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Assessment of the effects of physiological development of cocoa (*Theobroma cacao l.*) explant on somatic embryogenesis

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Abstract

Cocoa trees have shown a high degree of segregation for many traits when propagated by seeds. Somatic embryogenesis is an efficient *in vitro* propagation method which allows the production of several embryos capable of generating plants similar to the initial one from somatic tissues. The use of cocoa floral parts has been reported for regeneration of elite cocoa genotypes. This research is targeted in evaluating the effect of physiological development of the explants (staminode) and its response to two different cytokinase (kinetin and BAP) on embryogenesis. The experiment was laid in factorials with three replications in CRD. Three different physiological ages of the explant were examined: 1 week old (unopened, about 3-4 mm in length),2 weeks old (unopened, about 5-/6 mm in length) and 3 weeks old (unopened; matured flowers). Staminode was studied and explants were initiated for callus induction on Primary Callus Growth medium. The following data were scored for: Explants Induction Percentage, Percentage of callus induced and Percentage of Embryogenic callus. Results showed the interactions among the genotypes, hormones and the physiological age of the explants were significantly different at 0.05% probability level. Explants at 3 and 2 weeks respectively had higher efficiency for embryogenesis while the average performance was observed for explants at a week. Also, BAP recorded higher frequency 80% for embryogenesis compared to kinetin 70% under the present study. Physiological age of explants and the choice of callus development hormone have been found to play significant role in the embryogenesis of cocoa genotypes examined.

Keywords: *Theobroma cacao*; Somatic embryogenesis; Explants; Physiological; cytokinase; staminodes and segregation

1. Introduction

Theobroma cacao L. (chocolate tree) is grown in the humid tropics and constitutes an important source of incomes for many countries of the West and Central Africa regions. *Cacao* trees are predominantly propagated by seedlings, from seeds selected by farmers from their own materials (Chaidamsari*et al*., 2005). The seeds used for propagation are selected to be uniform of good quality and from seed derived clones. Propagation by seeds results in a high level of heterogeneity of the crop, and genetic variation of low yielding trees. Vegetative propagation is essential to produce true to type trees. Budding and the use of rooted cuttings are common practice throughout the cocoa growing regions (Eskes B., 2001). Propagation of *cacao* trees by rooted cuttings involves the use of orthotropic and plagiotropic shoots. Orthotropic (chuppons) shoots produce cocoa clones with the same morphology as seed derived trees, but only small quantity can be sourced.

Cacao breeding usually takes a long time and this is because of its long-life cycle and the narrow genetic background (Brown *et al*., 2007). Utilizing plant tissue culture technology is expected to accelerate constrain in achieving *cacao*

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improvement programs. Plant tissue culture relies on the fact that many plant cells have the ability to regenerate to a whole plant (totipotency).The flowers are hermaphroditic, with small sizes (diameters ranging from 0.5 to 1cm), regular and composed of 5 sepals, 5 petals, 5 staminodes, a pistil and an ovary. Pollination is predominantly entomophilous although it can be done manually in the experimental fields (Rodrigue *et al*., 2016). Flowering in cocoa is manifested by the production of a minimum of 50 000 flowers during the term with less than 5% of production pods (Lass, 1999).

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 - *Pimpinellapruatjan*Molk.) (Ajijah*et al.*, 2010). The present study was to examine the effect of physiological development of explants and the effect of hormone (for callus development) on the cocoa embryogenesis.

2. Materials and Methods

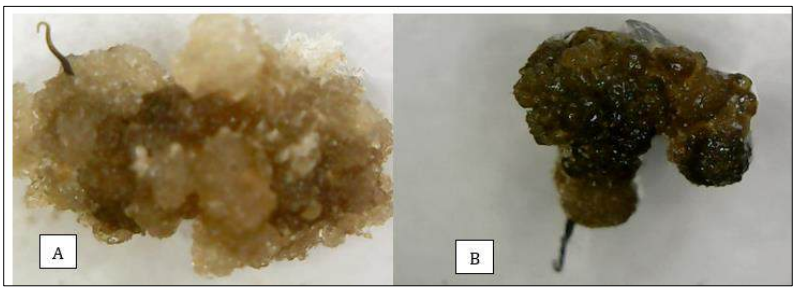
Five *cacao* genotypes (TC-1 (G5), TC-5 (G9),TC-8 (G12), Spec 54-1 (G2) and PA150 (G3)) were used. The *cacao* genotypes were tagged at the point of flower initiation, harvested for in-vitro culture initiation at: 1week, 2weeks and 3weeks respectively. A floral part (staminode) was studied. The experiment was laid in factorials with three replications in CRD. Three different physiological ages of the explants were examined: 1 week old (unopened, about 3-4 mm in length), 2 weeks old (unopened, about 5-/6 mm in length) and 3 weeks old (unopened; matured flowers).The explants were initiated (for callus induction) on PCG medium for all the ages of the flower for the period of fourteen days; respectively for the five genotypes under study. The callus produced were then transferred into two different Secondary Callus Growth medium (SCG: containing 1mg/ml kinetin and 1mg/ml BAP respectively) for fourteen days and Embryo Development (ED) medium for four weeks with daily maintenance on the media. SCG medium containing kinetin was prepared according to the recipes in the protocol manual (Penn.,2010), with filter-sterilized kinetin into an autoclaved medium after being cooled to a temperature of about 60^0C in a laminar-flow hood. The 0.2um filter was used. This was done to prevent the denaturing of kinetin during autoclaving.

2.1 Data collection

- Explants Induction Percentage was scored at twenty-four hours after explants initiation on induction medium, PCG
- Percentage of callus induced was scored from three to fourteen days after explants initiation on both the PCG and SCG (kinetin and BAP) medium respectively
- Percentage of Embryogenic callus was evaluated at 2 weeks to 4 weeks after initiation on ED medium

2.2 Data Analysis

Data were analysed using means and standard errors (S.E.M.) and statistical significance among values were assessed using ANOVA at a probability of B/0.05.



A = embryogenic callus developed on SCG (with BAP hormone); B = embryogenic callus developed on SCG (with Kinetin hormone)

Figure 1 Embryogenic callus of the *cacao* staminode tissue

3. Results

Table 1 showed the mean squares of inductions and callus formation of the five *cacao* genotypes (in staminode tissues) in three different physiological ages. Staminode tissues at 2 weeks of age showed significant differences among the five *cacao* genotypes tested for the explants induction at three days after initiation. Also, callus formation was significantly

different among the *cacao* genotypes at 3AI and 7AI for the physiological age at week1 and 2 respectively. In Table 2, there were significant differences among the genotypes, hormones, physiological age, and their interactions for the embryogenic callus formation for the five *cacao* genotypes tested. Table 3 revealed means of the embryogenic callus of the *cacao* genotypes in different hormones at different physiological age. The highest mean for embryogenic callus was observed in G3 at two weeks (81.39%) and four weeks (83.88%) while G12 had the least mean at two weeks (66.67%) and four weeks (67.22%) respectively. Also, BAP had the highest mean for embryogenic callus (83%, 83.89%) while the least mean was recorded for kinetin (71.22%, 73.22%) at two and four weeks respectively. For the physiological age of the floral explant (staminode), highest mean was recorded at week 3(99.50%, 100%), followed by week 2 (79.33%, 81.67%) while week 1 had the least mean (52.50%, 54%) at two and four weeks respectively. The interactions among the genotypes, hormones and the physiological age of the staminodes were significantly different at 0.05% probability level.

Table 1 Mean squares of induction and callus formation of the five *cacao* genotypes (in staminode tissues) in three different physiological ages

Treatment		**Induction**	**Callus formation**		
Source	**DF**	**3AI**	**C3AI**	**C7AI**	**C8AI**
1 week Genotype	4	40.42^{ns}	186.67**	563.55^{ns}	36.67^{ns}
Error	25	22.33	59.50	332.39	18.00
2 weeks					
Genotype	4	115.42**	123.75^{ns}	42.92**	11.25^{ns}
Error	25	18.33	111.67	12.33	7.67
3 weeks					
Genotype	4	40.42^{ns}	142.92^{ns}	5.42^{ns}	2.08^{ns}
Error	25	26.33	123.00	23.83	13.17

** Significant at P <_ 0.05; Ns = Non significant; AI = After initiation; C = Callus

Table 2 Mean squares of the embryogenic callus of the cacao genotypes and its response in two different hormones at different physiological age

Treatment		**Embryogenic callus**	
Source	**DF**	**2 Weeks**	**4Weeks**
Genotype(G)	4	633.06**	759.31**
Hormone(H)	1	3121.11**	2560.00**
PHYAGE	2	16678.61**	16087.78**
G*H	4	287.78**	317.64**
G*PHYAGE	8	458.47**	513.47**
H*PHYAGE	2	846.94**	693.33**
G*H*PHYAGE	8	478.19**	528.06**
Error	58	104.47	111.48

** Significant at P <_ 0.05; PHYAGE = Physiological age; 2 weeks = Embryogenic callus at 2 weeks; 4 weeks = Embryogenic callus at 4 weeks

Table 3 Means of the embryogenic callus of the cacao genotypes in different hormones at different physiological age

Treatment	**Embryogenic callus**	
Genotype(G)	**2 Weeks**	**4 Weeks**
G2	78.61a	79.44a
G3	81.39a	83.33a
G5	79.72a	81.94a
G9	79.17a	80.83a
G12	66.67b	67.22b
LSD	6.82	7.05
Hormones(H)		
BAP	83.00a	83.89a
Kinetin	71.22b	73.22b
LSD	4.31	4.46
PHYAGE		
1 week	52.50c	54.00c
2 weeks	79.33b	81.67b
3 weeks	99.50a	100.00a
LSD	5.28	5.46
G*H	**	**
G*PHYAGE	**	**
H*PHYAGE	**	**
G*H*PHYAGE	**	**
CV%	13.26	13.44

** Significant at P <_ 0.05; PHYAGE = Physiological age; BAP = Benzyl Amino Purine; Means with the same letter along the column are not significantly different at 5% level of probability. LSD = Least Significant Difference, CV% = Percentage of Coefficient of Variation

4. Discussion

Inspite of differences in the physiological age of the explants across the genotypes tested, over 90% induction within three days of culture initiation. Also, the development of the callus was not different from one another. Hence, explants at different physiological developments had a great potential for explants induction and callus development for the genotypes under study. Differentiation of the developed calli into embryogenic callus was different among the physiological development of the explants examined. Explant at 3 weeks gave the best frequency of embryogenesis (100%), followed by explants at 2 weeks (over 80%) while the explants at a week gave the least frequency (over 50%). Thus, explants at 3 and 2 weeks respectively had a higher efficiency for embryogenesis while the average performance was observed for explants at a week; for the genotypes examined. This was in line with the study of (Maximova *et al*.,2002 and Tan *et al*., 2002).

The assessment of the response of two different cytokinins (Benzyl Amino Purine (BAP) and Kinetin) on callus development revealed the better performance of BAP over kinetin across the three physiological developments of the explants examined. Though; the two hormones had a great potential in the callus development of the *cacao*; but BAP recorded higher frequency (over 80%) for embryogenesis compared to kinetin (over 70%). Among the five genotypes examined under this study, G12 had the least response for embryogenesis (over 60%) while the remaining genotypes were not significantly different from one another in embryogenic response (over 80%).

5. Conclusion

Physiological age of explant and the choice of callus development hormone have been found to play a significant role in the embryogenesis of cocoa genotypes examined. This could help in an effort to advance the improvement of *T. cacao* for further breeding research program.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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RESEARCH ARTICLE

ENHANCING THE EXISTING COFFEE GENETIC RESOURCES IN NIGERIA THROUGH *IN VITRO* CULTURE CONSERVATION.

