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EFFECTIVE CACAO SOMATIC EMBRYO REGENERATION ON KINETIN SUPPLEMENTED DKW MEDIUM AND SOMACLONAL VARIATION ASSESSMENT USING SSRs MARKERS

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ABSTRACT

This study aimed to develop the cacao (*Theobroma cacao* L.) *in vitro* regeneration system through somatic embryogenesis on kinetin supplemented DKW medium and somaclonal variation assessment using SSR markers. Callus were initiated from basal petal and staminoid explants cultured on callus induction (CI) medium contained DKW basalt salts and kinetin:2,4-D ratios of 1:15.5, 1:7.8 or 1:3.9 and then transferred onto secondary callus growth (SCG) medium contained WPM basalt salts and kinetin:2,4-D ratios of 1:7.8 or 1:3.9. The calli were then subsequently transferred onto embryo development medium contained DKW basal salts with or without the addition of amino acids, adenine or activated charcoal for the formation of somatic embryos. Nine cacao genotypes were tested for their ability to develop somatic embryos. Results of this study indicated DKW medium supplemented with Kinetin in combination with 2,4-D effectively induced cacao somatic embryogenesis. The highest somatic embryos formation was abtained from kinetin:2,4-D ratio of 1:3.9 and 1:7.8 in CI and SCG medium respectively. Cacao genotype responses were highly explant type dependent. The developed method resulted in a high percentage of somatic embryo formation (5.6-66.7%), germination (50%) and plantlet conversion (65%) and a medium percentage of somaclonal variations based on SSRs marker analysis.

Keywords: explants; genotypes; kinetin:2,4-D; SSRs; *Theobroma cacao* L.

INTRODUCTION

Cacao (*Theobroma cacao* L.) is the only plant widely used to produce chocolate. The world cacao demand continues to increase because of the increasing economic growth and public awareness about health benefit of eating chocolate. Cocoa product contains phenols, flavonoids and its antioxidant activity is higher than black tea, green tea or red wine (Subhashini *et al*., 2010). Eventhough global cacao demand has increased significantly, its production is not and there is a world deficit in cacao supply in recent years (International Cocoa Organization, 2014). Global climate change, pest and disease infestation and reduce plant productivity because of aging have caused world cacao production instability. Stabble cacao production is important for many cacao producing countries to maintain export stability and to ensure supply continuity of industrial raw materials to chocolate industries.

Cacao has an important economic role as a source of foreign exchange in Indonesia. However, the area number of constraints in cacao productionin Indonesia, such as: low yield, pest and disease attack, low bean quality and under develop downstream cacao industries. Recent cacao average productivity in Indonesia reaches 837 kg/ha/year (Indonesian Ministry of Agriculture, 2014), which is much lower than the average of expected cacao bean yield potential of 2000 kg

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ha^{-1} $year^{-1}$. The use of low quality planting materials, inappropriate agronomic technology, pest and plant disease infestation and the plant age are the major contributors to low cacao productivity in Indonesia (Rubiyo and Siswanto, 2012).

In general, seeds or grafted seedlings are used as cacao planting materials. Since cacao is naturally cross-pollinated, cacao planting materials from seeds are usually exhibits a highly heterogeneous genetic background. Therefore, agronomic performance of cacao plantation derived from seeds are highly variable (Li *et al.*, 1998). Cacao planting materials propagated clonally through grafting result in both low multiplication rate and undesirable bushy-like growth pattern. Micropropagation through plant tissue culture is expected to overcome these obstacles.

Cacao breeding usually take a long time and this is because of the long life cycle and the narrow genetic background (Brown *et al.,* 2007). Utilizing plant tissue culture technology and molecular biology is expected to accelerate constrain in achieving cacao breeding programs. *In vitro* techniques help cacao breeding by generating various genetic variation in a short period of times and in a small space because they are done at the cellular level. Genetic transformation also requires tissue culture technology to regenerate transgenic plants. For those reasons, availability of an efficient *in vitro* regeneration system of cacao is very important.

Somatic embryogenesis is an efficient *in vitro* method for regenerating plantlets because it has a high multiplication rate. *In vitro* plantlet regeneration via somatic embryos has been developed in many plants species, such as coffee (Ibrahim *et al.,* 2012; Ibrahim *et al.,* 2013a; Ibrahim *et al.*, 2013b), soybean (Widoretno *et al.*, 2003a) and one of the rare Indonesian medicinal plants (purwoceng - *Pimpinella pruatjan* Molk.) (Ajijah *et al.*, 2010). Regeneration methods of cacao through somatic embryo genesis have been developed using thidiazuron (TDZ) (Ajijah *et al*., 2014). However the success of cacao somatic embryo formation is still low (25 %) and limited to a particular clone (Sca 6). Although TDZ has been reported as effective for inducing cacao somatic embryogenesis (Li *et al.*, 1998), its price is too expensive to most of laboratories in developing countries. Therefore development of an alternative method of cacao somatic embryo regeneration using less expensive plant growth regulators (PGRs) is needed. The develop somatic embryogenesis method may be used to conduct either clonal propagation of desirable cacao clones, assist *in vitro* cacao breeding, or cacao genetic transformation.

