

## Anthocyanin and Chlorophyll Content in Flush as Selection Markers on Fine Flavor Cocoa (*Theobroma cacao* L.)

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### Abstract

Level of anthocyanin content in flush is different between fine flavor cocoa and bulk cocoa. This study was aimed to determine the potential of anthocyanin and chlorophyll content in flush as one of the selection indicator for fine flavor cocoa. Flush sampling was carried out at Cocoa Germplasm Collections in Kaliwining Experimental Station, Indonesian Coffee and Cocoa Research Institute, while anthocyanin and chlorophyll analysis was conducted at Laboratory of Ma Chung Research Center for Photosynthetic Pigments, Ma Chung University, Malang. The research utilized a completely randomized design consisted of four genotypes of fine flavor cocoa, namely DR 2, ICCRI 02, DRC 16, PNT 16 and five genotypes of bulk cocoa, namely Sulawesi 1, MCC 01, MCC 02, KW 617 and PA 191. Each treatment was repeated three times. Anthocyanin and chlorophyll content in flush was measured. Anthocyanin analysis was done by destructive methods, while flush color was observed based on Munsell Color Chart for Plant Tissues Data analysis used orthogonal contrast, multivariate and simple correlation. The results showed that there were differences in flush color on fine flavor cocoa and that of bulk cocoa. The flush of the fine flavor cocoa had a tendency towards yellow and that of bulk cocoa had a tendency toward red with different levels of variation among genotypes. The analysis results of chlorophyll content in flush showed that chlorophyll content of fine flavor cocoa was higher than bulk cocoa with a range of 2376-3692  $\mu\text{mol/L}$  for fine flavor cocoa and 1567-2628  $\mu\text{mol/L}$  for bulk cocoa. On the contrary, anthocyanin content in fine flavor cocoa was lower than bulk cocoa with a range of 3.82-5.72  $\mu\text{mol/L}$  for fine flavor cocoa and 6.01-11.88  $\mu\text{mol/L}$  for bulk cocoa. The analysis results of orthogonal contrasts showed that chlorophyll content value was more stable than anthocyanin. There was negative relationship between anthocyanin and chlorophyll in flush with the regression  $Y = -221,1X + 3935$  with a value of correlation  $r = -0.50$ . The anthocyanin and chlorophyll content in flush had a potential to be used as selection markers for fine flavor cocoa hybrid.

**Keywords:** anthocyanin, chlorophyll, flush, selection markers, *Theobromacacao* L.

## INTRODUCTION

A fine-flavor cocoa or better known as Java Cocoa is one of Indonesian specialty which has successfully entered world cocoa market. This type of cocoa has been planted in Indonesia since 1888 (van der Knaap, 1953 *cit.* Mawardi, 1982) and has been known as Java Criollo (Mawardi, 1982). This particular cocoa has unique character with its distinctive flavor and is generally better than bulk cocoa. Java Cocoa's distinctive feature is its white bean color, while fresh bulk cocoa bean possesses purple color (Anita-Sari *et al.*, 2014). Fine-flavor cocoa and bulk cocoa demand continues to increase each year. High demand of cocoa is related to the importance of maintaining chocolate products quality, especially in terms of flavor (Daniel *et al.*, 2012). This provides an opportunity for Indonesia to be able to continue increasing Fine-flavor cocoa production. However, production has decreased lately, one of which is caused by vascular streak dieback (VSD) disease.

Efforts to increase fine-flavor cocoa production is still continuing, but an obstacle encountered in the effort of assembling new planting material due to the lack of fine-flavor cocoa genetic resources which is under extinction threat (Anita-Sari *et al.*, 2014). Enriching genetic resources is the key factor in the breeding of the fine-flavor cocoa. The process of assembling the fine-flavor cocoa planting materials, in addition to genetic diversity requires a long periods of time. It is caused by the time spent waiting for cocoa plant to produce fruit before researcher could determine its seeds color. According to Sukartini & Shah (2009), another obstacle encountered is the amount of time required for selection process which is done during regenerative phase. Before making selections of characters which will be encountered at generative phase, it is required to look for criterias used to select breeds early.

