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Research article

Agronomic Performance and Genetic Fidelity of the Selected Elite Cocoa Clones Derived from Somatic Embryogenesis Culture

Gibson Entuni[1]* Hollena Nori[1], Rebicca Edward[1] and Ahmad Kamil bin Mohammad Jaafar[2]

[1]*Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, Kota Samarahan, Sarawak, Malaysia*
[2]*Malaysian Cocoa Board, Cocoa Research and Development Centre, Biotechnology Park, Kota Samarahan, Sarawak, Malaysia*

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Abstract

Keywords

agronomic performance;
genetic stability;
Theobroma cacao;
SSR primers;
mutation

This study was conducted to compare the agronomic performance of four elite cocoa clones (MCBC1, KKM22, KKM4 and PBC230) regenerated from staminode and immature zygotic embryo culture with conventional grafted cocoa clones. From the results, it was found that the KKM4 clone propagated from immature zygotic embryo culture exhibited variations in the fresh pod weight (339.6 g), fresh individual seed weight (4.13 g) and number of flat beans per pod (4 beans) compared with the rest of the regenerated clones. The genetic stability of the somatic embryogenesis cultured clones and the donor clones was then tested using fragment analysis with five SSR primers, i.e. mTcCIR7, mTcCIR18, mTcCIR22, mTcCIR33 and mTcCIR40. Four of these primers identified variations in the allele size and allele addition in KKM4 clone from immature zygotic embryo. Molecular analysis validated that the difference in agronomic performance of the KKM4 clone from immature zygotic embryo culture was due to genetic mutation created during the immature zygotic embryo culture process.

*Corresponding author: E-mail: gib5181@gmail.com

1. Introduction

Theobroma cacao L., or cocoa, is a tropical crop tree species. It nowadays has become an economic value as a main source of raw materials in the confectionary and cosmetic industries. Cocoa belongs to the Family Sterculiaceae and is one of the major agricultural export products for several countries in Africa, Latin America and Asia including Malaysia, Indonesia and India [1, 2]. According to the World Cocoa Foundation (WCF), 90% of the total cocoa beans in the world is produced by 6 million farmers from these continents. About 50 million people depend on this cocoa bean product for their livelihood [3]. It was estimated that cocoa bean generates the production of ~83 billion dollar per year from the chocolate making industry [4]. Hence, it is crucial to have enough supply of elite cocoa clone planting materials of high bean yield potential and quality to meet the demand.

Conventionally, cocoa is cultivated via seed and vegetative propagations such as rooted cutting and grafting. Propagation by seed is the cheapest and simplest method, but when open pollinated seed is used, cocoa plants in the field offer low agronomic performance [5]. Consequently, farmers turn to grafting and rooted cutting. Since grafting and rooted cutting are asexual propagation type, the regenerated cocoa clones are true to type. However, rooted cutting produces poorly rooted plants whereas grafting produces plants with undesirable bush-like growth pattern. These techniques require extensive labour in the field management such as pruning and harvesting [6]. Thus, somatic embryogenesis culture has been introduced recently as an alternative technique for cocoa propagation. Somatic embryogenesis is a process in which an embryo is induced from a single or group of somatic cells from leaf, stem, flower, petal and root. Under the manipulation of culture conditions and plant growth regulators, the embryo undergoes several stages of maturation before it develops into a whole cocoa plant. This culture technique, which can be used to produce uniform plants in a shortest time and reduced space, has been used to propagate many important crop species worldwide [7].

To date, some researchers such as Ajijah *et al*. [8] Maximova *et al.* [9], Masseret *et al*. [10], Miller [6] and Goenaga *et al*. [11] have successfully introduced somatic embryogenesis cultured cocoa plant into the field. The authors reported that the regenerated cocoa plants exhibited normal phenotype and growth characteristics similar to those propagated by conventional methods. Nevertheless, Lopez *et al.* [12] and Ajijah *et al*. [8] discovered some forms of mutation among cacao of somatic embryogenesis culture through molecular analysis. Additionally, *in vitro* cultured coffee [13] and maize [14] variants with abnormal leaves showed instability in their DNA content after molecular analysis. Regarding these findings, a detailed evaluation of the field performance followed by a validation via molecular analysis is critical before using somatic embryogenesis culture for the commercial use of important cash crops such as cocoa. The aim of this study was to assist plant scientists with the selection of superior clonal material and effective regeneration methods for cocoa plant micropropagation. In this study, the agronomic performance and genetic stability of somatic embryogenesis cultured cocoa clones at two years of transplanting were compared with clones derived from conventional method of grafting.