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Biotechnology, *Coffea* spp., Conservation, Genetic resources, *In vitro* culture, Somatic embryogenesis**.**

Abstract

This study aimed at enhancing and conserving existing coffee genetic resources in Nigeria through in vitro culture. In Nigeria there is an urgent need for proper maintenance of coffee genetic materials. This will pave way for sustainable improvement, safeguard against second collection of germplasm and loss of genetic resources. Establishment of easy, efficient and reliable *in vitro* library becomes paramount to complement field genebank which is the only form of conservation in Cocoa Research Institute of Nigeria. Callus tissues were generated from coffee leaf explant of C. *canephora* Pierre clone (C90). A DKW basal medium designed for culturing cocoa flower were used with three different hormonal combinations. Callus induction on the leaf was observed within 7days of culturing in the combination of Benzylamino Purine (0.5mg/ml) and Indole Acetic Acid (1mg/ml) and full callus development was reached at 14days. This callus was maintained in the second combination consisting of IAA (2mg/L) + Thidiazuron (25ug/L) for as long as 28days before changing to gray. The third combination, 2,4- Dichlorophenoxyacetic Acid (2mg/L) + BAP (1mg/L) + caseine hydrolysate (200mg/L) + coconut water (100ml/L) has the ability to convert gray callus to embryogenic or friable yellow callus which can be developed to plant prior converting to embryo in embryo development medium. This research has disclosed a proper and sustainable tissue culturing procedure of maintaining coffee germplasm and mass propagation of improved coffee variety for farmers.

Introduction:-

Coffee belongs to the plant family Rubiaceae. The two highly cultivated species are *Coffea arabica* and *C. canephora*. The latter has wide range of geographical distribution, from western to the central tropical and subtropical regions of the African continent (www.ico.org). It grows at low altitudes about 850m and of high yielding and low quality. Genetic improvement of this crop with regard to quality poses a great challenge to researchers in Africa which lack a good and sustainable conservation technology for its genetic resources.

Collection and maintenance of germplasm are highly expensive and paramount so as to avoid genetic erosion or lost of genetic resources which are already narrow (Omolaja and Fawole, 2004). A great number of genetic resources should be properly conserved in any research institute to enhance and sustain its breeding and improvement programmes. For long traditional ex situ conservation method in the field genebank which offers a satisfactory

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approach to conservation has been the only conservation strategy. This provides an easy access to genetic resources (Engelmann *et al*., 2007). However, there are drawbacks that limit its efficiency and threaten its security. They are exposed to pests, diseases and other natural calamities (climate change and vandalism). Therefore there is a need for an alternative and sustainable conservation strategy.

Biotechnology offers alternative strategies for generating new and improved coffee varieties, including those resistances to environmental extremes, pests, and diseases, low in caffeine, and with uniform fruit maturation and in vitro conservation. In vitro technique has a wide range of applications, in mass propagation, conservation and genetic improvement. Large improvement in bioreactor scale–up of micropropagation through somatic embryogenesis has been achieved (Ducos *et al*., 2007).

Steps of somatic embryogenesis are induction of embryogenic calli, multiplication of the cells, regeneration of large numbers of embryos from these cells, finally conversion of these embryos into mature embryos regenerating to plantlets (Ducos *et al*., 2007). Propagation of coffee through cuttings generates low multiplication rates as only the orthotropic shoot is used (Kumar *et al*., 2006). This research aimed at preserving callus generated from coffee leaf of *Coffea canephora var.* Pierre (C90).

Materials and Methods:-

Plant materials

Leaf explants was collected from C90 a clone of *Coffea canephora* from coffee seed garden at Cocoa Research Institute of Nigeria in Ibadan and subjected to *in vitro* .manipulation.

Methods:-

Young and fresh leaf from C90 was harvested using sterile blade and a clean 100 ml beaker filled with distilled water and transported to the culturing room for surface sterilization. Few drops of detergent was drop in the beaker containing the leaf and washed under sterile condition in a flow hood. Surface sterilization was carried out by added 70% of ethanol for 1 minute and 10% of household bleach (Sodium Hypochlorite) for 15 minutes by gently shaking every 5mins to ensure uniform sterilization. The explant was re-rinsed three times with sterile distilled water. Sterilized leaf explant was placed on a sterile petri dish and cut into smaller disc with sterile forceps and blade and placed on PCG medium which consisted of DKW micro supplemented with BAP (0.5mg/ml) and IAA (1mg/ml), medium was sealed with parafilm and kept in the dark at 30^{o}C. After 7 days the calli formed was transferred to another media CMA and CMB (Table 1) after 2 weeks and subsequent calli transferred to SCD A and SCD B media (Table 1).

Table I:-Hormonal combination

Plant hormones and other additives	Primary Callus Development (PCD)		Callus Maintenance (CM)		Secondary Callus Development (SCD)	
	A	B	A	B	A	B
BAP	1mg/ml		0.5mg/ml			1mg/ml
IAA	1mg/ml			1mg/ml	2mg/ml	
TDZ		0.2mg/ml		0.2mg/ml	25ug/ml	
2,4-D		1mg/ml	1mg/ml			2mg/ml
Caseine hydrolysate					200mg/ml	200mg/ml
Coconut water						100ml/L

Results:-

Friable callus (Fig.1C) which can be converted to plantlets prior embryo was generated on DKW medium containing 2,4-D, BAP, casein hydrolysate and coconut water (SCDB). However, SCDA medium lacking coconut water formed no friable callus.

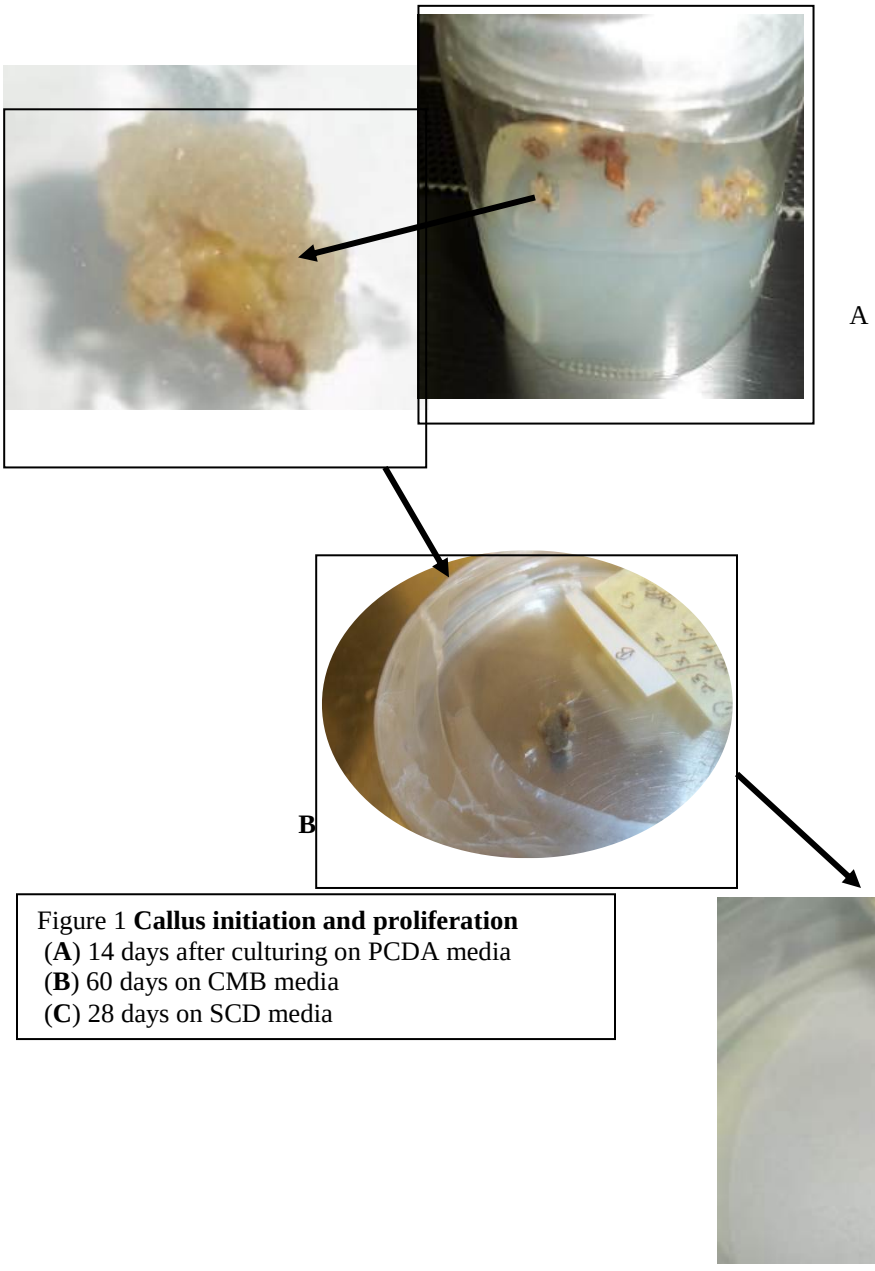

Figure 1 **Callus initiation and proliferation**
(**A**) 14 days after culturing on PCDA media
(**B**) 60 days on CMB media
(**C**) 28 days on SCD media

Also, CMA callus when transferred on SCDA medium reverted to white callus but no obvious change was observed when transferred on SCDB. However, when CMB callus was transferred to SCDA and SCDB they changed to yellow or friable callus but SCDB was more pronounced than the former. At initial culturing on both PCD A and PCD B, the former yielded callus at 1week after culturing. It can therefore be deduced that SCDB is best to serve as SCD than SCDA for coffee leaf.

Discussion:-

Callus production from coffee leaf offers a high potential in the future production of elite coffee variety (Ducos *et al*., 2007). *In vitro* conservation of coffee germplasm can be performed under two stages of development *in vitro*, either callus or plantlet stage. This has been demonstrated by this study where medium supplemented with IAA and TDZ (Table 1) was used in maintaining callus for 28 day.

The combination of BAP (2mg/L) and NAA (0.3mg/L) had yielded an increase in callus proliferation on Taxus baccata, the similar result was achieved in this experiment with BAP (0.5mg/ml) and IAA (1mg/ml). A DKW medium containing B5 Vitamin and supplemented BAP, 2,4-D and coconut water has the ability of converting gray callus to friable callus (Crocomo *et al*., 1986). The callus developed will be further transferred to a different medium to generate plantlets.

Conclusion:-

Maintaining or conserving genetic resources of coffee in the laboratory through the use of callus will serve as an alternative and cost effective way to compliment traditional field conservation so as to avoid the loss of genetic resources resulting from natural catastrophes.

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*Genetic diversity and re-classification of coffee (*Coffea canephora *Pierre ex A. Froehner) from South Western Nigeria through genotyping-by-sequencing-single nucleotide polymorphism analysis*

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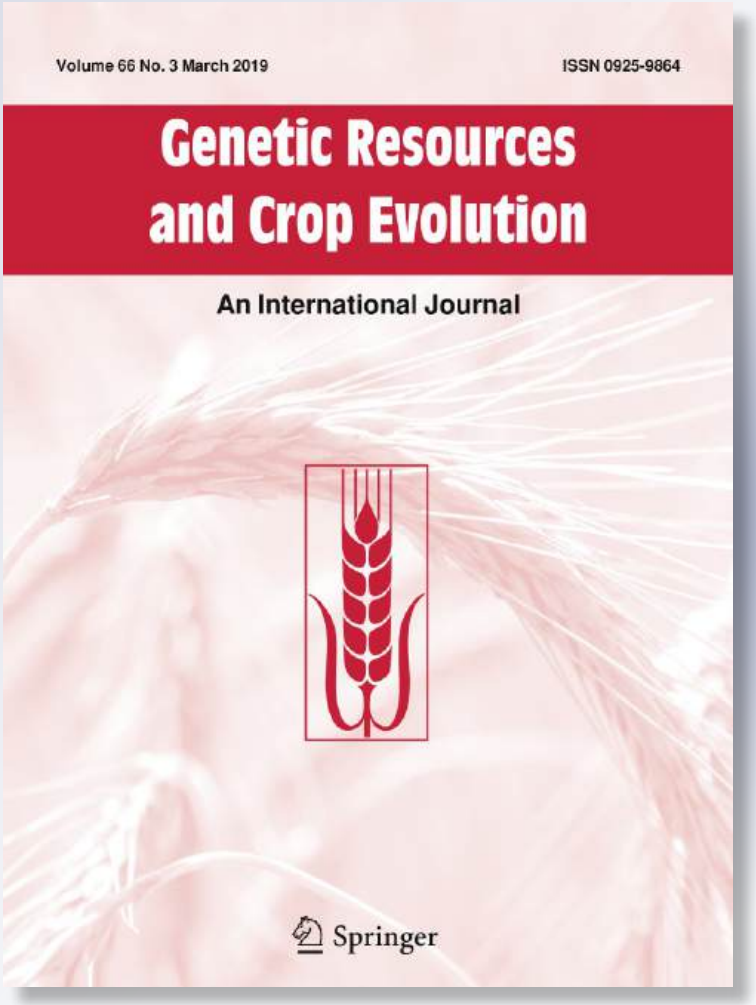

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RESEARCH ARTICLE

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Abstract *Coffea canephora* is an important economic crop in Nigeria, however, little is known about the diversity inherent within, and the genetic relationship among coffee grown and conserved in the country. We examined the genetic diversity and relatedness among 48 *Coffea* genotypes which included: (a) *C. arabica*, *C. abeokutae*, *C. liberica*, and *C. stenophylla*, (b) 14 *C. canephora* accessions conserved in the germplasm of Cocoa Research Institute of Nigeria (CRIN), and (c) 30 farmer-cultivated genotypes collected from South-Western Nigeria. By analyzing 433048 single nucleotide polymorphisms (SNPs) identified through genotyping-by-sequencing we discovered that previous characterizations of *C. canephora* based on morphological data were inconclusive. Here, we established the correct number of *C. canephora* varieties present in the CRIN genebank which was four and not six as previously described based on morphological characters. We found three distinct diversity structures within the *C. canephora* genepool that were dominated by a single genetic group determined from passport descriptors to most likely be of Congolese (Democratic Republic of Congo) origin. High uniformity was also found among the farmer-cultivated accessions with 99% of them representing *C. canephora* var. Niaouli as their ancestral background. The analysis showed that the genetic base of coffee germplasm in Nigeria is narrow compared to the large genetic diversity of *C. canephora.* Therefore, broadening this genetic base through future acquisition and hybridization is imperative. However, the relatively high genetic differentiation (F_{ST} estimate = 0.3037)

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identified between Java Robusta and Niaouli will be used as a starting point for our breeding program.