Fine-flavor cocoa selection methods have been developed since 1997 through the identification of flower colors and its influence on seed color (Winarno & Iswanto, 1997). However in its development, the theory was disproved because they discovered some flowers of bulk cocoa germplasm did not contain anthocyanin. On the other hand, *fine-flavor cocoa* flowers contain anthocyanin. Results of other studies (Adhiwibawa *et al.*, 2015) stated that of fine-flavor cocoa flush pigment reflectance differs from other cocoa. Anthocyanin level which is indicated by red pigment reflectance on bulk cocoa proved to be higher than that of fine-flavor cocoa. The results are consistent with the results of Anita-Sari *et al.* (2016) research which stated that anthocyanin content in fine-flavor cocoa flush is different with bulk cocoa. Fine-flavor cocoa anthocyanin content is relatively low compared to bulk cocoa. Research on other commodities exhibits anthocyanin pigment content in one of the organs will affect anthocyanin content in other organs. Anthocyanin is one of phenolic pigments which are expressed as red, blue (Lee & Kevin, 2002) and purple color (Close & Christopher, 2003). The pigments are located in the cell's vacuole and functions as antioxidants (Woodson *cit.* Sukartini & Shah, 2009). Anthocyanin content of cocoa plant can be found in the leaves, flowers, fruits and seeds (Anita-Sari *et al.*, 2015). According to Gould & Quinn (1996), anthocyanin found in cell palisade and mesophyll cork. Anthocyanin synthesis occurs during the growth of leaves (Woodall & Stewart, 1998), during senescence (Field *et al.*, 2001) and when plants respond to abiotic stresses (Close *et al.*, 2000).

Results of related studies mention that anthocyanin formation in plant parts can occur simultaneously with chlorophyll formation. It is formed in quantity should the plant color is green. Therefore it indicates that

plant part contains less anthocyanin compared to chlorophyll (Drumm-Herrel & Mohr *cit.* Sukartini & Shah, 2009). Anthocyanin formation is inhibited by chlorophyll formation (Woodall & Stewart, 1998). Stabilization of the selection criteria needs to be done to establish appropriate selection method to enable early selection process. Early selection method on assembling fine-flavor cocoa planting material is very useful, especially for fine-flavor cocoa and non-fine-flavor cocoa crosses. It would enable selection through organs other than flower such as fruit and seed. Accelerating selection process through certain criteria is particularly useful to increase genetic diversity of fine-flavor cocoa through crossbreeding. The purpose of this research is to find characters other than seed color to be used in selection method. It is done in order to enable early selection on fine-flavor cocoa.

## MATERIALS AND METHODS

The study was conducted in March to August 2016. Sampling process was conducted in Cocoa Germplasm Collection Garden of Kaliwining Experimental, Indonesian Coffee and Cocoa Research Institute, Jember. Anthocyanin and chlorophyll analysis was conducted at Ma Chung Research Center Laboratory for Photosynthetic Pigments, Universitas Ma Chung, Malang, East Java Indonesia. The experiment was conducted in a laboratory scale using a completely randomized design

with three repetitions. Each repetition consisted of five flush samples taken from plants in the field randomly. Five flush samples were taken from each of fifteen (15) plants of each genotype. Flush samples were put into the ice-filled cooler to be transferred to Ma Chung Research Center Laboratory for Photosynthetic Pigments. Table 1 presented the four accessions of fine-flavor cocoa and five accessions of bulk cocoa used in this research.

The parameters measured were anthocyanin and chlorophyll flush content of each genotype. Anthocyanin and chlorophyll content were analyzed using destructive methods referring to Sims & Gamon (2002) and Porra (2002). Color analysis was conducted based on Munsell Color Chart (Wilde, 2004) by matching flush color on each genotype with color on Munsell Color Chart. Data analysis was performed by calculating average concentration of anthocyanins and chlorophyll in each genotype tested. Variance analysis was conducted using orthogonal contrast analysis using SAS 9.1 program. Cluster analysis was conducted by utilizing multivariate-cluster analysis in STAR statistical program.