The objective of this study was to develop an *in vitro* regeneration system of cacao through somatic embryogenesis using DKW medium supplemented with Kinetin and 2,4-D. Effectiveness of several molar ratios of kinetin to 2,4-D, medium compositions, explants types and genotypes responses on cacao somatic embryogenesis induction was evaluated. The study also estimated the frequency of somaclonal variation among plantlets regenerated from cacao somatic embryos using SSR markers.

MATERIALS AND METHODS

The experiments were carried out at the Tissue Culture Laboratory, Indonesian Agency for Agricultural Research and Development (IAARD) and the molecular analysis was conducted at the Molecular Biology Laboratory of Indonesian Centre for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIO GRAD), Bogor. Closed flower buds of approximately 3 weeks old were collected from field-grown plants at Jampang-Sukabumi, West Java. Flower buds were surface sterilized for 10 min using 5% sodium hypochlorite solution and rinsed tree times with sterile distilled water for 10, 5 and 5 minutes, respectively. Stamenoids (Figure 3A) and basal petals (Figure 3B) were dissected using a sterile scalpel.

Explants were first cultured on callus initiation (CI) medium for 14 d, and then transferred for another 14 d in a modified secondary callus growth (SCG) medium. The CI medium consisted of DKW basal salts supplemented with 9 µM 2,4-D and either 0.58, 1.16 or 2.32 µM kinetin (kin).The molar ratios of those kin:2,4-D were 1.0:15.5, 1.0:7.8 or 1.0:3.9. The modified SCG medium consisted of WPM basal salts supplemented with 9 µM 2,4-D and either 1.16 or 2.32 µM kin.The molar ratios of those kin:2,4-D were 1.0:7.8 or 1.0:3.9. The kin:2,4-D ratio of 1.0:7.8 with 2 times of the standard culture period (2 X 14 d) in SCG medium was also evaluated.

Embryo development was initiated by transferring callus onto embryo development (ED) medium and the callus was sub-cultured every 2

weeks onto same medium until mature cotyledonary embryos were formed. The ED medium consisted of either DKW basal salts (ED1), DKW + mixture of amino acids (AA) (ED2), DKW + AA + 0.185 µM adenine (ED3), DKW + 0.1% (w/w) activated charcoal (ED4) or WPM basalt salts + AA + 0.185 µM adenine (ED5). The AA consisted of L-arginine (0.435 mg L^{-1}), L-glycine (0.187 mg L^{-1}), L-leucine (0.328 mgL^{-1}), L-lysine (0.456 mg L^{-1}) and tryphtopan (0.510 mg L^{-1}).

Nine cacao genotypes representing Forastero genetic group (Sca 6, Pa 300), Trinitario (UIT 1, ICS 13, GC 7, DR 2, ICCRI 2) and two genotypes of unknown genetic group (Cimanggu 1 and Cimanggu 2) were tested for their somatic embryogenesis responses using the various kin:2,4-D molar ratios of 1.0:3.9 in CI medium and 1.0:7.8 in SCG medium. Embryogenic callus was then transferred onto ED1 medium for the formation and development of somatic embryos.

Data were collected from at least 3 replicates with ten explants per experimental unit. The effects of treatments were determined by analysis of variance (ANOVA) using SPSS 20 statistic software.

SSR Analysis

The DNA was extracted from fresh leaves of 19 plantlets and the mother plant of Cimanggu 2 following the modified protocol for cacao DNA extraction as described by Obando and Manuel (2009). DNA polymorphisms were detected by polymerase chain reaction (PCR) using 20 SSR markers according to Pugh *et al.* (2004) which the primer sequences were re-designed by Kurniasih *et al.,* (2011). PCR reaction was performed in a volume of 12.5 µl, consisted of 2 µl DNA sample as a template, 6.25 µl of PCR mix, 2.75 µl MQ water and 0.75 µl each of forward and reverse primers. The DNA amplification was performed in a DNA thermocycler, using the following steps: a pre-denaturation step of 3 minutes at 95ºC; 35 cyclesof denaturation for 15 seconds at 95ºC, annealing for 15 seconds at 51 - 62ºC depend on microsatellite loci, and extension at 72ºC for 5 seconds; and a final extension at 72ºC for 10 minutes. PCR products were separated on 8% non-denaturing polyacrylamide gels using vertical electrophoresis at 80 volt for 150 minutes. Gels were documented using Chemidoc gel system after staining using ethydium bromide. Plantlets showing different SSR allele profiles than the mother plant ("off type") were reevaluated to make sure that it is variants.

RESULTS AND DISCUSSION

Callus formation occurs 5-7 days after culture and was preceded by swelling explants. Two weeks after culture, all responsive explants have formed callus. Two types of callus were observed in this study, namely non-embryogenic white-compact callus and embryogenic yellowish nodular callus (Figure 3C). Somatic embryos only formed from this nodular callus type.

The level of kin or kin:2,4-D ratiosin CI medium and its interaction with explants types did not significantly affect callus and nodular callus formation while the types of explants did. The average percentage of callus formation from stamenoid was significantly higher compared to that from basal petal (Table 1). Although it was not statistical significantly different, increasing kin level or kin:2,4-D in CI medium increased the callus and nodular callus formation (Table 1).