Keywords *Coffea canephora* · Genetic diversity · Genotyping-by-sequencing · Single nucleotide polymorphism

Introduction

Coffee (*Coffea* spp. Linnaeus) is a high-value crop globally. Over 2.25 billion cups are consumed daily (Dicum and Luttinger 2006) by more than a third of the world's population (Bolvenkel et al. 1993). It is also a source of income for millions of smallholder farmers who collectively produce over 70% of the world's coffee (Oxfam 2001).

The genus *Coffea*, has over 124 species. The two most cultivated are *C. arabica* Linnaeus and *C. canephora* Pierre ex A. Froehner (Zamir 2014), which make up 65% and 35% of the international coffee trade, respectively (Davis et al. 2006). *C. arabica* is prized for its cup quality attributes, while *C. canephora* is valued for its higher disease resistance and yield (Bertrand et al. 2003). *C. arabica* is an allotetraploid (2n = 4x = 44), while *C. canephora* is a self-incompatible diploid (2n = 2x = 22) and one of the progenitors of *C. arabica* (Pearl et al. 2004).

In Nigeria, *C. canephora* constitutes 95% of total coffee production while *C. arabica* contributes only 5%. Previous research activity has been limited to germplasm introductions, and agro-morphological characterizations of six *Coffea* spp. including *C. arabica*, *C. canephora*, *C. liberica* Bull. ex Hiern, *C. abeakutae* Cramer, *C. excelsa* Aug. Chevalier, and *C. stenophylla* G. Don (Omolaja et al. 1997). *C. canephora* has received the most attention, specifically, the six varieties: Gold Coast, Java Robusta, Uganda, Kouilou/Quillou, Java Robusta ex. Gamba and Niaouli. The true-to-type identity of genotypes/varieties cultivated by farmers in Nigeria is not known. The introduction of these genotypes was made in 1966 from Ghana, Indonesia, Zaire (Democatic Republic of Congo), Uganda and the Benin Republic (Williams 1989).

Information on the molecular characterization of *Coffea* in Nigeria is scarce. *Coffea* genotypes in this country have been traditionally distinguished using morphological characteristics (Omolaja et al. 2000), which is insufficient because of environmental influences on phenotype (Souza et al. 2013). Molecular markers can partition environmental from genetic influences on phenotype, thus providing a higher level of accuracy on the genetic relatedness of different genotypes (Mishra et al. 2011). First- and second-generation molecular markers have already been adopted for coffee genotyping (Achar et al. 2015; Hendre and Aggarwal 2007; Hendre et al. 2008; Lashermes et al. 1999; Silvestrini et al. 2008; Garavito et al. 2016). "Next-Generation Sequencing" technologies such as genotyping-by-sequencing (GBS) however, are preferred for genome-wide diversity studies because of their high efficiency compared with other single nucleotide polymorphism (SNP) discovery techniques (He et al. 2014; Kwok 2001; Poland et al. 2012). Also in coffee, GBS and diversity array technology sequencing (DArTseq) were used to discover large number of SNPs which could be useful in subsequent coffee breeding programs and in understanding genetic background of varieties of coffee produced (Garavito et al. 2016; Hamon et al. 2017). The availability of the draft genome of *C. canephora* (Denoeud et al. 2014) has facilitated the utilization of GBS for such diversity studies.

In this study we used GBS-SNPs to analyze a total of 48 coffee genotypes of importance to south-western Nigeria, the primary coffee-production area in the country. These comprised 30 accessions selected directly from farmers' fields and 18 accessions acquired from the Cocoa Research Institute of Nigeria (CRIN) germplasm repository. Our aim was to determine (i) the extent to which there was genetic uniformity among the farmer-cultivated coffee cultivars, (ii) the genetic diversity among the conserved germplasm, and (iii) the genetic background of the farmer-cultivated genotypes.

Materials and methods

Plant material

Forty eight coffee samples comprising of 18 accessions from the Cocoa Research Institute of Nigeria (CRIN) germplasm repository (Table 1) and 30 accessions from six farmers' plots with differing locations were used in this study (Table S1). The

CRIN accessions were obtained from five *Coffea* species: *C. arabica*, *C. abeokutae*, *C. liberica*, *C. stenophylla* and *C. canephora*. Each of the species was represented by one genotype except *C. canephora* species which was represented by six different varieties: Kouillou, Gold Coast, Java Robusta, Niaouli, Uganda and Java Robusta Ex Gamba (Table 1), where two to three genotypes were selected to represent each variety (Table 1). The farmers' fields were located in south-western Nigeria (Figure S1A), and the altitude and latitude of the locations were recorded using ArcGIS software (Redlands, California). This included three farms each in both Kogi (7°79′N 5°80′E) and Ekiti states (7°77′N 5°77′E), regions where coffee production is dominant within Nigeria (Figure S1B).

Genotyping-by-sequencing analysis of coffee genotypes

DNA extraction

Young leaves were harvested from all 48 genotypes and placed into zip-lock bags filled with silica gel (Rabbi et al. 2015). The material was lyophilized at the International Institute of Tropical Agriculture in Nigeria and DNA extraction performed using a cetyl trimethylammonium bromide (CTAB) method optimized for coffee (Santa Ram and Sreenath 2000) at the University of California Davis, USA.

DNA library preparation and sequencing

Genomic DNA was sent to the Cornell University Biotechnology Resource Center for GBS sequencing and analysis: http://www.biotech.cornell.edu/brc/brc/services/terms-and-policies. A GBS 96-plex protocol commonly used by the maize research community was applied in this study (Elshire et al. 2011). The restriction endonuclease, *Ape*KI (New England Biolabs, Ipswitch, MA) that recognizes a degenerate 5 bp sequence GCWGC (where W is A or T) and leaves 2 to 3 bp (CWG) overhangs was chosen. Oligonucleotides (Table S2) comprising the top and bottom strands of each barcode adapter and a common adapter, were diluted and annealed in a thermocycler according to Elshire et al. (2011). Adapters quantification and dilution, DNA and adapter plating, and DNA digest were all performed following the protocol developed for maize GBS (Elshire et al. 2011).

The digested DNA samples, each with a different barcode adapter, were combined (5 µL each) and purified using a commercial kit (QIAquick PCR Purification Kit; Qiagen, Valencia, CA) according to the manufacturer's instructions. DNA samples were eluted into a final volume of 50 µL. Restriction fragments from each sample were pooled and amplified by PCR in 50 µL volumes containing 2 µL pooled DNA fragments, 16 µL *Taq* Master Mix (New England Biolabs), and 25 pmol each of the primers (Table S3). These PCR primers were complementary to the ligated adapters, allowing the amplified product to bind the oligonucleotides that coat the Illumina

Table 1 The known background i.e. genome size, variety, and origin of the 18 coffee genotypes acquired from the CRIN Germplasm repository prior to GBS analysis

Species	Genome size	Variety	Origin	Sample name
C. arabica	1174	–	Kenya (Williams 1989)	Ara_18
C. abeokutae	587	–	Nigeria (Omolaja et al. 2000)	Abe_02
C. liberica	636	–	Nigeria (Omolaja et al. 2000)	Lib_02
C. stenophylla	587	–	Ivory Coast (Razafinarivo et al. 2013)	Ste_02
C. canephora	807	Kouillou	Zaire, DRC (Montagnon et al. 1998)	C90, C111, C36
		Gold Coast	Ghana (Williams 1989)	A111, A81
		Java Robusta	Indonesia (Williams 1989)	E106, E77
		Niaouli	Republic of Benin (Montagnon et al. 1998; Williams 1989)	M10, M36
		Uganda	Uganda (Montagnon et al. 1998, Williams 1989)	G129, G37
		Java Robusta ex Gamba	Zaire, DRC (Williams 1989)	T1049, T921, T797

sequencing flow cell, and to prime subsequent DNA sequencing reactions (Bentley et al. 2008). PCR cycling parameters were: 72 °C for 5 min, and 98 °C for 30 s, followed by 18 cycles of 98 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s, with a final step at 72 °C for 5 min (Elshire et al. 2011).

The amplified library was purifiied using a commercial kit (QIAquick PCR Purification Kit; Qiagen, Valencia, CA) and the DNA was loaded onto a capillary sizing system to evaluate the fragment sizes contained within the library (Experion® automated electrophoresis station; BioRad Experion® Hercules, CA). DNA fragment sizes between 170–350 bp were used for, single-end sequencing on a flow cell channel of HiSeq 2500 Illumina (Illumina, Inc., San Diego, CA) (Bradbury et al. 2007; Glaubitz et al. 2014). The pre-processing of the sequenced read data was performed on a TASSEL (Trait Analysis by Association, Evolution and Linkage)-GBS Pipeline (Bradbury et al. 2007; Glaubitz et al. 2014). Of the 267 million reads generated, 9.2% of the reads were acceptable and retained for SNP calling.

SNP Calling

The read or raw data (C81ECANXX_8_fastq.gz) was aligned to the reference genome, i.e. the *C. canephora*, pseudomolecules.fa.gz (http://coffee-genome.org), using the Burrows-Wheeler Alignment (BWA) (Li and Durbin 2009) within the TASSEL commands (net.maizegenetics.pipeline.TasselPipeline—Tassel Version: 3.0.173). After alignment, SNP calling was intitiated with Sequence Alignment Map (SAM) (Li and Durbin 2009) on aligned tags, using a default settings to generate a Hapmap genotype (Danecek et al. 2011; Etter et al. 2011). Genotype quality (GQ) score was calculated to the GATK version (http://gatkforums.broadinstitute.org/discussion/1268/how-should-i-interpret-vcf-files-produced-by-the-gatk). SNPs were filtered with VCFtools. Those with a minor allele frequency $> 1\%$, and, with less than 10% missing data per site across taxa, were retained. During this process Can_47 and *C. abeokutae* genotypes were removed because of their low SNP coverage (as a result of high missing data at $> 10\%$ missing sites) and the occurrence of SNPs at a low allele frequency of < 0.01. This reduced the number of genotypes from 48 to 46. All raw GBS sequencing data was submitted to the National Center for Biotechnology Information (NCBI), Sequence Read Archive (study accession number SRP096172: https://www.ncbi.nlm.nih.gov/sra/?term=SRP096172). The filtered SNPs were used for downstream statistical analyses.