## RESULTS AND DISCUSSION

Anthocyanin and chlorophyll content in four fine-flavor cocoa or Java Cacao genotypes flush was significantly different from that of the five non-fine-flavor cocoa genotypes (bulk). Chlorophyll content in the

Table 1. Fine-flavor cocoa and bulk cocoa genotypes for anthocyanin and chlorophyll study

Genotype	Information
Sulawesi 01	Bulk cocoa, exploration result in East Kalimantan Province
MCC 01	Bulk cocoa, exploration result in South Sulawesi Province
MCC 02	Bulk cocoa, exploration result in South Sulawesi Province
PA 191	Bulk cocoa, clone of Upper Amazon Forastero from Parinari
KW 617	Bulk cocoa, selection result of Sulawesi 01 x TSH 858 crossbreeding
DR 2	Fine-flavor cocoa result in Djati Roenggo
ICCRI 02	Fine-flavor cocoa, exploration result in Penataran, East Java
DRC 16	Fine-flavor cocoa, selection result of DR 53's line from Djati Roenggo
PNT 16	Fine-flavor cocoa, exploration result in East Java

Table 2. Variance analysis of chlorophyll and anthocyanin content of fine-flavor cocoa and bulk cocoa genotypes

Source of variations	df	Mean square			Calculated F		
		Chlorophyll	Anthocyanin	Chlorophyll/ anthocyanin	Chlorophyll	Anthocyanin	Chlorophyll/ anthocyanin
Genotype	8	2286972	0.77569	309686	2.34 *	8.88 **	3.97 *
Replication	2	288217	0.12986	49946.	0.29 ns	1.32 ns	0.64 *
<b>Contrast</b>							
Bulk vs Fine-flavor	1	902345	65.81	1756524	9.18 *	32.53 **	22.50 ns
(Sulawesi 1, PA 191) vs (DRC 16, PNT 16)	1	5736369	71.33	1233032	5.86 *	35.26 **	15.80 ns
(MCC 02, KW 617) vs (DR 2, ICCRI 02)	1	5372240	0.67014	505158	5.49 *	7.74 *	6.47 ns
MCC 01 vs DR 2	1	531418	0.21	14473	4.55 *	0.10 ns	0.19 *
MCC 01 vs ICCRI 02	1	5086834	0.1069	420034	5.19 *	0.96 ns	5.38 ns
MCC 01 vs DRC 16	1	4071524	0.21181	495417	4.16 *	2.30 ns	6.35 ns
MCC 01 vs PNT 16	1	5649568	0.34861	638323	5.77 *	3.86 *	8.18 ns
<b>Bulk vs Bulk</b>							
(Sulawesi 1, PA 191) vs (MCC 01, MCC 02, KW 617)	1	881478	12.11	14311	0.90 ns	5.99 *	0.06 **
Sulawesi 1 VS PA 191	1	1137487	51.54	137072	1.16 ns	25.48 **	1.76 *
(Sulawesi 1, PA 191) (MCC 01, KW 617)	1	854950	5.26	4889	0.87 ns	2.6 ns	0.18 * vs
<b>Fine-flavor vs Fine-flavor</b>							
(DR 2, ICCRI 02) vs (DRC 16, PNT 16)	1	1031614	0.22292	269677	1.05 ns	2.38 ns	3.46 *
DR 2 vs ICCR 02	1	2309153	0.06042	278570	2.36 ns	0.43 ns	3.57 *
DRC 16 vs PNT 16	1	158750	0.41	9043	0.16 ns	0.20 ns	0.12 *

Notes: \*\*) Significantly different according to F test at 1% level; \*) Significantly different and \*\*) Not significantly different according to F test at 5% level.

cross genotype fine-flavor cocoa's did not show significant differences, as well as bulk cocoa cross genotype. However, flush anthocyanin content exhibited variations for anthocyanin content in bulk cocoa flush. Fine-flavor cocoa flush anthocyanin content was significantly different compared to bulk cocoa (Table 2). These results concur with Adhiwibawa *et al.* (2015) and Anita-Sari *et al.* (2016) researches which found that anthocyanin content in fine-flavor cocoa flush has different patterns tendency compared to bulk cocoa. Anthocyanin content in fine-flavor cocoa flush was lower compared to bulk cocoa, either through color reflectance analysis (Adhiwibawa *et al.*, 2015) or destructive analysis.

