OBSERVATION ON THE AGRO-MORPHOLOGICAL, FIELD PERFORMANCE AND ASSESSMENT ON CLONAL FIDELITY OF STAMINODE-GENERATED COCOA CLONE IN COMPARISON WITH THEIR CONVENTIONALLY PROPAGATED COCOA CLONES

Lea Johnsiul
Centre for Cocoa Biotechnology Research, Malaysian Cocoa Board,
Kota Kinabalu Industrial Park (KKIP), 88460 Kota Kinabalu, Sabah.
e-mail: lea@koko.gov.my

Norasekin Tamchek
Centre for Cocoa Biotechnology Research, Malaysian Cocoa Board,
Kota Kinabalu Industrial Park (KKIP), 88460 Kota Kinabalu, Sabah.

ABSTRACT

Comparative observations focused on the phenotypic traits and field performance of mature cocoa trees regenerated through tissue culture, was carried out to evaluate the differences between a tissue culture generated cocoa (specifically via staminode-derived clones) and cocoa clone generated through conventional propagation method. These traits are compared against cocoa clones produced through traditional grafting methods. The main objectives of the research were to observe the agro-morphological, field performance and assess whether the tissue-cultured cocoa tree generated maintain clonal fidelity comparable to their conventionally propagated. Observation results on the agro-morphology of tissue culture generated cocoa compared to the cocoa clone generated conventionally through grafting shown some significant differences in the phenotypic and field performance. Tissue culture generated cocoa has jorquette branches, the usual characteristic of hybrid cocoa trees propagated from seeds, which make the tree taller than cocoa clone generated through conventional method. Cocoa trees generated from tissue culture technique also observed to have smaller girth circumference measurement compared to the conventionally propagated cocoa tree. However, early flowering was observed with tissue culture cocoa trees at approximately 12 months after planting compared to the conventionally propagated cocoa tree which is about 18 - 36 months. Assessment on the clonal fidelity of both set of clones by genotyping using the MCB cocoa SNP panel. Results from the DNA fingerprinting confirm that the tissue culture-derived cocoa trees are true-to-type clones, exhibiting identical genetic profiles to the plants generated through conventional grafting methods.

Keywords: staminode-generated cocoa, Theobroma cacao, agro-morphological traits, clone verification; SNP genotyping.

Introduction

Cocoa (*Theobroma cacao* L.) is one of the main commodities and the third most important crop grown in Malaysia, behind oil palm and rubber (Azhar and Lee, 2004). With an annual production close to 250,000 metric tonnes, Malaysia was one of the main cocoa beans producers in Southeast Asia the third largest producer in 1990s. However, by 2003, the total production had dropped to less than 50,000 metric tonnes (Azhar, 2007). The sharp increase in demand had resulted to farmers planting any cocoa planting materials available at the lowest cost of production they could get regardless of the poor-quality planting materials, disorganised pest and diseases management and low knowledge on technology usage. These problems had eventually set a decrease in planting cocoa and caused the decline in cocoa production.

In 2021, the total planting area decreases continuously, and Malaysia is left with total cocoa planting acreage of 6,000 hectares with annual production of 0.54 metric tonnes but with increased total export amount of RM 6.872 billion (Malaysian Cocoa Board, 2022) and the third largest producer in the Southeast Asia region.

Traditionally, cocoa is vegetatively propagated through grafting and rooted cuttings. However, due to the high demand for cocoa beans and the limited availability of cocoa beans for seedling production, alternative techniques of vegetative multiplication of cocoa that do not rely on the cocoa beans for seedling production are continually being researched. The objective of vegetative propagation is to create offspring plants with genotypes that are identical to those of a single donor plant. The biological procedure is called ‘cloning’, and the population of plants that results is referred to as a "clone." Clones are extremely important in cocoa propagation and other elements of agriculture. This is due to both the benefits and the issues that must be resolved for the process to be successful. An alternate way of plant vegetative propagation is by the use of tissue culture (TC) techniques. One significant benefit of clonal cultivars in commercial production is the ability to establish homogenous individual plants within a clone population by clonal multiplication through tissue culture. However, it is also well known now that genetic variations occur in undifferentiated cells, isolated protoplasts, calli, tissues and morphological traits of the regenerated plants (Alizadeh *et al.*, 2015, Entuni *et al.*, 2022, Ulrika, 2019, Uma *et al*., 2021, Tongtape *et al*., 2023, Adero *et al*., 2023).