Table 1. Effect of kin:2,4-D ratios in the CI medium and explants types of Sca 6 clone on percentage of callusing explants and nodular callus formation

Factors Evaluated	Callusing explants (%)	Nodular callus (%)*
Kin:2,4-D		
1: 15.5	85.00 ± 6.19	80,23 ± 8.34
1:7.8	87.46 ± 4.40	92,63 ± 4.66
1:3.9	90.00 ± 3.65	94.58 ± 3.56
P-val	0.370	0.131
Explants		
Basal petals	79.42 ± 2.91	83.85 ± 3.94
Stamenoids	95.56 ± 2.42	94.44 ± 5.56
P-val	0.001**	0.139

Remarks: *) Data were observed 14 days after explants were sub cultured on SCG medium with kin:2,4-D ratio of 1.0:7.8. Value represent mean ± SE of at least three replicates. ** Significantly different according to t test (P< 0.05).

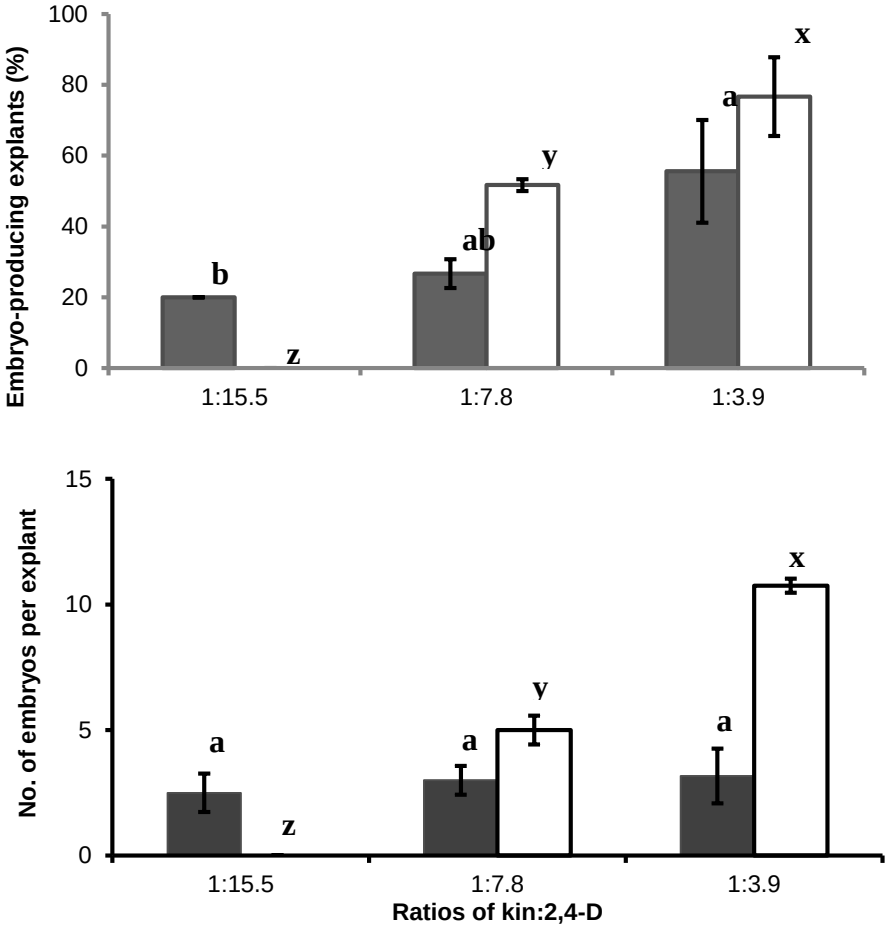

Figure 1. Effect of kin:2,4-D ratios in CI medium on induction of somatic embryogenesis from basal petals and stamenoids explants of Sca 6 cacao clone. The same letter(s) for each type of explant indicated the responses was not significantly different according to DMRT (P< 0.05). Bars indicate standard errors (SE) of at least three replicates □: stamenoids, ■ basal petals explants.

Kin level or kin:2,4-D ratios in the medium, explants types and interaction effects among the two factors in CI medium significantly affected the somatic embryo formation. Two types of explant responded differently to a given kin:2,4-D ratios. Stamenoid explant seemed to be more responsive to higher kin:2,4-D ratio than that of basal petal. No somatic embryos were formed from stamenoid explants when kin:2,4-D ratio of 1.0:15.5 was used in the medium. On the other hand, the somatic embryos were formed from basal petals at this kin:2,4-D ratio (Figure 1).