The analysis of chlorophyll content of cross-fine-flavor cocoa did not show

significant differences, as well as cross bulk cocoa. On the other hand, anthocyanin content in cross bulk cocoa exhibited significant differences (Table 2). Cross bulk cocoa anthocyanin content variance exhibits that anthocyanin content could not be used as indicator for fine-flavor cocoa early selection due to its characteristic instability among tested cocoa groups. While chlorophyll content showed good stability in cross fine-flavor cocoa and cross bulk cocoa respectively, it was not significantly different in fine-flavor cocoa and bulk cocoa groups. It has a tendency to be more stable. Thus, flush chlorophyll content can be used as one of fine-flavor cocoa early selection criteria.

Anthocyanin and chlorophyll content ratio in flush did not exhibit good stability in both

types of tested cocoa. Contrast analysis result exhibited significant differences on chlorophyll and anthocyanin ratio parameter for bulk cocoa and fine-flavor cocoa. Thus chlorophyll and anthocyanin ratio parameter can not be used as indicator for fine-flavor cocoa early selection process. These results correspond with Sims & Gamon (2002) research result which stated that anthocyanins have a strong influence on chlorophyll; therefore anthocyanin has relatively no effect on chlorophyll content. Red/green ratio has a weak connection to anthocyanin and chlorophyll content. In contrast Gamon & Surfus (1999) found that red/green ratio indicated good connection to anthocyanin and chlorophyll content. Gamon & Sims (2002), stated that each species had red/green ratio, anthocyanin content and reflectance difference, even with chlorophyll absence depending on cell chemical environment.

The average value of the anthocyanin content in fine-flavor cocoa flush was lower than bulk cocoa with a range of 3.82–5.72  $\mu\text{mol/L}$  for fine-flavor cocoa and 6.01–11.88  $\mu\text{mol/L}$  for bulk cocoa. In contrast, fine-flavor cocoa had higher average flush chlorophyll content compared to bulk cocoa with a range of 2376.47–3692.35  $\mu\text{mol/L}$  for fine-flavor cocoa and bulk cocoa amounts to 1567.33–2627.91  $\mu\text{mol/L}$  (Table 3). Different leaves color exhibits pigment content difference, especially chlorophyll and anthocyanin (Markham, 1988).

Bulk cocoa possess higher anthocyanin content in flush organ compared to fine-flavor cocoa. The existence of positive connection for anthocyanin content in flush and seeds was researched in ‘mango plant phenomenon’ conducted by Sukartini & Shah (2009) which states anthocyanin content in mango leaf have positive connection to anthocyanin content in mango fruit color. Anthocyanins are calculated based on cells mesocarp palisade exterior and mesophyll of darker leaves (Lightbourn *et al.*, 2008). According to Lee & Kevin (2002), anthocyanin phenolic pigment expressed as red, blue and purple color code (Close & Christopher, 2003; Tanaka *et al.*, 2008). The pigment exists in vacuole cell and function as antioxidants (Tanaka *et al.*, 2008). According to Lightbourn *et al.* (2008), color is formed from several pigments, including chlorophyll, carotenoids, flavonoids and betalains. Betalains was divided into two classes, betaxanthins and betacyanins which produce yellow to orange or violet color (Harris *et al.*, 2011). Flavonoids can be divided into colorless and color (anthocyanin). Chlorophyll is located at chloroplasts within cytoplasm cell. Carotenoid exists in chromatoplast while flavonoids and betalain are in vacuole cell.