Multivariate analyses

The genetic diversity and relatedness of 46 coffee genotypes, both conserved and cultivated, were assessed using TASSEL software version 5.0 (Bradbury et al. 2007) to generate a tree based on the Neighbour-Joining dendrogram approach; PCA and hierarchical clustering on 43,3048 SNPs were carried out using SNPRelate software (http://www.Rproject.org) (Zheng et al. 2012) to estimate the genetic relatedness among 46 genotypes. Pairwise analysis of Identity by State (IBS) distance matrices was used to relate genetic distance to genetic diversity among all genotypes (Purcell et al. 2007). F-statistic (F_{ST}) was calculated using VCFtools (Danecek et al. 2011) based on Weir and Cockerham estimation, to identify the genetic differentiation between and among populations (Balloux and Lugon-Moulin 2002). Admixture analysis was carried out using STRUCTURE (structureHarvester.py v0.6.94) (Dent and Bridgett 2012).

Results and discussion

To our knowledge, this is the first GBS analysis of *C. canephora* genotypes in Nigeria to assess the extent of genetic diversity and genetic uniformity among conserved genotypes and farmer-cultivated coffee cultivars, and to determine the genetic background of the farmer-cultivated genotypes.

SNP distribution/characterization and heterozygosity of alleles

The number of raw SNPs detected was 440,481 while 433,048 SNPs were obtained after filtering for low ($< 10\%$) minor allelic frequency (MAF; 0.01), across 46 genotypes. Of these SNPs, 329,577 were distributed across the 11 coffee chromosomes. The remaining 103,471 SNPs were anchored on "chromosome 0", because they could not be assigned to any of the known 11 chromosomes. Most SNPs were recorded on chromosome 2, which had three times

more SNPs compared to Chromosome 9, which had the least number (Table S4).

The highest number of heterozygous alleles (106,365) was observed in *C. arabica* (Ara_18). The genotype classified as *C. liberica* (Lib_02), the farmer-cultivated accession Can_19, and the *C. canephora* var Kouillou accession C90, also had high number of heterozygous alleles (more than 60,000), but the *C. canephora* genotypes var. Java Robusta, (E106) had the highest number of heterozygous alleles (between 60,000 and 80,000). In contrast, the farmers' accessions Can_20, Can_38, and Can_44 had the fewest alleles i.e. 6697, 4462 and 4721 respectively (Figure S3), almost 10-fold fewer compared with the genotypes with the highest number of heterozygous alleles.

Twice as many heterozygous alleles were found in *C. arabica* compared to *C. canephora.* This is likely due to the polyploid nature of *C. arabica,* and the presence of polymorphisms existing between the loci of the two homeologous genomes of the tetraploid *C. arabica*. Lashermes et al. (1999) also found a high level of fixed heterozygosity in the *C. arabica* genome and regarded the level of its internal genetic variability to be twice that present within its diploid relatives.

Relatedness of the 46 genotypes (conserved and cultivated)

There was a high degree of similarity in SNP polymorphisms between M10 and each of the farmer-cultivated accessions, irrespective of where they were grown in Nigeria (Fig. 2 and Figure S4). The genetic uniformity observed among the cultivated accessions is an indication that they may have been propagated vegetatively. This suggests a production system characterized by a high level of clonal multiplication and cooperation among Nigeria coffee farmers. The lack of genetic diversity in these accessions contradicts previous report that there were as many as 26 varieties of *C. canephora* in the distribution zone that encompasses Nigeria (Montagnon et al. 1998; Gomez et al. 2009).

Of the 30 farmer-cultivated accessions analysed, only one (Can_19), was genetically distinct. This exceptional genotype (Can_19) resembled Lib_02 and C90, both of which were conserved in the CRIN coffee germplasm. The Lib_02 genotype labelled as *C. liberica,* was introduced from farmers' field to the CRIN coffee germplasm (Omolaja et al. 1997). This genotype seemed to be divergent from the other farmers' accessions (Figure S4).

Previous morphological characterizations of the CRIN coffee germplasm classified C90 and Lib_02 as *C. canephora* and *C. liberica* respectively (Omolaja et al. 1997). The floral morphology of C90 and Lib_02 appeared to be different from *C. canephora* and even *C. liberica* (Fig. 2b). However, the GBS-SNP analysis in this study showed a high level of divergence in the SNPs detected among C90/Lib_02/Can_19 (Figures S4 and 3A) and *C. canephora*. Our analysis found C90 and Lib_02 (G1; Tables 2, 3) to be closer to *C. canephora* than to *C. arabica.* First, the average genetic distance was 0.2014 with *C. canephora,* compared with the higher value of 0.3346 found with *C. arabica* (Table S4). Second, the mean F_{ST} estimate of 0.50006 with *C. arabica* indicates higher genetic differentiation, while the lower values (0.1321–0.2501) found with *C.canephora* indicate greater similarity (Tables 2, 3). Finally, genetic structure analysis (Fig. 3) confirmed C90 and Lib_02 to be *C. canephora*.

Assessment of genetic diversity and reclassification of coffee germplasm

The multidimensional scaling (MDS) using SNPRelate analysis is generally used in assessing diversity and relatedness of genotypes. This analysis grouped the 46 genotypes into four diverse clusters on a principal component plot, showing the first (PC1) and the second principal components (PC2) which explained 36.2% of the variation (Fig. 1a, b). The genotypes belonging to a cluster are more genetically similar compared to those in other clusters. This clustering differentiated *C. canephora* into 3 subgroups (II, III and IV). Also, an admixture analysis discovered three populations among the *C. canephora* genotypes, (Q1, Q2 and Q3), of which two (Q2 and Q3) were regarded as being sub-populations by STRUCTURE (Fig. 3 and Table S7).

Identity By State (IBS) distance matrix and principal component analysis (PCA) clustering were used to identify both between and within-species diversity. The analysis revealed that the average genetic distances of *C. canephora* from (i) *C. arabica*, (ii) the *C. canephora* variety once labeled as *C. liberica*, and (iii) the farmers' accessions were 0.3346, 0.2014, and 0.1867, respectively (Table S5). These data may be

Table 2 The grouping of genotypes based on hierarchical clustering

G1	Lib_02	Can_19	C90					
G2	Ara_18							
G3	T979	T921	T1049	E106				
G4	C36	C111	G37	G129	E77	A81	A111	Ste_02
G5	M36	M10	Can_46	Can_45	Can_44	Can_43	Can_42	Can_41
	Can_40	Can_39	Can_38	Can_37	Can_36	Can_35	Can_34	Can_33
	Can_32	Can_31	Can_30	Can_29	Can_28	Can_27	Can_26	Can_25
	Can_24	Can_23	Can_22	Can_21	Can_20	Can_18		

Table 3 Pairwise F_{ST} estimation of the five groups identified from HC (Hierarchical Clustering) analysis

Comparing group	Total sample	Mean F_{ST} estimate	Weighted F_{ST} estimate
All groups	46	0.3648	0.35337
G1_vs_G2	4	0.50006	0.59913
G1_vs_G3	7	0.1321	0.22404
G1_vs_G4	11	0.17983	0.3079
G1_vs_G5	33	0.25018	0.38704
G2_vs_G3	5	0.25351	0.43656
G2_vs_G4	9	0.29954	0.51683
G2_vs_G5	31	0.3037	0.6111
G3_vs_G4	12	0.14999	0.27821
G3_vs_G5	34	0.24287	0.39527
G4_vs_G5	38	0.13977	0.26971

Key is as follows: 0–0.05 indicates little genetic differentiation; 0.05 and 0.15, moderate differentiation; 0.15 and 0.25, great differentiation and above 0.25, very great genetic differentiation. For the interpretation of F_{ST} estimate see Balloux and Lugon-Moulin (2002)

interpreted as follows: firstly, there is high genetic distance between *C. canephora* and *C. arabica*, revealing inter-species diversity even though *C. arabica* resulted from a recent hybridization between *C. canephora* and *C. eugenioides* (Lashermes et al. 1999). Secondly, there is low genomic variation between *C. canephora* and the genotype formerly classified as *C. liberica*, revealing intraspecific diversity. This is evidence that the formally classified *C. liberica* is actually a *C. canephora* genotype because it shares more common alleles with *C. canephora* (Fig. 1b) and belong to the same sub-population with Java Robusta (*C. canephora* variety). If Lib_02 was a *C. liberica* genotype it would have a higher genetic distance compared with *C. canephora.* This was shown by Steiger et al. (2002) using AFLP markers. They reported that *C. canephora* and *C. arabica* were more genetically similar, while *C. canephora* and *C. liberica* were more genetically distinct. The low value (0.17983) detected with F_{ST} estimation between Lib_02 (G1) and *C. canephora* (G3 and G4) confirmed Lib_02 to be a *C. canephora* genotype. Thirdly, there is low genetic distance between *C. canephora* and the farmers' accessions (0.1867). From both hierarchical analysis and IBS genetic distance values, it is possible to assume that coffee farmers in south-western Nigeria are cultivating one variety of *C. canephora*, specifically the Niaouli variety. This was illustrated by the low genetic distance (IBS value) of 0.1194 between *C. canephora* var. Niaouli (M10) and the farmers' accessions (Table S5), and the formation of a cluster (II) between the farmers' accessions and M10 (Fig. 1b). Also the ancestral inference detected with Structure analysis grouped farmers accessions into the same population structure as M10 (Fig. 3). The reason for this widespread adoption of a single genotype among farmers is not known. It is possible that there was an exchange of coffee seedlings from the

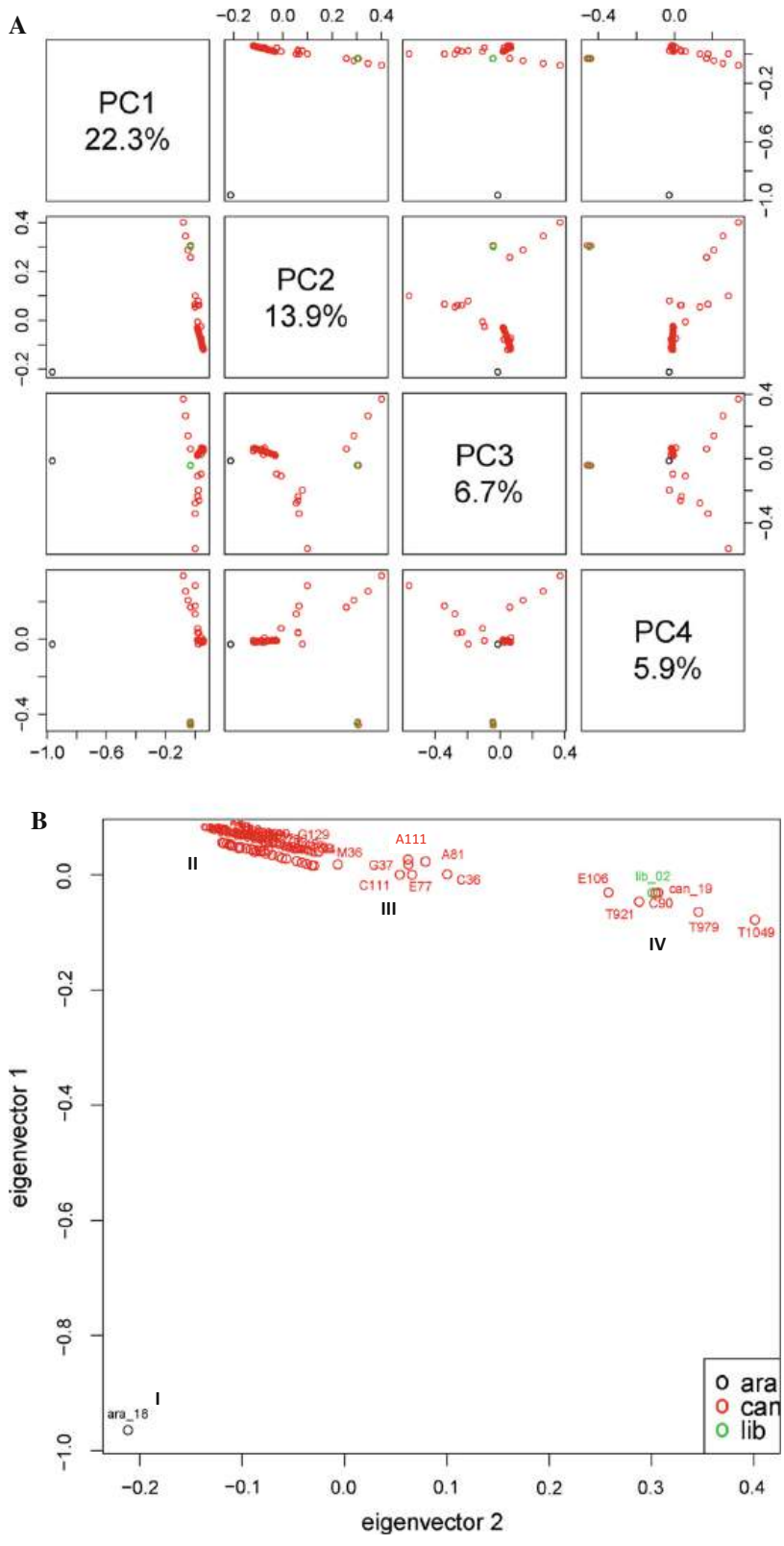

Fig. 1 **a** Principal component analysis of 46 coffee genotypes categorized by SNPRelate software, **b** The multi dimensional scale (MDS) plot of PC1 (22.3%) and PC2 (13.9%) of 46 genotypes (cluster I: *C. arabica*, II -IV: *C. canephora*)

neighboring country of Benin (Gomez et al. 2009) the center of origin of *C. canephora* var. Niaouli (Montagnon et al. 1998), leading to its introduction into Nigeria, and its subsequent cultivation was due to farmer preferences based on its unique characters or due to its availability.