When kin:2,4-D ratios were increased from 1.0:15.5 to 1.0:3.9, the average percentage of explants forming somatic embryo from stamenoids and basal petals and the number of embryos per stamenoid explants increased 76, 2.7 and 10 folds, respectively. However, increasing the kin:2,4-D ratios did not significantly increase the average number of embryos per basal explant (Figure 1). Different responses of the two explant types are likely due to differences in the endogenous hormone content. Endogenous hormone levels are considered as major factors in determining the cellular response (Feher *et al*., 2003) and crucial factors in influencing explant embryogenic potential (Jimenez, 2005). The different responses of the two types of cacao explants on somatic embryogenesis have been previously reported (Ajijah *et al*. 2014). Results from this study showed supplementing kin:2,4-D ratio of 1.0:3.9 in CI medium was the best for inducing somatic embryos from either basal petal or stamenoid explants (Figure 1).

The low kin:2,4-D ratio of 1.0:7.8 in SCG medium was required to increase formation of cacao somatic embryos. The average number explant forming somatic embryo and number of somatic embryos per responsive explant decreased when the kin level or kin:2,4-D ratio in SCG medium was increased from 1.0:7.8 to 1.0:3.9 and the culture period was extended to 2 X 14 d (Figure 2). There was no interaction effect between kin:2,4-D ratios in SCG medium and explant types. The low kin:2,4-D ratio of 1.0:7.8 for 14 d in SCG medium was enough to induce somatic embryos from either basal petal or stamenoid explants (Figure 2).

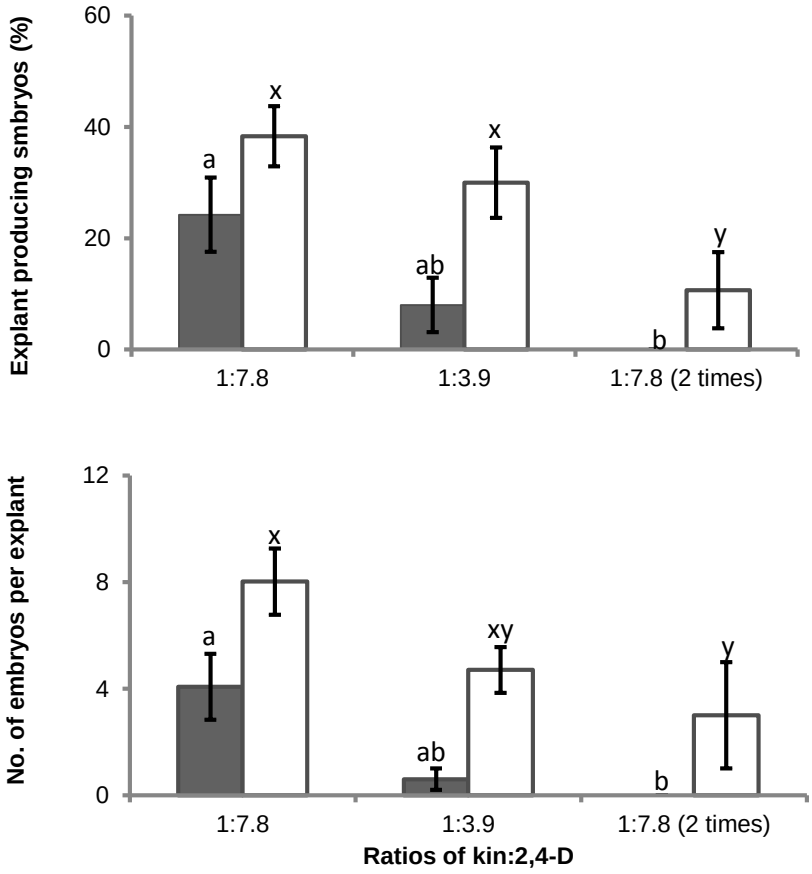

Figure 2. Effect of kin:2,4-D ratios and culture period in SCG medium on somatic embryogenesis from stamenoids and basal petals of Sca 6 clone. Same letter(s) for each type of explant were not significantly different according to DMRT ($P< 0.05$). Bars indicate standard errors (SE) of at least three replicates. □ : stamenoids, ■ : basal petals.

The use DKW medium supplemented with kinetin for inducing cacao somatic embryogenesis has not been reported. The present study found that kinetin in combination with 2,4-D was also effective for inducing cacao somatic embryogenesis using a DKW medium-based protocol. The basal salts formulation affected callus quality in relation to plant regeneration capacity (Al Khayri, 2011) and affected culture response to 2,4-D and kinetin (Dalila *et al.,* 2013). DKW basal salts medium was reported more appropriate for cacao somatic embryogenesis than MS basal salts medium (Li *et al.*, 1998) since it contained higher level of calcium, sulphur and magnesium. Those elements may be crucial for somatic embryogenesis and differentiation of cacao explants (Emile *et al*., 2010).

Kinetin is an aminopurine (adenine) derivative type of cytokinins. Together with auxin, cytokinins have a very important role in plant somatic embryogenesis. Exogenous hormone especially auxin and cytokinin are needed for the activation of somatic cell and for the entry to the division cycle (Pasternak *et al,.* 2002) and for sustained cell division (Feher *et al*., 2003). Tang *et al*. (2000) reported that auxin: cytokinin ratio affect-ted the formation of somatic embryos in three cultivars of sour cherry (*Prunus cerasus* L.). Auxin:cytokinin ratios also influenced the intensity of embryo formation, germination and conversion of Indian chicory (*Cichorium intybus* L.) as reported by Abdin and Ilah (2007). Present results showed the molar ratio of kinetin to 2,4-D also had significant effect on cacao somatic embryogenesis. The high kin:auxin ratio of 1.0:3.9 on CI medium and the low kin:auxin ratio of 1.0:7.8 on the SCG medium gave better results for cacao somatic embryo formation than other ratios.