2. Materials and Methods

2.1 Somatic embryogenesis culture establishment

A randomized complete block design (RCBD) experiment with seven replicates was initiated on four superior clones (MCBC1, KKM22, KKM4 and PBC230) of Trinitario variety derived from staminode and immature zygotic embryo culture. These cocoa clones were collected from the Malaysian Cocoa Board (MCB) Research Station, Kota Samarahan, Sarawak to initiate somatic

embryogenesis culture. Following surface sterilization using 20% Clorox (a commercial bleach containing 5.25% sodium hypochlorite as active ingredient) for 15 min, the explants were then rinsed three times with distilled water and cultured onto DKW medium supplemented with 1.0 mg/l 2,4-Dichlorophenoxyacetic Acid (2,4-D) and 25 µg/l Thidiazuron (TDZ) for the callus induction. All cultures were incubated inside the laboratory at room temperature (25-26˚C) and in dark condition for two weeks. After that, the callus was inoculated onto another medium with 2.3 g/l McCown's salts, 1.0 mg/l B5 vitamins, 2.0 mg/l 2,4-D and 50 µg/l 6-Benzylaminopurine (6-BA) for the embryogenic callus induction. All cultures were maintained at room temperature (25-26˚C) and in light conditions at 1,850 lux (25.9 µE $m^{-2}s^{1}$) provided by cool white-fluorescent lamps. To develop somatic embryo, two-week-old embryogenic callus was transferred onto embryo development DKW medium containing 30 g/l sucrose and 1.0 g/l activated charcoal without plant growth regulator. Only somatic embryos with fully developed cotyledons were transferred onto culture medium with half strength MS macro, DKW micro and vitamins, 0.01 mg/l NAA (1-Naphthaleneacetic acid) and 0.02 mg/l GA3 (Gibberellic acid) for maturation and germination. After six weeks, the fully developed somatic embryos with complete roots and shoots were transferred onto medium with half strength MS medium, DKW micro and vitamins, 10 g/l glucose, 5 g/l sucrose without plant growth regulators for the plantlet regeneration. These plantlets were continuously sub-cultured every four weeks onto the same development medium until their acclimatization. The acclimatization of the plantlets was accomplished in 25-26ºC inside the laboratory with light intensity of approximately 1420 Lux. The plantlets were covered with transparent plastic bags to protect them against mechanical damage and insect and manually misted with 100 ml water twice a day.

2.2. Field performance

Stem height and diameter were collected for a period of 25 months following transplanting. The stem diameter was measured at 10 mm from the soil using a calliper (OEMTOOLS 25363, Malaysia) with an accuracy of 0.1 mm. The stem height was measured using a measuring tape (McKenic measuring tape, Malaysia). The date of first flowering was quantified using a calendar day. The fresh fruit and seed weight were measured using a digital analytical balance (Mettler TOLEDO XS105DU, USA). The number of flat beans per pod was counted manually. Statistical analysis was performed using VassarStats online Statistical Computation [15]. Means for each variable were reported and variation was established by Tukey HSD test at $p<0.05$. Two-Way ANOVA was conducted to test the differences and interaction among cocoa clones and propagation type.

For genetic analysis, one young and healthy leaf from each somatic embryogenesis culture and grafted donor trees were collected from the MCB Research Station for the analysis of somaclonal variation. DNA was extracted from each treatment using a slightly modified CTAB-SDS method from Everaert *et al*. [16] and Johnsiul and Awang [17]. Freshly extracted DNA (5 µl) was then electrophoresed on 1% Agarose gel, stained with ethidium bromide, and visualised under ultraviolet transilluminator for the quality and yield assessments. These DNA concentrations were quantified with a NanoDrop® ND – 1000 UV Vis Spectrophotometer (Thermo Fisher Scientific) at 260 nm. Six internationals standard SSR primers which were commonly used for DNA sequencing of cocoa clones [18] were chosen for this study. These primers were purchased from Integrated DNA Technologies (IDT, Coralville, Iowa). The forward primers were labelled at the five-prime end with the fluorescent CEQ blue and green dyes (D4, Beckman Coulter) to allow the detection of PCR products. From these, mTcCIR7, mTcCIR40 and mTcCIR22 primers were labeled with 6-FAM (blue dye) whereas mTcCIR18 and mTcCIR33 primers were labeled with HEX (green dye) (Table 1). The thermal cycling protocol for the amplification was based on a modified method

described by Everaert *et al*. [16]. All the PCR amplification was carried out in a GeneAmp® PCR System 9700 (Applied Biosystems, Inc.).