While comparing *C. arabica* with the four *C. canephora* varieties (Table 2, 3), it was observed that var. Java Robusta grouped closer to *C. arabica* (G2) having a mean F_{ST} estimate of 0.25351 (Tables 2, 3), while *C. canephora* var. Niaouli was more distant with mean F_{ST} estimate of 0.3037 (Table 3 and S5). *C. canephora* var. Java Robusta is known to have good cup quality and leaf rust resistance, and has the shortest genetic distance from *C. arabica* (high quality coffee) and should be utilized for for intraspecific hybridization, and interspecific hybridization after confirming their reproductive compatibility.

The intra-species diversity among different genotypes of the six *C. canephora* varieties used in the study was compared in a pairwise manner, and the average genetic distance was 0.1867 (Table S6). The highest IBS value of 0.2552 (Table S6), pairwise F_{ST} estimate of 0.24287 (Table 3) and unrelated population structures of Q2 and Q1 (Fig. 3) were found between var. Java Robusta ex Gamba (T1049) and var. Niaouli (M10), suggesting that these two varieties are the most diverse. The heterosis within these *C. canephora* varieties may therefore be utilized in intra-specific crossing, to develop improved varieties of coffee. In addition, hierarchical clustering (Fig. 2) disclosed four inter-genetic diversity among *C. canephora* constituting four varietal levels: (1) Java Robusta and Java Robusta ex Gamba (Group III), (2) Kouilou/Quillou and Java Robusta (Group IV), (3) Uganda and Gold Coast (Group V) and (4) Niaouli (Group VI). Interestingly, the results from the population structure (Fig. 3) and PCA analysis (Fig. 1b) are in conformity, since both revealed three genetic units in the *C. canephora* germplasm from the CRIN. The varieties of *C. canephora* identified here are of Congolese' origin (Montagnon et al. 1998; Dussert et al. 1999; Gomez et al. 2009; Musoli et al. 2009; Razafinarivo et al. 2013; Leroy et al. 2014), and thus represent a very narrow genetic pool. There has been little acquisition of new coffee genetic resources at the CRIN. The need to broaden this genetic base for continued coffee improvement in Nigeria becomes imperative. Low genetic diversity has also been observed by Omolaja and Fawole (2004) using morphological characteristics.

The low number of heterozygous alleles found in different *C. canephora* genotypes was surprising, since it is a self-incompatible species. A study by Souza et al. (2013) on *C. canephora* cultivated in Brazil detected high genetic diversity. Low genetic diversity in self-incompatible *C. canephora* genotypes maintained and cultivated in Nigeria indicated little or no major breeding efforts targeting these genotypes, which may be a reason for their current low yield and quality. To ensure proper utilization of genetic resources, a full knowledge of the inherent genetic diversity and relationship within the genepool of interest is necessary (Li and Durbin 2009). All analytical approaches used in the current study revealed the existence of misclassified genotypes conserved in the CRIN germplasm repository. It is recommended that the two varieties currently described as 'Uganda' and 'Gold Coast' be merged into a single variety (Uganda/Gold Coast) since no significant genetic difference was detected between them (Figs. 1b, 2a, b). Although the selected 14 *C. canephora* conserved genotypes from the CRIN germplasm repository were previously categorized into six varietal groups based on morphological characteristics (Table 4), our genomic analyses using next-generation sequencing method (GBS) confirmed that these actually belonged to four varietal groups (Table 4). The comprehensive nature of the SNPs-GBS genomic analysis instilled a high degree of confidence with respect to the classification of these varieties. A similar result was achieved with EST-SSR markers for proper grouping of different populations and varietal groups in coffee from Brazil (Souza et al. 2013).

The two admixture genotypes, M36 and G129 detected with STRUCTURE (Fig. 3) contain almost equal proportion of Niaouli and Kouillou. These genotypes will be incorporated into var. Java Robusta genome through conventional hybridization thereby generating F_1 with the combined genomic composition of all the three genetic structures (Niaouli, Kouillou and Java Robusta). From this study it can be deduced that the analysed genotypes of germplasm representative of the CRIN collection which was started in 1966, comprised of three genetic structures. Some of the nomenclature of the accessions were assigned based to the donor countries' name, and

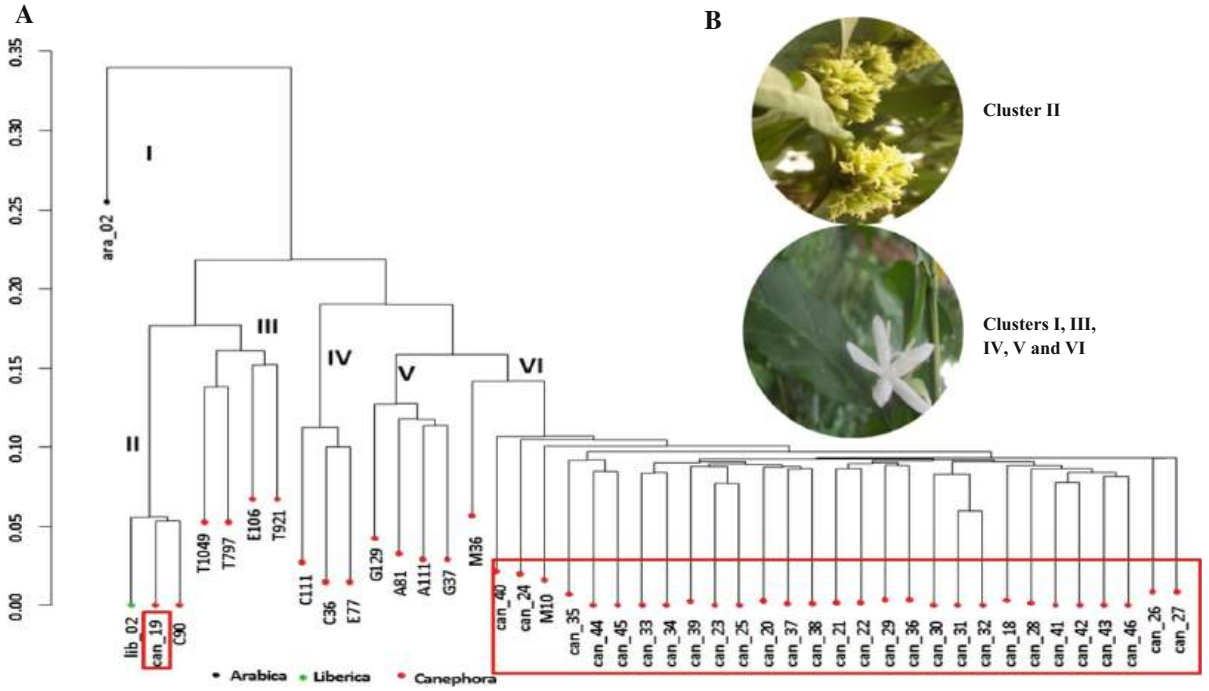

Fig. 2 **a** Hierarchical clustering of the genotypes. Cluster I: A *C. arabica* genotype; II: Unknown *C. canephora* genotypes, III: Java Robusta ex Gamba and Java Robusta (E106); IV: Kouillou and Java Robusta (E77); V: Uganda and Gold Coast; VI: Niaouli and farmers' accessions. The samples in red rectangle are the accessions from farmers' field except M10. **b** Morphological differences in the flowers produced by genotypes in Cluster II and I, III, IV, V and VI. (Color figure online)

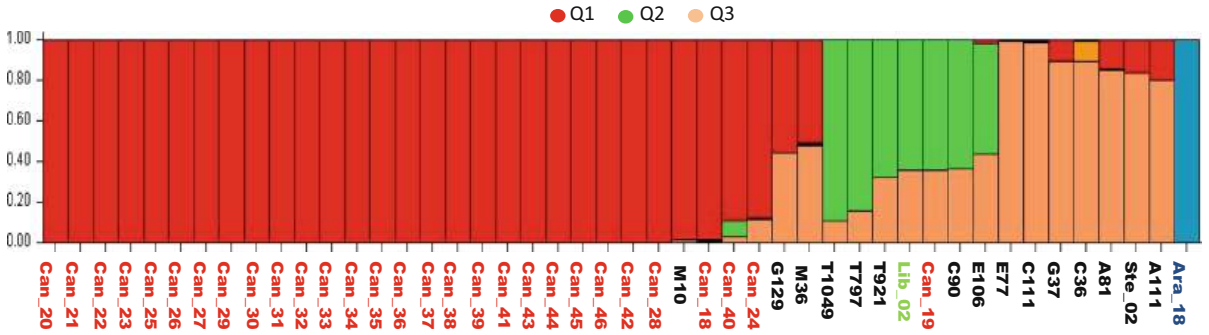

Fig. 3 The Genetic Structure analysis of the 46 genotypes between K = 1 and K = 10 with3 populations Q1, Q2 and Q3. Q2 and Q3 are regarded as sub-populations. The Q1 population represents the farmers' accessions, while Q2 and Q3 are from the CRIN conserved genotypes (Black are conserved genotypes of *C. canephora*, green is formerly *C. liberica,* blue is a genotype of *C. arabica,* while red are cultivated accessions). (Color figure online)

collection was by seeds. These three genetic structures were grouped based on the clustering of the genotypes into Niaouli, Gold Coast/Kouilou and Java Robusta.

Improvement in the CRIN germplasm collection should be chanelled towards acquiring more genetic material for *C. canephora* genotypes from Ivory Coast and Uganda as they have high yield components, quality traits and tolerance to biotic and abiotic stresses. In order to improve the organoleptic quality, the introduction of var. '126' should be prioritized, as it is the best accession for quality and yield traits (Leroy et al. 2014). This accession has been widely distributed in Togo, Guinea and Cameroon. Also, accessions '410', 'A03 and '466' all have specific genotypic values between Guinean and Congolese groups (Leroy et al. 2014) and should be targeted for

Table 4 Grouping of the *C. canephora* genotypes based on SNP-GBS genotyping. The number decreased from six varieties to four. (a) Hierarchical clustering formed the following varietal groups: Java Robusta ex Gamba and Java Robusta (E106), Kioullou and Java Robusta (E77), Uganda and Gold Coast, and Niaouli alone (Fig. 2). (b) Similar classification was confirmed using PCA and IBS (Fig. 1 and Table S5)

Species	Genotypes	Varietal Grouping based on Morphological Markers	Varietal Grouping based on SNP-GBS
C. canephora	T1049	Java Robusta Ex Gamba	Java Robusta Ex Gamba
C. canephora	T797	Java Robusta Ex Gamba	Java Robusta Ex Gamba
C. canephora	E106	Java Robusta	Java Robusta Ex Gamba
C. canephora	T921	Java Robusta Ex Gamba	Java Robusta Ex Gamba
C. canephora	C111	Kouillou	Kouillou
C. canephora	C36	Kouillou	Kouillou
C. canephora	E77	Java Robusta	Kouillou
C. canephora	G129	Uganda	Uganda/Gold Coast
C. canephora	G37	Uganda	Uganda/Gold Coast
C. canephora	A81	Gold Coast	Uganda/Gold Coast
C. canephora	A111	Gold Coast	Uganda/Gold Coast
C. canephora	M10	Niaouli	Niaouli
C. canephora	M36	Niaouli	Niaouli

possible incorporation into the farmer's germplasm. The high variability found within the Java Robusta and Niaouli should also be utilized in crosses to obtain hybrids.