Nodular embriogenic callus induced from basal petals of Sca 6-1 clone on CI medium supplemented with kin:2,4-D ratio of 1.0:3.9 and SCG medium supplemented with kin:2,4D ratio of 1.0:7.8 were sub cultured on several compositions of embryo development medium. In this study embryo development medium was also used as maturation medium. Results of this study indicated medium composition had significant effect on differentiation of embryogenic cells to form somatic embryos. Selection of the appropriate composition of the basal medium is very important in this phase.

The average percentage of explant forming embryos and number of somatic embryos per explant was higher in DKW medium alone (ED1) than those in DKW medium enriched with amino acids, adenine or activated charcoal (Table 2). The average percentage of explant forming embryos and number of embryos per explant was signify-cantly decreased when WPM medium was used, with or without enrichment with amino acids and adenine (ED5) (Table 2). WPM medium has lower content of all macro and micronutrients than that of DKW medium (da Costa Pinto*,* 2007).

Table 2. Effect of medium compositions on formation of somatic embryos and number of embryos per explant of Sca 6-1 clone

ED medium	Embryo forming explants (%)	Number of embryos per explants
DKW	23.99 ± 5.25a	1.36 ± 1.09a
DKW + amino acids	21.25 ± 9.73a	1.01 ± 0.41a
DKW + amino acids + 0.185 µM adenine	21.94 ± 6.44a	0.74 ± 0.20a
DKW + 0.1% activated charcoal	10.13 ± 4.13ab	0.41 ± 0.19ab
WPM + amino acids + 0.185 µM adenine	1.67 ± 1.67b	0.03 ± 0.03b
P-val	0.010	0.023

Remarks: The value represent mean ± standard errors (SE) of at least three replicates. For each variable, means with different letters are significantly different according to DMRT ($P < 0.05$)

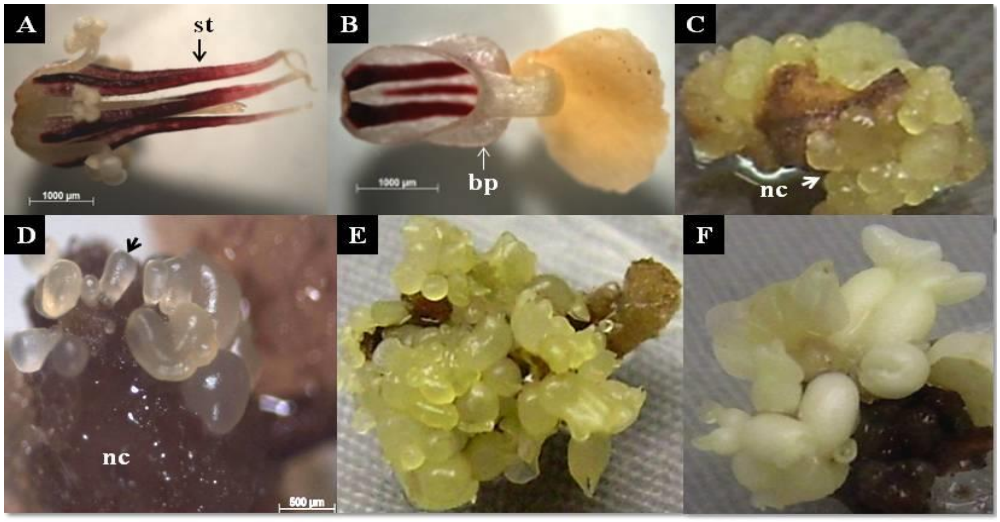

Figure 3. Formation of primary somatic embryos of Sca 6 clone induced using kinetin. (A) Stamenoids (st) and (B) basal petal (bp) were dissected from cacao flower bud.(C) Formation of embryogenic nodular callus (nc) from basal petal, 4 weeks after culture.(D) Formation of pro-embryo (indicated by black arrow) from the surface of nodular callus, 5 weeks after culture.(E) Stamenoid explant covered with somatic embryos at various development stages, 9 weeks after culture and (F) Various stages of cotyledonary embryos, 12 weeks after culture

Somatic embryos were only formed from the surface of nodular callus type which turn brown after sub-cultured on medium without growth regulators (Figure 3D). Based on the microscope observation approximately 200 µm translucent pro-embryos began to develop after 5 weeks of culture (Figure 3D), or one week after the nodular callus was sub cultured on ED medium. Globular embryo further developed to heart, torpedo and cotyledonary shapes (Figure 3E, 3F). The young somatic embryos were yellowish or white in color (Figure 3E, 3F) and changed to milk white color (sometimes with pink cotyledons) after reaching somatic embryo maturation (Figure 3F). In the culture medium, the embryos developed asynchronously and in the same culture vials contained various stages of somatic embryos development (Figure 3E, 3F).