For the Fragment Analysis and scoring, 3 µl of each PCR product was mixed with 6.95 µl of deionized formamide (SLS, sample loading solution) and 0.05 µl GeneScan 400HD ROX (Applied Biosystems) for analysis. Each mixture was centrifuged at 13,000 rpm for 5 min, incubated at 95ºC for 5 min and placed on ice for another 5 min. Each mixture was visualised by capillary electrophoresis on ABI PRISM 310 Genetic Analyser (PE Biosystems). After electrophoresis, all fragment sizes were scored to two decimal places with the Local Southern Method option of GeneMapper Software Version 5.0 (Applied Biosystems, Inc.). For DNA samples in which fragment size differences could be attributed to mutations, PCR amplification and Fragment Analysis were repeated thrice for validation. The alleles were mutated when >2 bp mismatches were found in the somatic embryogenesis cultured clone alleles compared to the size of the original alleles from donor clones from grafting.

Table 1. The characteristics of the SSR primers [18] where T = Annealing temperature for Polymerase Chain Reaction (PCR) amplification, F = 5' Forward, and R = 3' Reverse

Locus name	Primers sequences	Allele size range (bp)	T (°C)
mTcCIR7-Y16981	F:ATGCGAATGACAACTGGT R:GCTTTCAGTCCTTTGCTT	150-167	51
mTcCIR18-Y16991	F:GATAGCTAAGGGGATTGAGGA R:GGTAATTCAATCATTTGAGGATA	333-357	51
mTcCIR40-AJ274913	F:AATCCGACAGTCTTTAATC R:CCTAGGCCAGAGAATTGA	262-288	51
mTcCIR22-Y16995	F:ATTCTCGCAAAAACTTAG R: GATGGAAGGAGTGTAAATAG	276-301	46
mTcCIR33-AJ271826	F:TGGGTTGAAGATTTGGT R:CAACAATGAAAATAGGCA	265-348	51

3. Results and Discussion

3.1 Field performance

In this study, stem height and diameter gradually increased from the first to 25 months after transplanting. The growth progression in stem height and diameter are important to show a typical development of a cocoa plant. These traits indicated a transition from juvenile to adult phases of development. During the period, all clones had achieved height above 1 m and their diameter exceeded 50 mm (Table 2). Miller [6] and Goenaga *et al*. [11] also reported comparable results for somatic embryogenesis cultured clones after two years of transplanting. However, both stem height and diameter were found to be different in clones of MCBC1, which had the maximum measurements for the traits of stem height and diameter (stem height = 1.20 mm and stem diameter = 60.1 mm). These findings were congruent with the previous findings [11, 19] where variation among clones was correlated with genotype. Efron *et al*. [19] reported that stem height and diameter were under genetic control in polyclonal varieties such as Trinitario. This variety, which was produced from a natural hybrid of Criollo and Lower Amazon Forastero family [20], inherited the traits of both family groups. Thus, clones from Trinitario variety were heterogeneous and displayed

extreme diversity in the field [21-23], such as MCBC1 clone which developed significantly different measurements of stem height and diameter when compared to other clones.

Table 2. Field performance of all cocoa trees following 25 months of field planting

Cocoa clones	Propagation type	Field Performance					
		Ph (mm)	Sd (mm)	Fpw (g)	Nfb	Fsw (g)	Tff (day)
MCBC1	ST	1.22	61.4	399.4	2	4.30	527
	IZ	1.20	62.2	403.5	2	4.33	531
	G	1.17	56.8	408.0	2	4.26	555
	Mean	1.20	60.1	403.6	2	4.30	538
PBC230	ST	1.14	57.2	408.9	2	4.28	543
	IZ	1.09	58.1	406.0	2	4.30	536
	G	1.08	55.4	405.0	2	4.26	545
	Mean	1.10	56.9	406.7	2	4.28	541
KKM22	ST	1.14	56.2	396.6	2	4.33	588
	IZ	1.10	58.9	393.1	2	4.34	592
	G	1.10	57.7	380.8	2	4.31	595
	Mean	1.11	57.6	390.2	2	4.32	592
KKM4	ST	1.11	57.8	404.0	2	4.32	560
	IZ	1.15	58.4	339.6	4	4.13	610
	G	1.08	58.8	403.0	2	4.31	554
	Mean	1.11	58.3	382.2	3	4.26	575
	P	0.222	0.064	0.720	<0.001	0.473	0.992
	T. HSD (5%)	0.07	2.24	20.57	0.31	0.06	94.82
	C	<0.05	<0.05	0.053	<0.001	0.139	0.556
	T. HSD (5%)	0.09	2.85	26.12	0.40	0.08	21.01
	P*C	0.952	0.193	<0.05	<0.05	<0.05	0.993
	T. HSD (5%)	0.20	6.40	58.18	0.88	0.17	23.86