Conclusion

The ability to capture and efficiently use abundant genetic resources are considered essential for sustainable coffee production in Nigeria. The utilization of information on the diversity, relatedness, and consistency of the coffee genetic resources found in this study will help in planning a worthwhile coffee improvement program in Nigeria. Despite the limited number of genotypes used, we were able to determine that the previous characterization performed only with morphological characters was inconclusive and that the efficient utilization of genetic resources has been lacking. There is a need to broaden the genetic base of *C. canephora* and, generally, the *Coffea* species in Nigeria. Collaboration with other scientists from the Ivory Coast and Uganda, countries that harbor large collections of *C. canephora* genotypes, becomes imperative, in order to acquire new genetic material, and to ascertain their true genetic identity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Somatic Embryos Development among some Nigeria Cocoa (Theobroma cacao L.) Genotypes

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Abstract

Clonal propagation is an alternative method of maintaining the genetic purity of superior cocoa plants (*Theobroma Cacao* L.). The somatic embryogenesis technique is likely the most effective and efficient method for plant propagation. This study aimed at investigating the callogenesis and embryogenic potential of some Nigeria cocoa clones on culture media added with various concentrations of antioxidants and variation in the concentration of auxin hormone combined with ascorbic acid. The superior clones used in this study were N38, TC1, C77, TC3, and PA150. The parameters observed in this study were percentage of embryogenic callus formation, number of secondary somatic embryos, and percentage of embryo somatic cells that develop into planlets. The addition of 2,4,D combined with ascorbic acid affected the initiation of cocoa secondary somatic embryogenesis. Treatment combination of ascorbic acid 100 $mg.L^{-1}$ and 2,4,D 1 $mg.L^{-1}$ had a significant effect on the initiation of secondary somatic embryogenesis of cocoa in the embryogenic callus phase (4.73%), globular embryos (2.53%), torpedo embryos (4.67%) and sprouts (0.47%). Three clones i.e N38, TC3 and PA150 clones showed a higher percentage (3.0%, 2.10%, and 1.80%, respectively) of embryogenic callus growth than the other clones. Meanwhile, on C77 clone, all treatments did not affect the regeneration of embryogenic callus. The combination treatment of organic acid and adenine showed a low embryogenic callus response in TC1 and N38 clones. However, TC1 clone also did not show a response in form of globular, torpedo, and germination phase somatic embryo regenerations. This indicates that different plant cultivars show different responses to the addition of organic acids. Treatment combinations of adenine 0.0125 $mg.L^{-1}$ + ascorbic acid 100 $mg.L^{-1}$ gave the best response to the regeneration of somatic embryos for the globular, torpedo, and germination stages, started 3 weeks after being transferred to regeneration medium.

Keywords: somatic embryogenesis, cocoa, *in vitro*, somatic embryo, ascorbic acid, browning

INTRODUCTION

Theobroma cacao L. is one of the important plantation commodities that has significant economic value for the chocolate industry in the world (Squicciarini & Swinnen, 2016). ICCO (2020) shows that world cocoa production in 2019-2020 decreased by 4.750 million tons compared to 2018-2019 (4.780 million tons). The reason behind this decline in production is caused by the reduced number of cocoa plantations and the decrease in crop productivity.

Along with its development, in addition to the aging of cocoa trees and high input costs, there are several obstacles in cocoa

cultivation related to abiotic stress problems such as drought (Towaha & Wardiana, 2015) and biotic stress such as pests and diseases attack that affect cocoa productivity. Development of superior cocoa clones that have resistance to biotic stress in form of pests and diseases can be obtained through a breeding program carried out periodically and continuously from genetic materials, which may provide opportunities for genetic conservation of cocoa plants. Environmental stress might be caused by both biotic and abiotic factor.

Procurement of superior planting materials conventionally often facing problems because it is difficult to get hight quality seedlings in large quantities and in a short time. According to Pancaningtyas (2013), cocoa somatic embryogenesis is a tissue culture technique that produces primary embryos from cocoa plant organs, i.e. the flower parts (staminodes and petals). This technique has several advantages over conventional propagation techniques for large-scale plant propagation while maintaining genetic stability and integrity, i.e., obtaining plants with a uniform genotype in large numbers and in a short time (Maximova *et al*., 2002; Quainoo & Dwomo, 2012).

The development of this technology to date has produced plantlets with a conversion rate of 65% and relatively low percentage of somaclonal variation (Lopez *et al*., 2010). Modifications related to the growth medium for somatic embryogenesis (SE) have been reported recently by Kouassi *et al*. (2017) and Modeste *et al*. (2017). Further development of SE protocol modified by various commercial companies has been outlined in various international patent documents (Wickramasuriya & Dunwell, 2018). Despite continuous progress, the low reproducibility and regeneration efficiency of the developed methods and the differences in responses between genotypes in the SE process are still challenges for mass propagation of elite cocoa plants on a commer

cial scale (da Silva *et al*., 2008). The diversity of responses to somatic embryogenesis is mainly due to differences in genotypes (Issali *et al*., 2008; Kouassi *et al*., 2017).

However, variation of somatic embryos for each cocoa genotype is one of the obstacles that must be overcome. The application of somatic embryogenesis in cocoa plants is considered to be quite complicated due to the recalcitrant character of cocoa seed and plant genetics that affect the ability of gene expression to regenerate. Recalcitrant is defined as the inability of plant cells, tissues, and organs to thrive in tissue culture (Benson, 2000). One of the components that affect the disability in vitro is the high phenol content and phenol oxidase compounds. Oxidation of this compound is an inhibiting factor for multiplication and tissue regeneration. The ability of embryogenesis is also influenced by the concentration and distribution of polyphenolic compounds (Gallego *et al*., 2016). High concentrations of phenolic compounds are associated with non-regenerating responses of somatic embryos (Alemanno *et al*., 2003). These compounds can further inhibit the development of plant tissue in vitro. The addition of polyvinylpyrrolidone (PVP) and antioxidants such as ascorbic acid and citric acid is a way to inhibit the production of phenolic compounds or reduce their accumulation in tissue culture media (Krishna *et al*., 2008). Overcoming this bottleneck requires optimization of existing protocols.

Application of the somatic embryogenesis protocol to new superior cocoa clones requires optimization at the secondary embryogenic callus regeneration stage, which is the most important stage in somatic embryo production. Therefore, this study aimed to evaluate the callogenesis and embryogenic potential of new superior cocoa clones on culture media equipped with various concentrations of antioxidants and variations in the concentration of auxin hormone.

MATERIALS AND METHODS

The genotypes used in this study were N38, C77, Tc1, PA150, and Tc3. Each clone used 100 budding flowers. The used as explants material were cocoa flower parts.

This research was carried out at the Biotechnology Laboratory of the Cocoa research institute of Nigeria, taking place from February 2019 to December 2020. Research on cocoa propagation through the somatic embryogenesis technique was carried out in integrated procedures, starting from mother tree selection, explant collection, flower induction, primary embryo production, embryogenic callus initiation, embryogenic callus multiplication, secondary embryo production, germination, and planlet maturation. The initial stage of laboratory works begans with the orientation of the medium used for each stage of development. Different stages require different culture mediums, as well as different genotype, require different medium.

The growing medium used in the cocoa genotypes protocol included modified Murashige and Skoog, and Driver and Kuniyuki basal medium, PGR (2,4-D, 2,4,5-T, kinetin, adenine sulfate, NAA, IBA), ascorbic acid, glucose, sucrose, gelrite, aquadest, sodium hypochlorite 10%, aluminum foil, NaOH, HCl, alcohol.

Embryogenic Callus Initiation

Primary embryos obtained from the previous stage were re-initiated to produce secondary embryogenic callus, incubated for eight weeks in the dark at 25^{o}C. Secondary embryogenesis was used to increase the number and quality of somatic embryos produced (Maximova *et al*., 2002). The culture medium used consisted of a combination of basic media Murashige and Skoog (1962) and Driver & Kuniyuki (1984), adenine sulfate 0.25 mg.L^{-1}, glycine 2 mg.L^{-1}, L-lycine 0.40 mg.L^{-1}, L-leucine 0.40 mg.L^{-1}, L-arginine 0.40 mg.L^{-1}, L-tryptophan 0.20 mg.L^{-1}. Embryogenic callus from each cocoa clone had a different regeneration rate. Therefore, the addition of ascorbic acid antioxidant treatment and variations in the concentration of auxin (2,4,5-T) in the growing media were as following:

M0 (Control/embryogenic callus initiation medium - SEC).

M1 (ascorbic acid 100 mg.L^{-1} + 2,4,5-T 0.5 mg. L^{-1}).

M2 (ascorbic acid 100 mg.L^{-1} + 2,4,5-T 1 mg.L^{-1}).

M3 (ascorbic acid 200 mg.L^{-1} + 2,4,5-T 0.5 mg.L^{-1}).

M4 (ascorbic acid 200 mg.L^{-1} + 2,4,5-T 1 mg.L^{-1}).

The study was arranged according to a factorial completely randomized design, were the first factor was five cocoa clones (N38, C77, Tc1, PA150, and Tc3) and the second factor was five types of media with three replications. The total treatment combinations were 75. Furthermore, analysis of variance was carried out using Duncan multiple range test at 5% significant level. The characteristics of embryogenic callus are having a friable structure and forming crystals like dew. The formation of the embryogenic callus is an important key in the somatic embryogenesis process of propagation. The optimal time for secondary embryogenic maintenance is 21 to 26 weeks after initiation of the primary embryo (Maximova *et al*., 2002).

Somatic Embryo Regeneration

This stage is a process for the formation of somatic embryos derived from secondary embryogenic callus. Multiplicated embryogenic callus was sub-cultured on expression media with balanced hormone composition. This process lasted for four weeks in the dark. Embryos that have been formed were maintained until they were ready to be harvested. The characteristics of normal embryos that are ready to be harvested are embryos in the torpedo-cotyledon phase. At this stage, there is still a diversity of various stages of embryonic growth produced, including the globular phase to the cotyledon phase. Embryos before the torpedo phase and the cotyledon phase were sub-cultured on the same medium.

Phenolic oxidation is one of the obstacles in the propagation of cocoa embryogenesis. To overcome this problem, organic acid was added to increase the potential of somatic embryos from the resulting callus biomass. The formulation of the media for enhancing the regeneration of embryos from biomass with the addition of organic acids, as follows:

Media C0 (Control /expression medium)

Media C1 (adenin 0.025 $mg.L^{-1}$ + ascorbic acid 50 $mg.L^{-1}$)

Media C2 (adenin 0.0125 $mg.L^{-1}$ + ascorbic acid 50 $mg.L^{-1}$)

Media C3 (adenin 0.025 $mg.L^{-1}$ + ascorbic acid 100 $mg.L^{-1}$)

Media C4 (adenin 0.0125 $mg.L^{-1}$ + ascorbic acid 100 $mg.L^{-1}$)

Media C5 (adenin 0.025 $mg.L^{-1}$ + ascorbic acid 150 $mg.L^{-1}$)

Media C6 (adenin 0.0125 $mg.L^{-1}$ + ascorbic acid 150 $mg.L^{-1}$)

The study was arranged according to a factorial completely randomized design (CRD), the first factor was four cocoa clones (N38, C77, PA150, and Tc1) and the second factor was seven types of media with three replications. The total treatment combinations were 84. Further, analysis of variance will be carried out using DMRT at 5% level.

Observation Method

Several parameters were observed in this study. Observation of secondary embryo-genic callus percentage was carried out started at eight weeks after culture on secondary embryogenic callus initiation media (SEC).

$$\% \text{ embryogenic callus} = \frac{\text{total embryogenic callus}}{\text{total explants}} \times 100$$

Percentage of secondary somatic embryos was observed when ± 3 g of embryogenic callus clusters were cultured on the expression medium for somatic embryo formation. Observations were carried out qualitatively using a microscope and quantitatively by counting the number of embryos produced by each embryogenic callus cluster. Observations were made at four weeks after culture in embryo regeneration medium.

$$\% \text{ somatic embryos} = \frac{\text{total somatic embryos}}{\text{total explants}} \times 100$$

RESULTS AND DISCUSSION

Embryogenesis Process

The process of embryogenesis in cocoa is secondary somatic embryogenesis, where the resulting primary somatic embryo is reinitiated to produce secondary somatic embryos. At this initiation stage, there are regeneration constraints that are mainly caused by the oxi-

dation of phenolic compounds which causes browning in the explants (Figure 2). Browning is one of the obstacles in in-vitro culture caused by the accumulation of phenolic compounds due to polyphenol oxidase (PPO) oxidation (Jones & Saxena, 2013). Browning in explant tissue is the main obstacle at this stage. In addition, variations in the content of endogenous hormones also affect the rate of regeneration of embryogenic callus. Based on Pancaningtyas (2015) the highest content of phenolic compounds was at the embryogenic callus stage. The addition of organic acids in in-vitro culture media was carried out to overcome the inhibition of phenolic compounds so that the explants could increase their regeneration to produce somatic embryos.