Genotypes Responses

The nine cacao genotypes tested showed different ability to induce somatic embryogenesis and their responses were explant types dependent. The GC 7, ICCRI 2 and Cimanggu 2 cacao clones showed higher responses of somatic embryogenesis induction when basal petals were used as explants than that of stamenoids. On the contrary, stamenoid explants of Sca 6, ICS 13 and DR 2 cacao clones showed higher responses of somatic embryogenesis than that of basal petal (Table 3). Such results indicated that selection of appropriate explants is necessary to obtain somatic embryos from explants originated from different cacao genotype. This study also showed that Sca 6 was the most responsive genotype for somatic embryo regeneration among the nine cacao genotypes tested (Table 3).

Somatic embryogenesis is a process largely determined by genetic factors. Different somatic embryo genesis response among genotypes were reported in a number of plant species (Li *et al*., 1998; Widoretno *et al.,* 2003a; Ajijah *et al.,* 2014). Genotype dependent response of cacao somatic embryogenesis has been reported previously (Li *et al*., 1998; Ajijah *et al.* 2014). This study also indicated that Forastero cacao group (Pa 300, Sca6) is more responsive than Trinitario (UIT 1, GC 7, ICS13, ICCRI2, DR2) (Table 3) and this finding supports previous report by Ajijah *et al.* (2014). Criollo cacao group was not tested in this study because the explant for this cacao group was not available. Both petal and stamenoid can be used as explants for inducing cacao somatic embryogenesis. However, their responses to somatic embryo induction medium is generally genotypes and explant types dependent.

Table 3. Differences in somatic embryogenesis response of petal and stamenoid explants of nine cacao genotypes

Genotypes	Genetic Group	Explants producing embryos (%)		No. of embryos per explants	
		Basal petals	Stamenoids	Basal petals	Stamenoids
Sca 6	Forastero	20.0 ± 2.9abB	66.7 ± 6.7aA	3.0 ± 0.6aB	24.0 ± 2.4aA
Pa 300	Forastero	22.0 ± 11.3abA	13.1 ± 2.3bcA	4.3 ± 2.5aB	15.6 ± 2.1bA
UIT 1	Trinitario	18.0 ± 3.4abA	26.7 ± 13.7bA	3.2 ± 1.0aA	1.0 ± 0.5cA
ICS 13	Trinitario	6.7 ± 3.3bA	22.9 ± 4.0bA	2.7 ± 2.2aB	11.5 ± 4.4bA
DR 2	Trinitario	8.3 ± 5.0abA	24.2 ± 3.4bA	0.7 ± 0.5aA	4.3 ± 1.50cA
GC 7	Trinitario	5.6 ± 4.4bA	0.0 ± 0.0cA	2.7 ± 2.1aA	0.0 ± 0.0cA
ICCRI 2	Trinitario	26.0 ± 3.9aA	0.0 ± 0.0cB	2.7 ± 0.7aA	0.0 ± 0.0cA
Cimanggu 1	Unknown	15.0 ± 7.6abA	23.3 ± 5.1bA	2.0 ± 1.0aA	1.7 ± 0.7cA
Cimanggu 2	Unknown	19.2 ± 1.8abA	0.0 ± 0.0cB	2.9 ± 1.0aA	0.0 ± 0.0cA

Remarks: The value represent mean ± SE of at least three replicates. For each variable, means in a column with different lower letters and those in a row with different capital letter are significantly different according to DMRT (P< 0.05)