ST = staminode culture trees; IZ = Immature zygotic embryo culture trees; G = Grafted trees; T. HSD = Tukey Honestly Significance Difference; P = Propagation type; C = Cocoa clones Ph = Plant height; Sd = Stem diameter; Fpw = Fresh pod weight; Fsw = Fresh individual seed weight; Nfb = Number of flat beans per pod; Fsw = Individual fresh seed weight; Tff = Time to first flowering

For the fruit and seed traits, an interaction between propagation type and cocoa clones was significant. Only KKM4 clone from immature zygotic embryo culture had the significantly highest number of flat beans per fruit (4 beans). For the lowest fresh fruit weight measured in KKM4 clone of immature zygotic embryo culture (339.6 g) compared with other treatment, the highest number of flat beans per fruit (4 beans) and reduce fresh seed weight (4.13 g) are the main factors. On the industrial scale, an increased number of flat beans per fruit will have a significant effect on cocoa plant yield. Thus, it is suggested to conduct a long-term study on the impact of elevated number of flat beans per fruit on the yield of the regenerated cocoa clones. The variations in fruit and seed traits of somatic embryogenesis cultured coffee [13] and oil palm [24, 25] have been reported which are similar with this study. The evaluation of both fruit and seed traits could facilitate identification of cocoa clones which become unstable following somatic embryogenesis. Traore and Guiltinan [26] reported that cocoa clones from various genotypes responded differently to tissue culture conditions. It is recommended to identify cocoa clones which are prone to variation. After such

identification, the propagation type protocols for the clones could be modified. Another developmental process evaluated in this study is the time of first flowering. Our results are comparable with those found by Maximova *et al*. [9]. The period to first flowering averaged 561 days (within two years) for all cocoa trees. In contrast, an early time of flowering was reported for somatic embryogenesis culture derived mangosteen [27], guava [28] and rice [29]. Genotype effect, agronomic practices during field managements and environmental conditions including soil nutrient and status could be possible attributes that account for the differences in first time of flower setting among plants [13]. The findings in this study suggest that the optimization of culture protocol is necessary to reduce the possibility of variation, especially for the clones which are susceptible to such variation.

3.2 Molecular evaluation

Five SSR primers (mTcCIR7, mTcCIR40, mTcCIR22, mTcCIR18 and mTcCIR33) were used to detect polymorphism between the somatic embryogenesis cultured clones and grafted mother clones. The effectiveness of SSR primers compared with the rest of DNA markers, i.e., RAPD and RLFP to assess genetic fidelity from different cocoa genotype has been confirmed previously [8, 12, 30]. SSR markers have also been used to detect the genetic fidelity of numerous true to type [31-33] and off type regenerated tissue cultured plants including coconut [34], sugarcane [34] and banana [35]. SSR primers were used for genetic stability studies due to their characteristics of high genomic abundance throughout genome, specific locus, high level of polymorphism and strong discriminatory power [36]. In our study, no variation was detected in the allelic profiles for all clones except for KKM4 clone. Notably, only the KKM4 clone from the immature zygotic embryo culture was observed to have allele mutations such as allele size variation and addition. For instance, one addition of a mutant allele was found in both mTcCIR40 (size = 272.14 bp) and mTcCIR22 (size = 289.83 bp) primers (Figure 1). In contrast, three cases of allele size variations were observed in mTcCIR7 (from 157.34/159.40 bp to 155.02/157.03 bp), mTcCIR18 (from 333.95/345.57 bp to 331.46/347.22 bp) and mTcCIR33 (from 290/346.65 to 274.22/309.55).

The incidence of slippage mutation such as allele size change and addition in this study was similar to those found by Lopez *et al*. [12] and Ajijah *et al*. [8]. The researchers reported that allele addition originated from the extra repeat units at the SSR locus produced from abnormal mitotic recombination of the interchromatid unequal crossing over and intrachromatid exchange during a long callus proliferation period of indirect somatic embryogenesis culture [37, 38]. On the other hand, allele size variation was produced from sample contamination and locus duplication. This allele size change, which was also observed in tissue cultured cork oak [37], coffee [13], maize [14] and pineapple [39], was reported to be due to cell lineage mechanism which reduced cellular competence for proofreading and maintenance of DNA sequence integrity [40]. These then led to the allele size change in some tissue cultured plants [14]. A similar process was also encountered by the KKM4 clone during the immature zygotic embryo culture process.