In the first secondary embryogenesis observation (3 weeks after initiation), N38 Tc3, and PA150 clones showed a higher percentage of embryogenic callus growth compared to the other clones (3.0%, 2.10%, and 1.80%). Meanwhile, in the PA150 clone, all treatments did not affect the regeneration of embryogenic callus. In the further regeneration of secondary somatic embryo-genesis, C77 clones showed a higher percentage of secondary somatic embryo formation both at the globular and torpedo stage and even at shoot growth at the 9th week of observation (Figure 2). Based on these data, genotype differences greatly influence the initiation of secondary somatic embryogenesis. The effect of genotypes had a significant effect

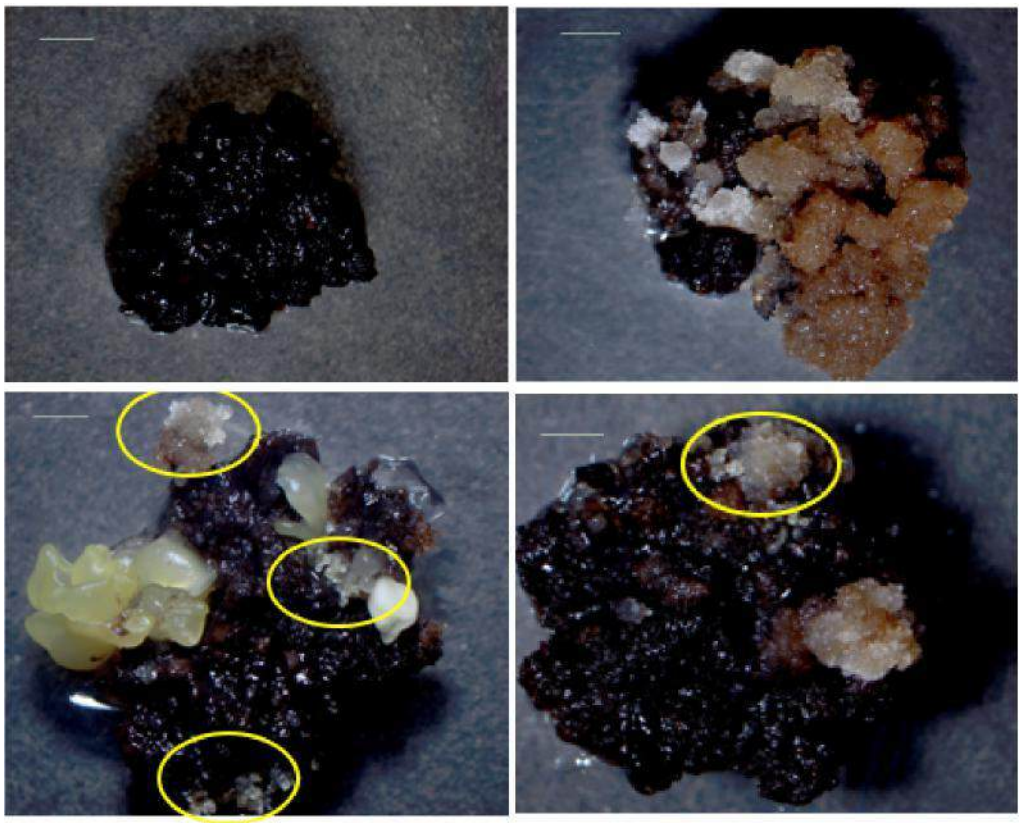


Figure 1. Browning on callus biomass culture of cocoa (before treatment) (A & B). Embryogenic callus after the addition of ascorbic acid treatment (Observations using an Olympus microscope *SZ61* equipped with a DP25 camera) (C & D). Bar = 3,75 mm

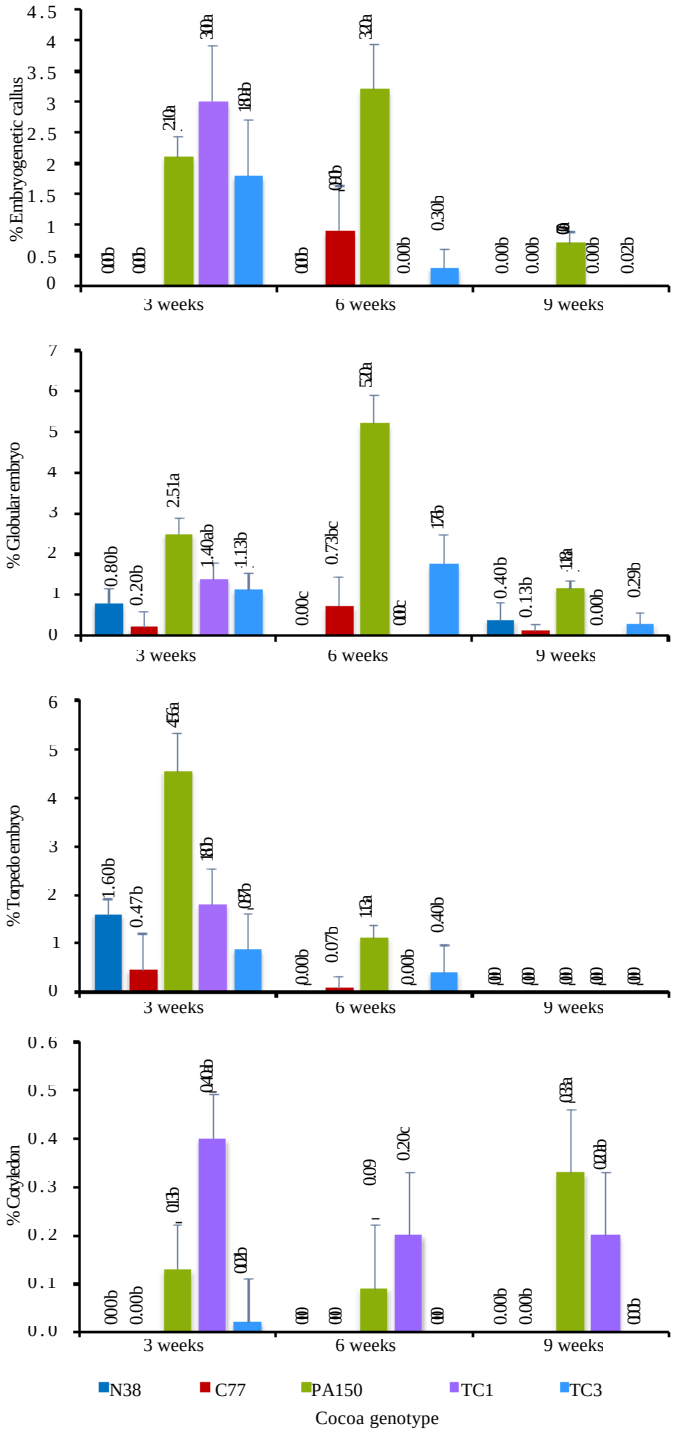


Figure 2. Response of some cocoa clones to the addition of organic acids and hormones 2,4D at the initiation stage of secondary embryogenesis

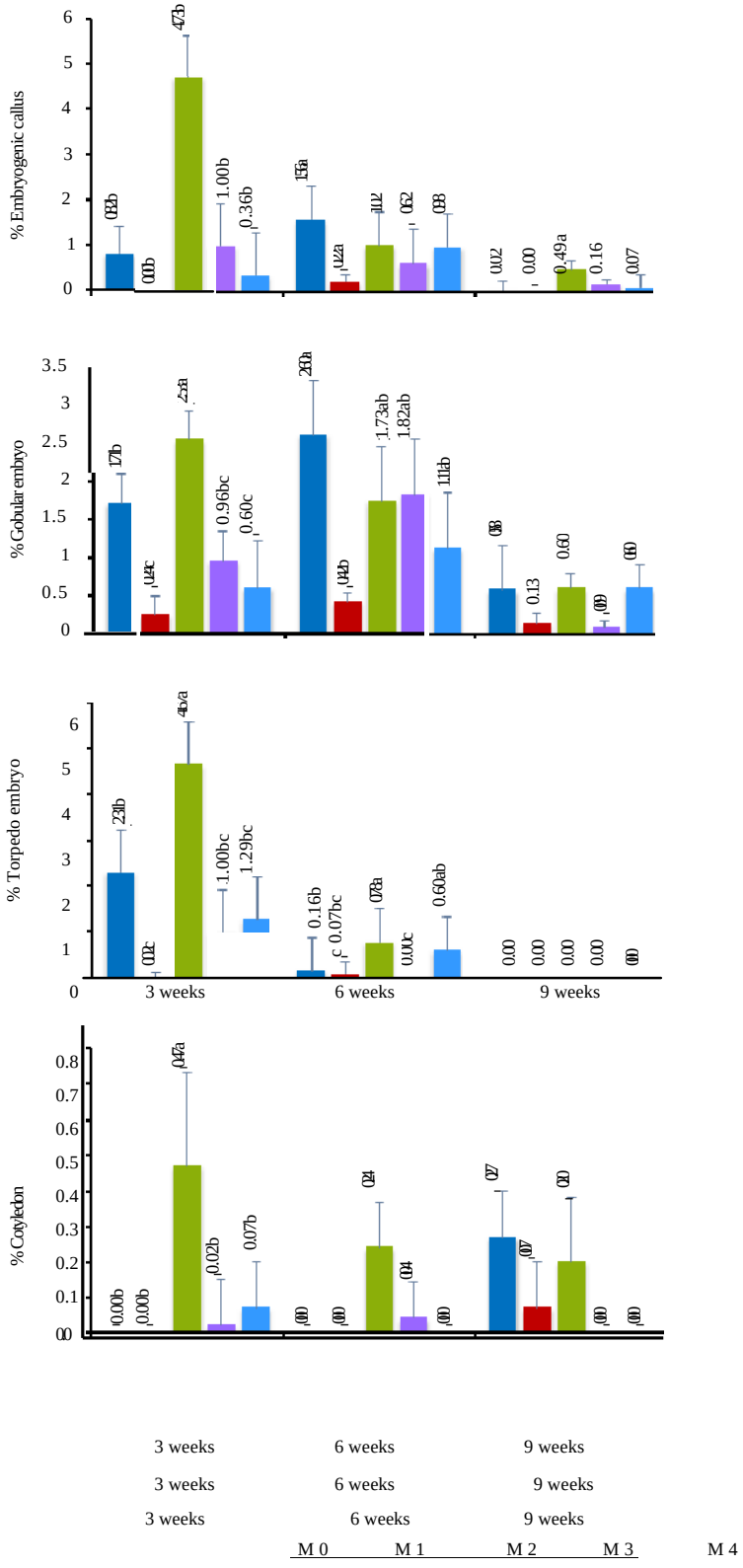


Figure 3. Effect of ascorbic acids and 2,4,D concentration on the initiation of secondary embryogenesis

Notes: M1 (ascorbic acid 100 mg.L^{-1} + 2,4,D 0.5 mg. L^{-1}); M2 (ascorbic acid 100 mg.L^{-1} + 2,4,5-T 1 mg.L^{-1}); M3 (ascorbic acid 200 mg.L^{-1} + 2,4,D 0.5 mg.L^{-1}); M4 (ascorbic acid 200 mg.L^{-1} + 2,4,5-T 1 mg.L^{-1}).

on the regeneration response of cocoa secondary somatic embryos. Nonetheless, there is no interaction between genotype and culture media. This is also related to the physiological conditions of the cultured cells or tissues.