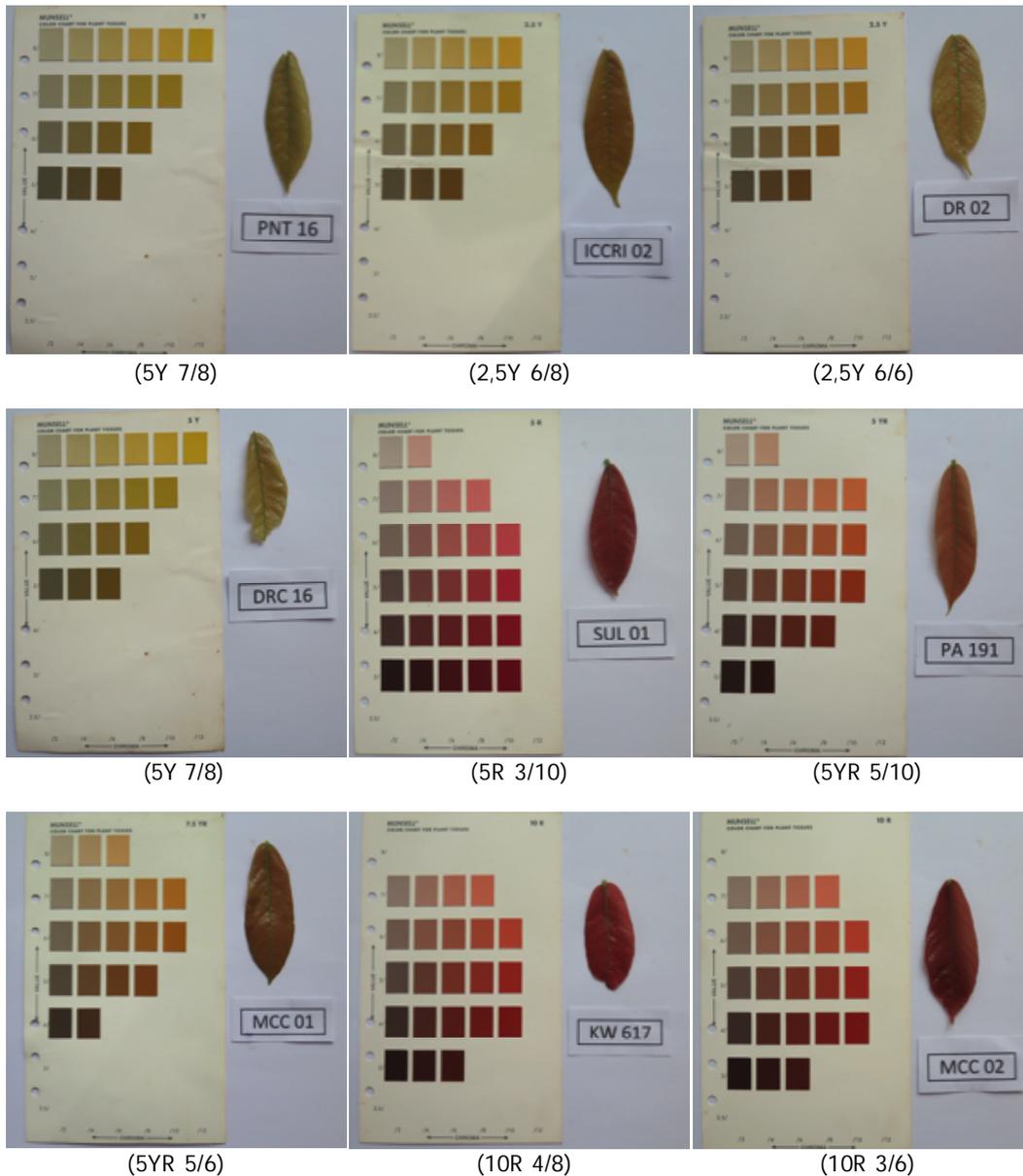
Determining flush aside from utilizing destructive method is using Munsell color chart. Analysis result using Munsell color chart showed that fine-flavor cocoa possessed

Table 3. Average of chlorophyll and anthocyanin content on flush of fine-flavor cocoa and bulk cocoa

Genotype	Chlorophyll concentration ( $\mu\text{mol/L}$ )	Anthocyanin concentration ( $\mu\text{mol/L}$ )
	Means $\pm$ standard deviation	Means $\pm$ standard deviation
Sulawesi 01	1757 $\pm$ 142	11.88 $\pm$ 1.15
PA 191	2628 $\pm$ 61	6.01 $\pm$ 0.96
MCC 01	1776 $\pm$ 46	6.09 $\pm$ 0.32
MCC 02	1567 $\pm$ 130	8.39 $\pm$ 0.52
KW 617	1750 $\pm$ 60	6.85 $\pm$ 0.98
DR 2	2376 $\pm$ 78	5.72 $\pm$ 0.64
ICCRI 02	3617 $\pm$ 288	4.96 $\pm$ 0.17
DRC 16	3423 $\pm$ 161	4.33 $\pm$ 0.78
PNT 16	3692 $\pm$ 277	3.81 $\pm$ 0.37