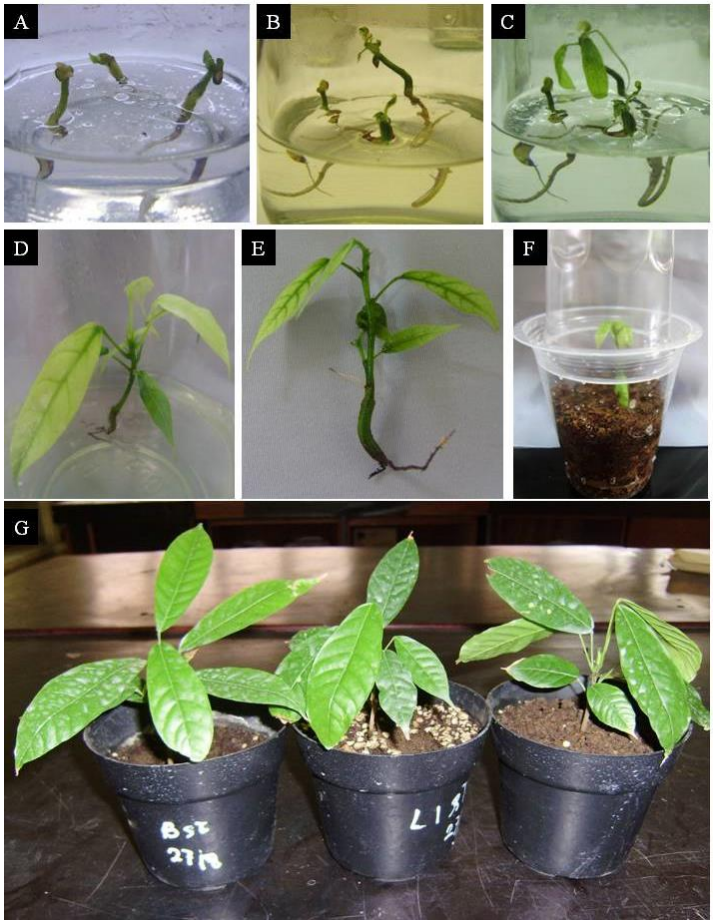

Figure 4. Germination of somatic embryos and conversion somatic embryo into plantlets. Germination of somatic embryos after 1 month (A), 1.5 months (B) and 2 months (C) on germination medium. Plantlets regenerated from somatic embryos with taproot and three leaves, 6 months after culture (D, E); Cacao plantlet hardening and seedling potted in soil (F, G) derived from somatic embryos.

Germination and Conversion

Mature somatic embryos at cotyledonary stage were transferred onto the germination medium consisted of DKW basal salt and amino acids. The germinated cacao somatic embryos were maintained under 25-26℃ and 16-h (light) photo period under cool-white tubular fluorescent lights (40W, 220V, Philips) of approximately 1800 lux and sub cultured onto the same medium every month until they form leaves. This procedure resulted in 50% somatic embryo germination and plantlets regeneration (Figure 4A-C). The low rate of somatic embryo conversion into plantlets is still a problem in cacao somatic embryogenesis. In this study the rate of somatic embryo germination reached 50%.

Several factors were associated with successful somatic embryo germination and plantlet conversion, such as: the somatic embryo quality and the carbon source in the germination medium. Result of our study also showed the composition and concentration of basal medium also affected the rate of somatic embryo germination (data not showed).

Hardening

Plantlets with a minimum of three leaves were rinsed thoroughly under tap water to remove residual medium from the plantlets and soaked in a 2% fungicide solution for 15 minutes. Plantlets were transplanted to plastic pots containing sterilized mix of soils, sands and coco pits (1:1:1 v/v). The plastic pots were covered with another transparent plastic pots and maintained in the culture room for acclimatization under cool white tubular fluorescent light at 25-26℃. After 1 month, acclimatized plantlets were transferred to room temperature (27-29 ℃) and plastic glasses were gradually removed. One month later, plantlets were transplanted to larger plastic pot containing sterilized mix of soil and manure (2:1 v/v). Hardening of the plantlets with survival rate of 65.3% (64/98) when maintained at the temperature of 28-30℃ (Figure 4F-G) was obtained using those procedures.

Somaclonal Variation Assessment

Positive PCR amplification in 19 out of 20 SSR loci were observed using mother plant and plantlets regenerated from cacao somatic embryos of Cimanggu 2. We are unable to obtain the PCR product from mTcCIR 95 locus using DNA of mother plant and plantlets regenerated from cacao somatic embryos of Cimanggu 2. The total of 646 alleles was generated from 19 plantlets regenerated from cacao somatic embryos. Three regenerated plantlets (3/19 – 15.8%) exhibited different SSR alele paterns than the originated mother plant. Four allele polymorphisms were found in those 3 plantlets, indicated there was plantlets carrying more than one mutations. Two types of polymorphisms were observed, such as allele losses and new allele formation. The frequency of observed mutations based on SSR marker analysis was 6.2 X 10^{-3} (4 out of 646 events). Allele loss (Figure 5) was the most frequent polymorphisms found (3 out of 4 events) among plantlets, followed by new allele formation (1 out of 4 events). Cluster analysis among regenerated plantlets based on 19 SSR loci resulted in 0.97 – 100% level of similarity (Figure 6).

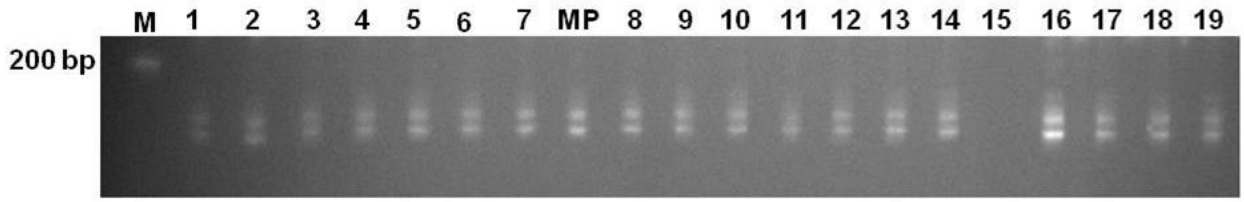

Figure 5. Example of PCR amplification products using DNA template of plantlets regenerated from primary somatic embryosno (1-19) and mTcCIR 213 SSR primer pairs. We used non-denaturing polyacrylamide gels electrophoresis (8%) to separatePCR products and ethydium bromide to stain the DNA fragments. The figure showed alleles loss in plantlet no. 15. M: 100 bp DNA ladder, MP: mother plant (Cimanggu 2), 1-19: plantlet samples regenerated from primary somatic embryos of Cimanggu 2.