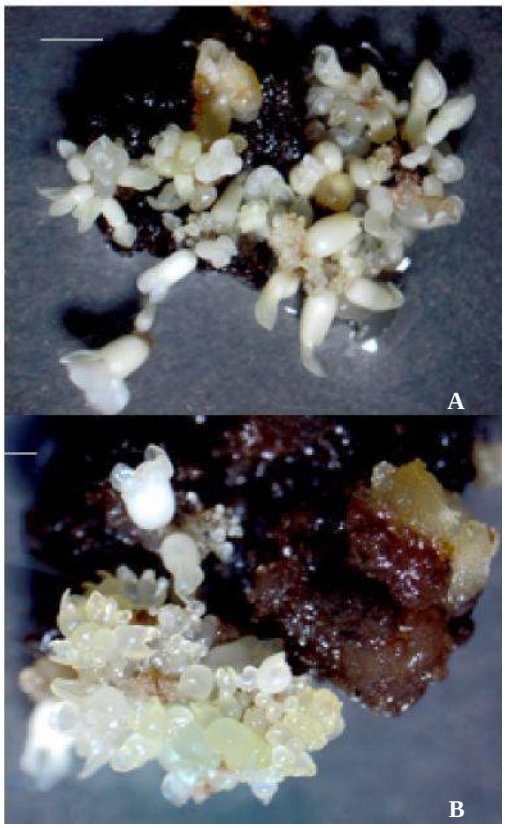
The addition of 2,4,D hormone combined with ascorbic acid affected the initiation of cocoa secondary somatic embryo-genesis. The treatment combination of ascorbic acid 100 mg.L^{-1} and 2,4,D 1 mg.L^{-1} had a significant effect on the initiation of secondary somatic embryogenesis of cocoa in both embryogenic callus phase (4.73%), globular embryos (2. 53%), torpedo embryos (4.67%) and planlets (0.47%) (Figure 3). That treatment medium still showed good results even up to the observation at week nine. In other treatment mediums, embryogenic callus and globular embryos showed an increase in the 6th week. The combination of ascorbic acid 100 mg.L^{-1} and 2,4,5-T 0.5 mg.L^{-1} treatment showed the lowest values in all parameters (embryogenic callus, globular embryos, torpedo embryos, and planlets).

Browning intensity in culture is influenced by plant species and varieties, tissues or organs, plant development phase, tissue or organ age, nutrient media, and other tissue culture variables (Huang *et al*., 2002; Chandra *et al*., 2005). Based on Jakhar *et al*. (2013), the addition of 100 mg.L^{-1} ascorbic acid can suppress browning intensity and increase shoot induction in vitro. The addition of ascorbic acid to the culture medium was also used to overcome browning in some in-vitro cultures of banana plants (Ko *et al*., 2009; Munguatosha *et al*., 2014).

Embryogenic Callus Regeneration and Somatic Embryo

Browning in tissue greatly affects the decline in-vitro regeneration of callus and organ cultures, including the process of somatic embryogenesis. The addition of organic acid (ascorbic acid) to the culture media to over-come browning was also given to the embryo regeneration medium to increase the number of somatic embryos produced. In the first observation (3rd weeks), N38 clones followed by C77 clones showed the percentage of embryogenic callus formation (2% and 1.7%), globular embryos (4.67% and 3.08%), and torpedo embryos (4.56% and 3.47%) which were higher than the other clones. In PA150 clones embryo-genic callus still produced a high percentage until the 9th week (Figure 6). TC1 clone showed the lowest embryogenesis response for all observation parameters.

Figure 4. Callus biomass after treatment with C4 medium (Observations using an Olympus microscope SZ61 equipped with a DP25 camera); A. Bar = 3,75 mm, B. Bar = 3 mm



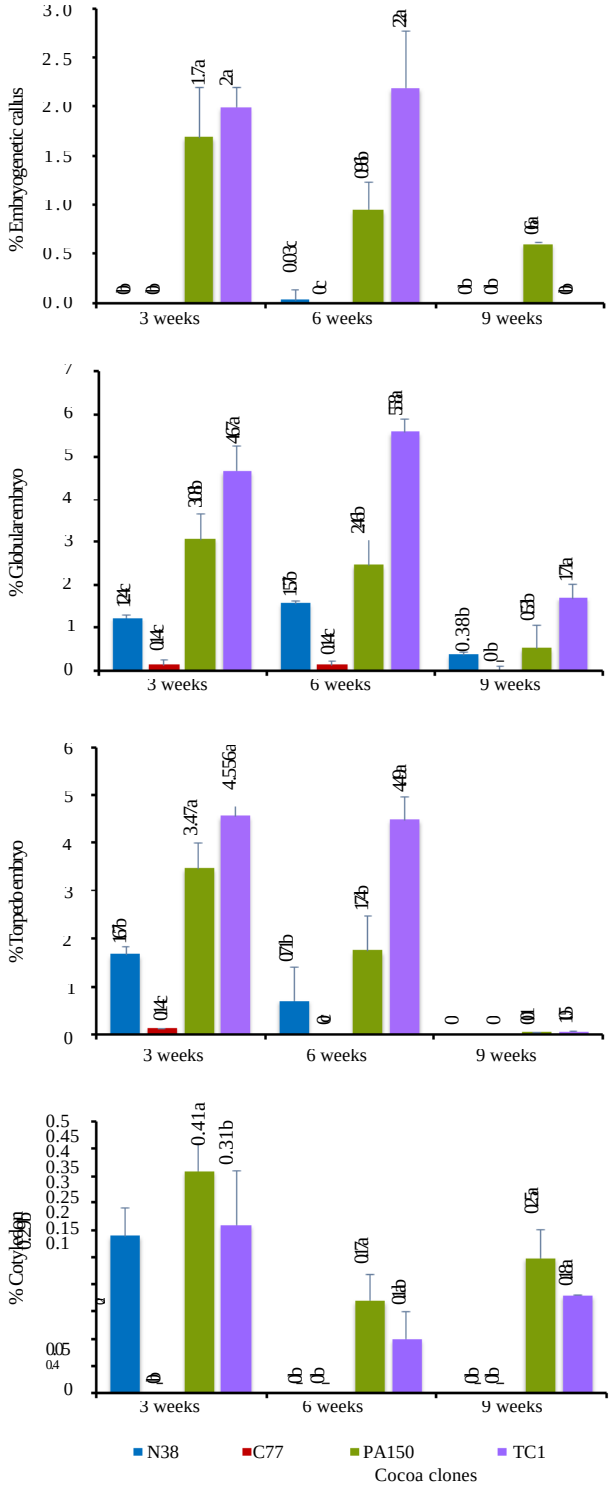


Figure 5. Response of various cocoa clones in terms of embryogenic callus and secondary somatic embryo regeneration

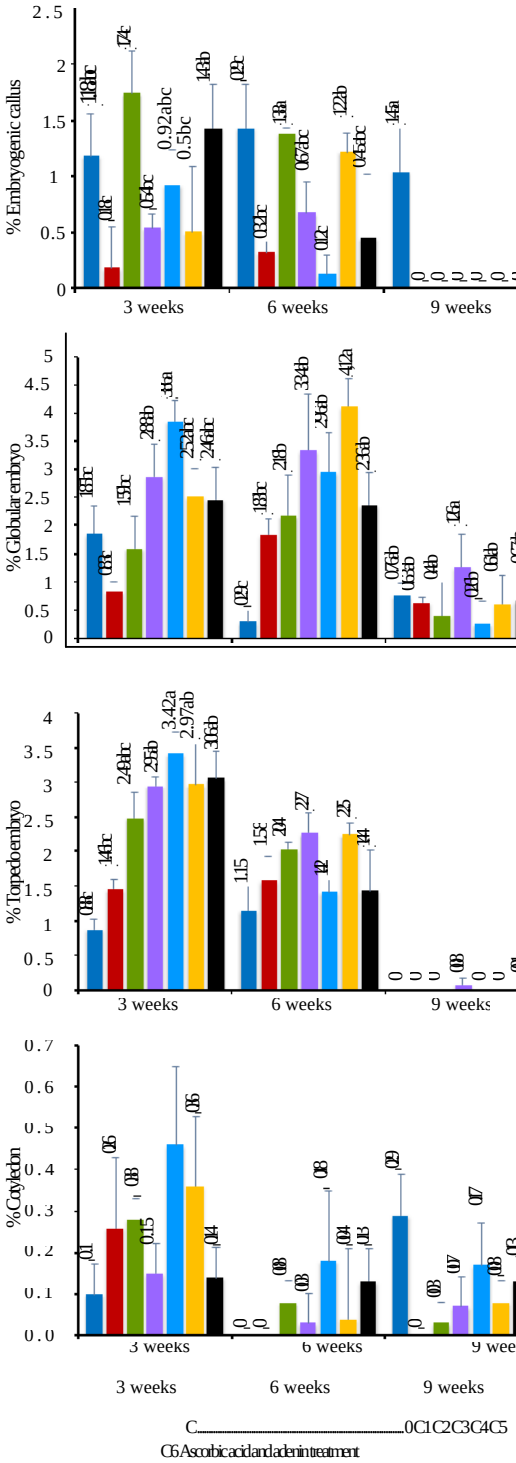


Figure 6. Effect of ascorbic acid and adenine concentration on the regeneration of embryogenic callus and secondary somatic embryos

Notes: Media C0 (Control /expression medium); Media C1 (adenin 0.025 mg.L^{-1} + ascorbic acid 50 mg.L^{-1}); Media C2 (adenin 0.0125 mg.L^{-1} + ascorbic acid 50 mg.L^{-1}); Media C3 (adenin 0.025 mg.L^{-1} + ascorbic acid 100 mg.L^{-1}); Media C4 (adenin 0.0125 mg.L^{-1} + ascorbic acid 100 mg.L^{-1}); Media C5 (adenin 0.025 mg.L^{-1} + ascorbic acid 150 mg.L^{-1}); Media C6 (adenin 0.0125 mg.L^{-1} + ascorbic acid 150 mg.L^{-1}).

The combination of treatments of ascorbic acid and adenine showed a low embryogenic callus responses in TC1 and C77 clones. However, N38 clones also did not show a response to globular, torpedo, and shoot growth phase somatic embryo regeneration. This indicates that different plant cultivars show different responses to the addition of organic acids. The treatment combination of adenine 0.0125 mg.L^{-1} and ascorbic acid 100 mg.L^{-1}) gave the best response to the regeneration of somatic embryos in the globular, torpedo, and shoot growth phases starting 3 weeks after being transferred to regeneration media. Increasing the concentration of organic acids did not affect the increase in the percentage of embryogenic callus.

Dubravina *et al*. (2005) in their research stated that high phenolic compounds in callus can cause explant tissue death. In guava plant micropropagation, browning can inhibit morphogenesis activity (Chandra & Mishra, 2007). The high activity of POD and PPO can suppress or even inhibit the development of somatic embryos in undifferentiated callus (Beruto *et al*., 1996).

The addition of ascorbic acid antioxidants to in vitro culture media can reduce the occurrence of browning. In this case, ascorbic acid does not interact directly with PPO but inhibits browning by reducing substrate oxidation (Arias *et al*., 2007). Oxidation due to the exudation of phenolic compounds can cause browning of the culture media which will inhibit nutrient uptake in explant tissues. In the study of Jakhar *et al*. (2019), ascorbic acid treatment of 150-200 mg.L^{-1} can increase the proliferation of embryogenic callus in *Commiphora wightii* plants.

CONCLUSIONS

Combination of ascorbic acid 100 mg.L^{-1} and 2,4,D 1 mg.L^{-1} had a significant effect on the initiation of secondary somatic embryogenesis of cocoa in both embryogenic callus phase (4.73%), globular embryos (2.53%), torpedo embryos (4.67%) and germination stages (0.47%). Tc3, C77, and Tc1 clones showed a higher percentage of embryogenic callus growth than the other clones. Meanwhile, on N38 clone, all treatments did not affect the regeneration of embryogenic callus. The combination treatment of organic acid and adenine showed a low embryogenic callus response in C77 and N38 clones. However, PA150 clones also did not show a response to globular, torpedo, and germination phase somatic embryo regeneration. This indicates that different plant cultivars show different responses to the addition of organic acids. The treatment combination of adenine 0.0125 mg.L^{-1} and ascorbic acid 100 mg.L^{-1} gave the best response to the regeneration of somatic embryos in the globular, torpedo, and germination stages, started 3 weeks after being transferred to regeneration medium.

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