flush color tendency toward yellow color (Y = yellow) with varying cross genotype intensity, unlike bulk cocoa which had tendency to red color (R = red) with varying cross genotype intensity (Figure 1). Flush color of the MCC 01 clones were included in bulk cocoa group in which visually exhibited

pale color, but it possessed a tendency toward red instead of yellow. Based on Munsell color chart analysis on both cocoa groups, there was good stability in flush color pattern. Fine-flavor cocoa possessed yellow color tendency (Y), while bulk cocoa had red color tendency (R).



Gambar 9. Variasi warna *flush* pada empat genotipe kakao mulia dan lima genotipe kakao lindak

Anthocyanins normally exist in young leaves, roots and stems. Anthocyanins, especially those at leaf upper surface or in epidermal cells play a role in plant physiological processes (Jordheim, 2007). Anthocyanin synthesis occurs during leaves growth (Woodall *et al.*, 1998). Anthocyanin biosynthesis started from cinnamic acid production of phenylalanine enzyme in shikimic acid cycle conducted by phenylalanine amoniliase enzyme (PAL) which then converted to fumaric acid and modified into malonil CoA. Anthocyanin biosynthesis is controlled by enzyme activity that in certain conditions can be affected by extreme environmental conditions (Woodall *et al.*, 1998).

Leaf pigment is integrally associated to the leaf functions (Sims & Gamon, 2002). Leaf color is indicated by three color combination namely compounds that produce green chlorophyll, carotenoids and flavonoids. They exhibit yellow and purple anthocyanins which show up as blue color. Young leaves or flush is dominated by anthocyanin content. Anthocyanins are more involved in the early stages of the leaf formation. It is caused by sugar content formed in plant before it could photosynthesize (Alkema & Seagerl, 1982; Mlodzinska 2009).

Simple correlation analysis result exhibits that high levels of anthocyanin content associated with lower chlorophyll content in cocoa flush with regression equation  $Y = -221.1X + 3935$ , and correlation  $r$  value = -0.50. Anthocyanin formation in plant can occur simultaneously with chlorophyll formation in quantity should a plant is green in color, therefore that section contains less anthocyanin compared to chlorophyll (Drumm-Herrel & Mohr *cit.* Sukartini & Shah, 2009) Anthocyanin formation could be inhibited by the formation of chlorophyll (Woodall *et al.*, 1998). Sims & Gamon (2002) stated that there was an association between chlorophyll content with other formed colors.

There was a correlation between red/green ratio on anthocyanin and chlorophyll content in the leaves. Anthocyanin content depends on plant growing environment.

Cluster analysis result based on chlorophyll and anthocyanin characteristics shows separate grouping between fine-flavor cocoa and bulk cocoa (Figure 3). In general, the pattern of chlorophyll and anthocyanin content distribution for fine-flavor cocoa and bulk cocoa could be separated visually, destructively and non destructively. Related research results explained that anthocyanins play an important role in morphology indicator for plant selection process produced by crossbreeding (Wang, 2007; Dai *et al.*, 2016). It can be used as indicator for pigmentation pattern and plant gene expression (Albert *et al.*, 2014). There is a correlation between fruit and petal color. Cultivars with red fruit color will have dark red petals (Holland *et al.*, 2009). There is stable pattern tendency between anthocyanin and chlorophyll content of *fine-flavor cocoa* and bulk cocoa flush. Both of these characters can be used as morphological indicators in selecting fine-flavor cocoa especially those produced from crossbreeding.