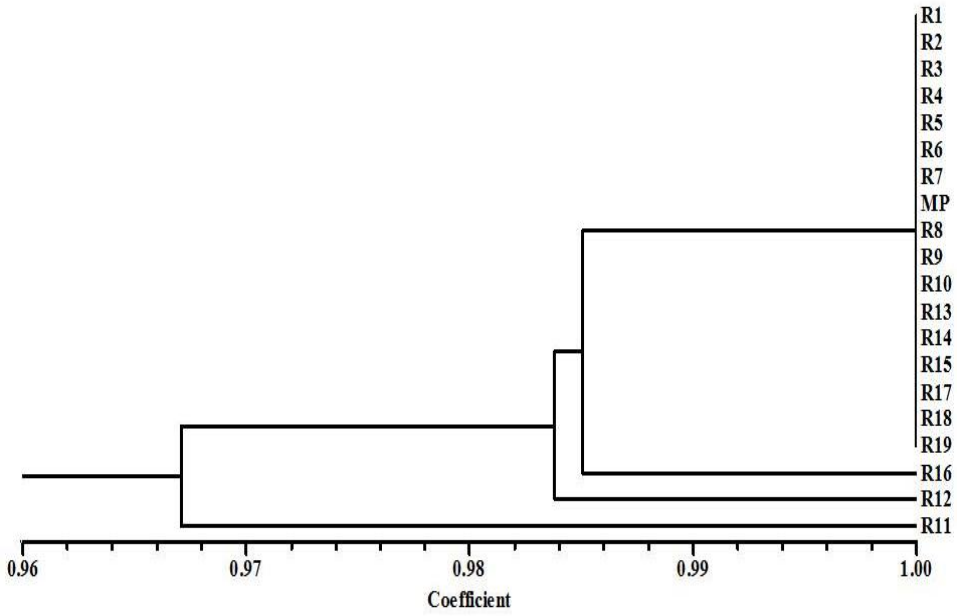

Figure 6. Results of cluster analysis among plantlets regenerated from primary somatic embryos of Cimanggu 2 based on 19 SSRs markers. We used the UPGMA using NTSYSpc version 2.02 to conduct cluster analysis. R1-R19 werethe plantlets of Cimanggu 2 regenerated from somatic embryos and MP was the mother plant (Cimanggu 2).

The observed frequency of somaclonal variation in this study was lower than that reported by previous study (Lopez *et al*., 2010). Lopez *et al.* (2010) reported that the frequency of somaclonal variation in cacao primary somatic embryogenesis ranged from 35-50%, depending on the genotypes. Those frequencies of somaclonal variation differences may have been because of the genotype diferences or the PGRs used for inducing cacao somatic embryogenesis. Lopez *et al.,* (2010) used TDZ to induce cacao somatic embryos while in this study used kinetin. According to Bidabadi *et al.* (2010) and Jose *et al.,* (2012), the types and concentrations of PGRs may have caused different level of somaclonal variations. The regenerated cacao plantlets carrying high frequency of allele losses and multiple mutations found in this study support previous finding reported by Lopez *et al.* (2010).

Tissue culture induced mutation in plants is known as somaclonal variation. Eventhough somaclonal variation is a potential problem in clonal propagation, it has been used to regenerate genetic variation in a number of plants (Yusnita *et al*., 2010; Yuliasti and Sudarsono, 2011; Widoretno *et al*., 2003c; Purwati *et al*., 2007).

According to Larkin and Scowcroft (1981), tissue culture derived planting material is a rich and unexpected source of genetic variations. Somaclonal variation induced by tissue culture process is useful to plant breeding since it can regenerate novel characters that can be identified through *in vitro* selection (Yuliasti and Sudarsono, 2011; Widoretno *et al*., 2003c; Widoretno *et al*., 2002; Purwati *et al*., 2007; Rahayu *et al*., 2005).

Cacao plantlet regeneration through somatic embryogenesis can be used as method for cacao clonal propagation. In such case, it is necessary to reduce the frequency of somaclonal variation to below 10%.On the other hand, it can also be used to support *in vitro* breeding. In the later case, higher percentage of somaclonal variation is more desirable. In various crops, somaclonal variation among somatic embryos has been used to identify drought tolerant (Rahayu and Sudarsono, 2015), quantitative and qualitative (Widoretno *et al*., 2003b) and diseases resistance variants (Yusnita *et al*., 2005).

Similarly, cacao somatic embryogenesis has the potential to induce variants that are resistance against *Phytophthora palmivora* (Ajijah *et al.*, 2012).

CONCLUSIONS AND SUGGESTION

DKW medium supplemented with Kinetin in combination and 2,4-D effectively induced cacao somatic embryogenesis. Kinetin to 2,4-D at 1.0:3.9 molar ratios in callus induction medium and 1.0:7.8 in secondary callus growth medium showed the best result for inducing somatic embryos from cacao explants. Cacao genotype responses were highly explant dependent. The results of this study provide an alternative method of inducing high rate (5.6-66.7%) cacao primary somatic embryogenesis using 2,4-D and Kinetin with a medium frequency of somaclonal variations (16%)

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