In the past three to four decades, using large-scale propagation tools has evolved into a serious strategy for many economically significant plant species, such as cocoa, in an effort to produce enough plant material for planting. Because of this, somatic embryogenesis-based in vitro micropropagation techniques have demonstrated several possible benefits over traditional propagation techniques. (Nandhakumar *et al*., 2017, Ulrika, 2019, Entuni *et al.*, 2021, Entuni *et al.*, 2022, Uma *et al*., 2021, Tongtape *et al*., 2023, Adero *et al*., 2023). However, there is always a chance of producing so many mutated regenerants that the method's viability from an economic standpoint is compromised (Etienne and Bertrand, 2016). Keeping the true-to-type

characteristics of *in vitro* propagated plants during commercial and marketing processes is essential for maintaining specific agronomic and horticultural features especially when using planting materials with desirable traits. (Alizadeh *et al*., 2015, Ulrika, 2019, Tongtape *et al*., 2023).

The Malaysian Cocoa Board (MCB) has successfully planted several cocoa plants regenerated through tissue culture techniques (Nik Aziz *et al.*, 2016; Norasekin *et al.*, 2022) and many of the trees were planted and growing for more than 3 years. To date, the TC regenerated cocoa plants have been observed on their agro-morphological characteristics but not evaluated on their clonal fidelity. The main purpose of this study was to observe the agro-morphological, field performance and determine the clonal fidelity of TC regenerated cocoa plant using the cocoa core SNP panel. Many techniques (such as morphological and physiological assessment, biochemical analysis, and karyotyping) are available for the detection of somaclonal variation, although nearly all of them have their limitations. In cocoa, several researchers have reported that TC regenerated plants showing normal phenotype and growth in the field similar to cocoa plants propagated through traditional methods (Maximova *et al*., 2009, Goenaga *et al*., 2015 and Masseret *et al*., 2009). Somatic embryogenesis often needs a very long rotation time and hormones treatment that can likely cause somaclonal variation. The production of true-to-type plants is the fundamental requirement of tissue culture plant propagation; thus, clonal fidelity verification is essential for long-term economic importance (Chittora *et al*., 2015). Uma *et al*., (2021) reported that based on field observation of their banana in vitro plants, it showed that there were no negative effects on the vegetative and yield parameters. However, Ulrika (2019) stated that there were phenotypic variations such as dwarfism, fasciation, variegated patterns, height alterations, modified branch angle and bushy shape, detected in several agricultural crops, although the frequency is low.

Several reports on the somatic embryogenesis (SE) of cocoa shown some somatic variation based on molecular markers (Entuni *et al.*, 2022, Entuni *et al.*, 2021, Ajijah *et al.*, 2016, Lopez *et al*., 2009). Most of the TC regenerated cocoa plants were molecularly verified using RAPD (random amplified polymorphic DNA) and microsatellite markers (Entuni *et al.*, 2022, Entuni *et al*., 2022, Henao-Ramírez *et al*., 2021, Maximova *et al*., 2008, Goenaga *et al*., 2015 and Masseret *et al*., 2009, Ajijah *et al*., 2016). Tongtape *et al.* (2023) assessed the genetic stability of their rubber somatic embryo generated *in vitro* plantlets by RAPD and SSR markers which resulted with no variation between mother plant and TC generated plantlets. Uma *et al.* (2021) postulated large-scale in vitro propagation of their banana in vitro plants would affect the genetic fidelity and used Inter Simple Sequence Repeats (ISSR) markers technique to assess their banana in vitro plants. Their results indicated that there were overall variations between the bananas in vitro plant and conventional propagated plant (Uma *et al*., 2021). Recently, the Malaysian Cocoa Board (MCB) has developed a cocoa core SNP (Single Nucleotide Polymorphism) panel which was developed specifically for verification and identification for verification of the MCB cocoa commercial clones (Johnsiul, Tamchek and Mat Yazik, 2022). This MCB cocoa SNP panel have been proven to be useful in identifying and verifying successfully the Malaysian cocoa commercial clones (Johnsiul *et al*., 2022 and Johnsiul *et al*., 2021). As the MCB tissue culture plants were generated from the Malaysian cocoa commercial clones, assessment of clonal fidelity was accomplished using the MCB cocoa core SNP panel specifically developed for the Malaysian cocoa commercial clones.