The potency of anthocyanin and chlorophyll measurement in fine-flavor cocoa flush to be used as morphological indicators for selection criteria needs to be supported in molecular level. It is caused by the fact that anthocyanin and chlorophyll content is influenced by environmental conditions. Molecular analysis approach of genes controlling anthocyanin and chlorophyll in flush organ such as chloroplast genes are necessary to support the establishment of the selection criteria. *Maturase K (matK)* gene is one of genes existing in chloroplast is regarded as the most accurate gene used to detect kinship of a species (Virgilio *et al.*, 2012). Gene analysis of *matK* is one approach to

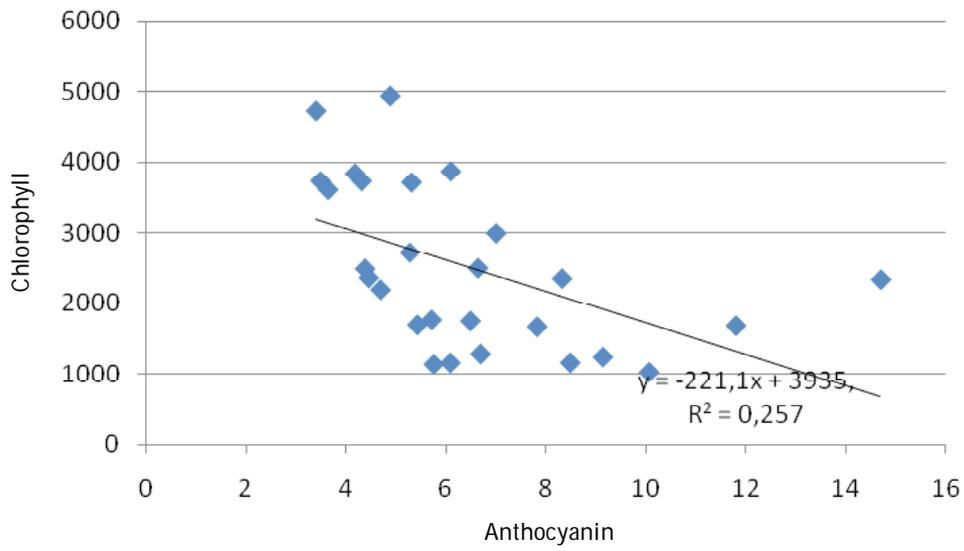


Figure 2. Relationship between chlorophyll and anthocyanin content in flushes of cocoa plants

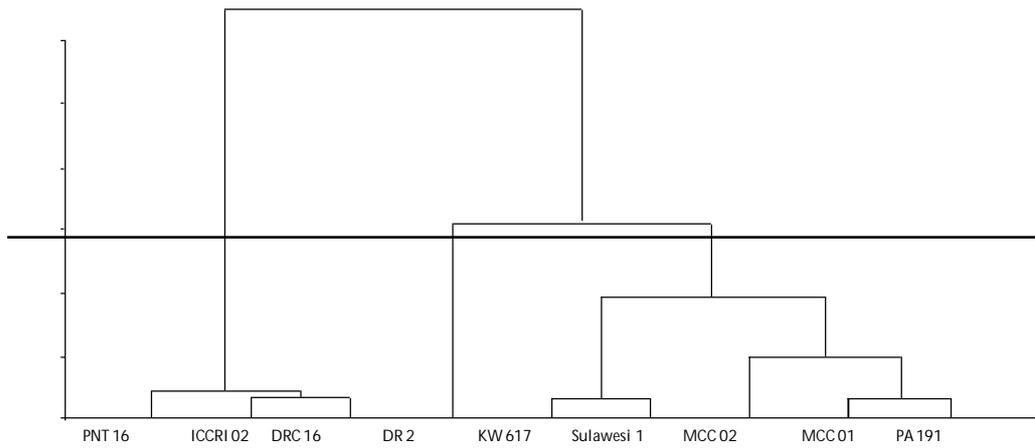


Figure 3. Dendrogram of fine-flavor cocoa and bulk cocoa genotype based on anthocyanin and chlorophyll content

find out anthocyanin and chlorophyll characteristics in fine-flavor cocoa and bulk cocoa flush organ without any environmental influences.

### CONCLUSION

Chlorophyll content in fine-flavor cocoa flush is higher compared to bulk cocoa. On the other hand, anthocyanin content in bulk cocoa was higher than fine-flavor cocoa. Chlorophyll content showed good stability in both cocoa types therefore chlorophyll could be used as one of selection criteria with undetermined threshold intensity. Fine-flavor cocoa flush color had yellow color tendency (Y = yellow) and bulk cocoa tended to possess red color (R = red) with different levels of variation among genotypes. Flush color characteristics can be used as an indicator of early selection for fine-flavor cocoa in seedling/vegetative stage.

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