perform genetic expression of the resistance. Brodribb *et al.* (2017) reported the mechanism on water distribution in plant were governed with a strong coordination system between xylem tissue and stomata activity for controlling evapotranspiration. This mechanism could be connected to the mechanism of VSD resistance as the symptom of fungus infection will defect the xylem bundle sheet that affecting water transpor-

tation in which directly connected to the stomata activity. Furthermore, there should be more studied on the interaction mechanism between xylem-bundle sheet defect to the stomata activity induced by VSD infection.

To develop criteria for selection the VSD-resistant genotypes using leaf stomata characteristics there should be defined the specific value of those characteristics refer to resistant classification as manual guidance for breeders. This result just informed in general overview that there was any negative relationship between leaf stomata characteristics to VSD damage scores but there is no group of those values regard to resistant classification. Therefore it should be clustered of the tested genotypes refer to the width of

opening stomata and the score of VSD damage that could be defined the range value representing the resistant genotype. By using the range values of width of opening stomata refer to the resistant genotype then breeder can testing the effectiveness for selecting VSD-resistant genotype.

CONCLUSIONS

Most of the leaf stomata characteristics positively correlated to VSD damage scores in which the coefficient of correlation varied in the range of 0.18 - 0.49. The width of opening stomata performed the highest value of the coefficient indicating close related to VSD susceptibility and also endorsed with direct positive effect of the characteristics contribute to VSD damage scores according to path analysis.

The estimated value of heterosis and heterobeltiosis for VSD damage scores varied among F_1 hybrids ranged between -50.42–27.87% and -33.58–1,762%; respectively indicated differences on the effectiveness of resistant inheritance among VSD-resistant clones. Refer to the estimated values of heterosis and heterobeltiosis for VSD damage scores and width of opening stomata, and the mean value of F_1 hybrid that could be selected the best crosses for generating VSD-resistant genotypes in order of KW 617 x KW 264, TSH 858 x Sulawesi 1 and TSH 858 x Sulawesi 3. It was also concluded the best parental clones for crosses are KW 617, Sulawesi 1, KEE 2, Sulawesi 3 and Sca 6.

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