MATERIALS AND METHODS

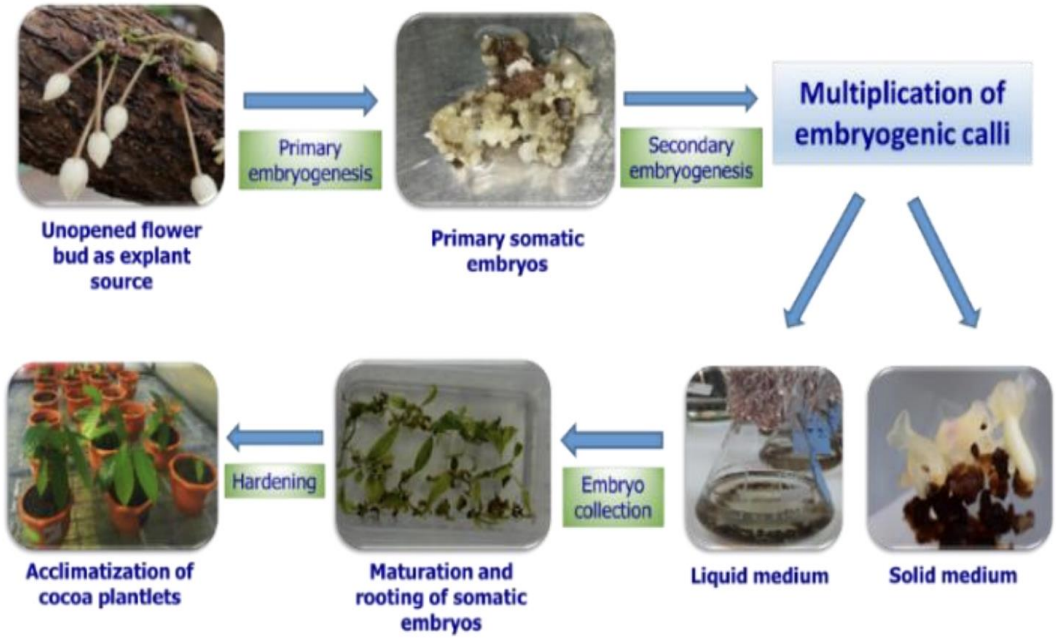
CONVENTIONAL COCOA PLANT PROPAGATION BY GRAFTING IN THE FIELD

Cocoa trees selected were from the Cocoa Biotechnology Research Centre' cocoa field for both the floral materials for SE and the cocoa clonal genotype of KKM1 from the 10-year-old clonal collection (previously verified using microsatellite markers). Each plant from the clonal garden was identified and labelled with tree marking label (individual number and verified genotype). Matured leaves samples were collected and kept in envelopes filled with silica gel beads to keep dry and prevent decomposition to ensure high quality DNA can be extracted from the leaves.

PLANT MATERIAL PROPAGATED BY SOMATIC EMBRYOGENESIS

Between 2014 and 2016, Cocoa clone KKM1 was propagated *in vitro* through somatic embryogenesis. Floral buds were harvested from the cocoa field at the Cocoa Biotechnology Research Centre and transported to the Tissue Culture Laboratory in sterile water. The sterilisation procedure used was that described by Nik Aziz et al in 2016. Staminodes and petals were extracted from the basal portion of the flower bud, and different phases of SE (induction, expression, maturation, conversion) were induced according to Norasekin, Ahmad Kamil and Rosmin (2021) protocol (Figure 1).

Figure 1: Steps of somatic embryogenesis and cocoa plant regeneration, from primary embryogenesis through secondary embryogenesis, embryo collection and finally hardening. (Norasekin, Ahmad Kamil and Rosmin, 2021).



The SE regenerated cocoa plant studied was planted in August 2016 without shade tree but located near building which provide some shades to the SE regenerated cocoa tree. The study employed a longitudinal data collection approach, spanning the first three years following planting, to evaluate the plant's growth and developmental milestones.

Stem Diameter
One of the key parameters monitored was the stem diameter, specifically measured at a height of 30 cm above the soil level. Data were collected on a six-monthly basis throughout the first three years.

Main Stem Height
The height of the main stem was measured from the soil surface up to the point where the first jorquette branch appeared. Similar to stem diameter, these measurements were taken every six months during the first three years of growth.

First Flowering and Fruiting.
The occurrence of the first flowering and fruiting event was observed and documented. The date was recorded, and the flowers' characteristics were examined for features like petal colour and size to confirm typical cocoa flower characteristics.

SAMPLE COLLECTION AND SNP GENOTYPING

Samples of mature leaves were taken from the conventionally produced cocoa tree (a grafted cocoa tree with the same clone as the SE material source) as well as the SE regenerated cocoa plant. The LGC DNA extraction service (https://www.biosearchtech.com) was used to extract DNA, and the KASP™ assays from LGC Genomics (http://www.lgcgroup.com/kasp) were used to genotype SNPs. Single nucleotide polymorphisms (SNPs) and insertions and deletions (Indels) at certain loci can be bi-allelic scored using KASP™ genotyping assays, which are based on competitive allele-specific PCR. The raw data were examined with LGC's in-house Kraken™ software, and each DNA sample's genotype was given a score on a Cartesian plot (also called a cluster plot) using the SNPViewer programme.

SNP GENOTYPING DATA ANALYSIS

Raw data was imported and organized in Microsoft Excel for each of the SNP locus and sample call. The approach used to identify mislabelling (off-types) in the collection was to directly compare the samples fingerprint profiles. Samples with non-matching SNP patterns were considered off-types.