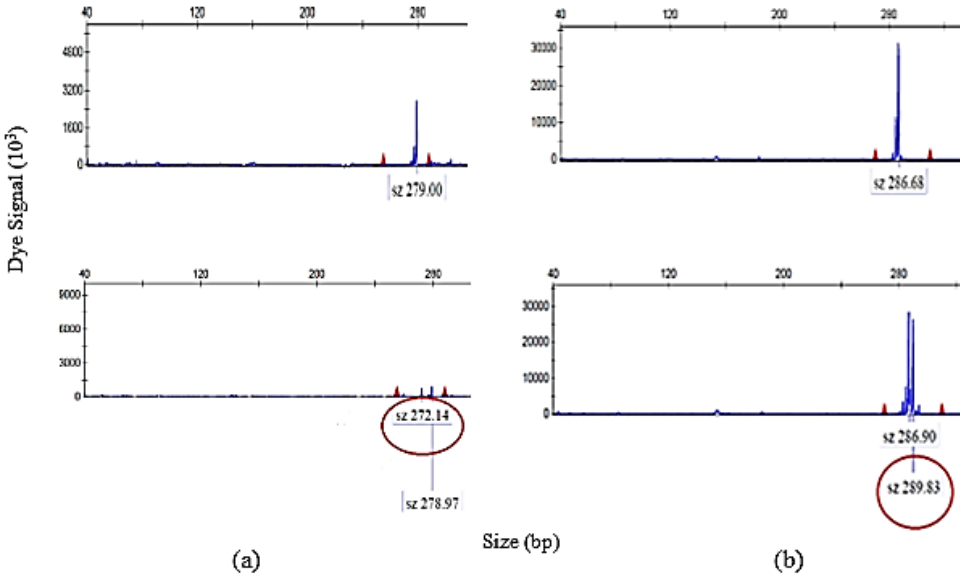

Figure 1. Amplification profiles represented one allele addition for (a) MTcCIR40-AJ274913 primer where the upper electropherogram corresponded to a homozygous individual of KKM4 clone from grafting with one allele of 279.0 bp. The lower electropherogram corresponded to a heterozygous individual of KKM4 clone from immature zygotic embryo culture with one normal allele of 278.97 bp and one mutant allele of 272.14 bp and (b) mTcCIR22 primer in which the upper electropherogram corresponded to a homozygous individual of KKM4 clone from grafting with one allele of 286.68 bp. The lower electropherogram corresponded to a heterozygous individual of KKM4 clone from immature zygotic embryo culture with one normal allele of 286.90 bp and one mutant allele of 289.83 bp. Top scale indicates fragment size in nucleotide. The left scale indicates fluorescence intensity measured in relative fluorescence units. Red circle = mutant allele

Interaction between propagation type and clones was a factor that create variation in the KKM4 clone from immature zygotic embryo culture. Fragment analysis that was congruent with field performance validated that some clones, i.e., KKM22, PBC230 and MCBC1 remained invariant whereas the KKM4 clone exhibited variation after somatic embryogenesis culture. This finding was also supported by Ajijah *et al*. [8] and Lopez *et al*. [30], whose work clarified the important effects of the initial genetic constituents of donor plants for somatic embryogenesis culture. This is because the difference in genetic constituency means the differences in stability of genetic make-up whereby some plants with unstable genomes become mutated during the stress conditions induced by somatic embryogenesis culture [41]. The limitations of using immature zygotic embryo explant for cocoa propagation have been reported in several studies [4, 42]. According to those authors, immature zygotic embryo explant carried additional DNA methylation genes compared to explants from the leaf, root and flower. Maximova *et al*. [42] reported that the higher expression of ethylene and flavonoid related genes during immature zygotic embryo culture was due to the embryogenic tissues experiencing high levels of stress. These tissues then initiated an alternative regulatory mechanism such as reprogramming of gene expression to develop into functional somatic embryos [43]. The result from this study suggested that for the KKM4 clone which had unstable genetic constituency, the use of immature zygotic embryo explants increased the chances of variation.

4. Conclusions

Somatic embryogenesis could be a promising culture technique to mass propagate elite cocoa clones such as PBC230, KKM22 and MCBC1 for commercial use. Nevertheless, there was considerable variation found in both agronomic performance and genetic fidelity using four SSR primers (mTcCIR7, mTcCIR18, mTcCIR33 and mTcCIR40) for the KKM4 clone that had been regenerated from immature zygotic embryo culture. Such variation, however, could possibly be reduce through the optimization of the culture protocols including the culture conditions and plant growth regulators.

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