RESULTS AND DISCUSSION

The cocoa tree propagated by somatic embryogenesis was transplanted to the field after four months of acclimatization in the nursery. The performance of the tree was monitored monthly. There were no significant growth differences observed on the somatic embryo (SE) plantlet compared to the grafted cocoa tree. No substantial growth differences were observed between the SE-derived plant and the grafted cocoa tree, which substantiates the effectiveness of the tissue culture method in replicating conventional growth patterns. Importantly, the SE plant exhibited morphology identical to that of seed-propagated trees, notably the presence of a jorquette and orthotropic growth patterns (Figure 2).

Young cacao trees' height and timing can serve as indicators for the normal growth of a cocoa tree and the appearance of a jorquette is important as it marks the cocoa tree's transition from the juvenile to adult stage of development (Maximova et al., 2008; Masseret et al., 2009). Hence, measurements were conducted at bi-annual intervals focusing on the main stem's height from the soil to the first jorquette once the tree had created a jorquette. The trend of stem height data from six to 36 months is shown in Figure 3. The stem's height of the cocoa tree generated by somatic embryogenesis increased steadily from 80.1 cm at six months after transplantation to 135.8 cm at 36 months. Compared to the grafted tree, the stem height from the soil to the first jorquette was much shorter. However, no significant differences were recorded in both methods at the data collection of six to 36 months, indicating that the cocoa tree generated by tissue culture demonstrated the typical growth of the cocoa tree and the change from the juvenile to adult stages of development.

Figure 2: Architecture of the (A) SE cocoa tree similar to a seed propagated tree with five branches jorquette and (B) Cocoa tree with double side grafted.

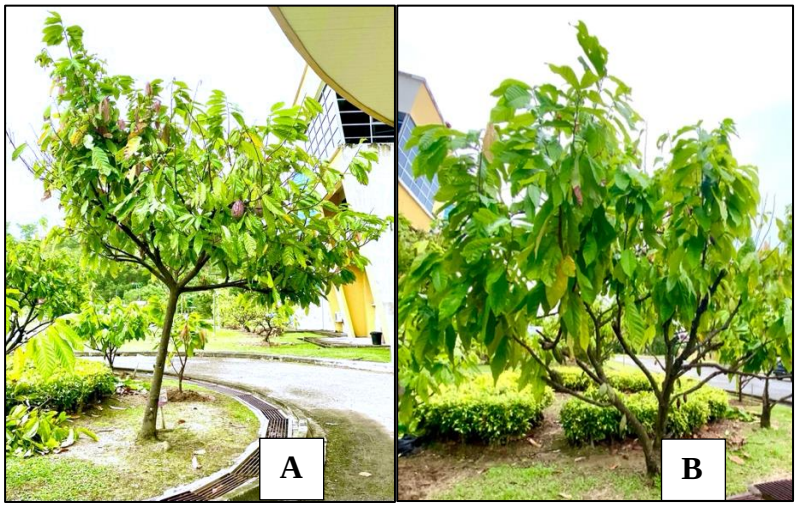
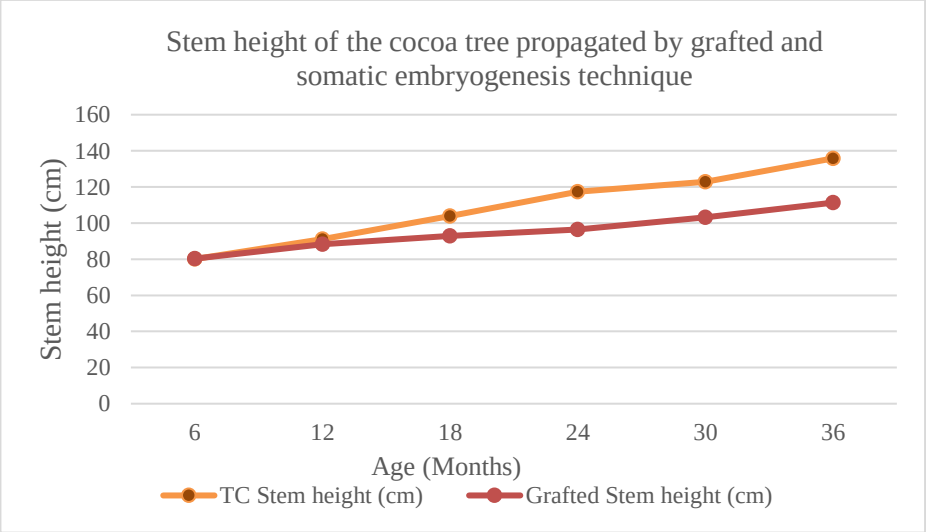
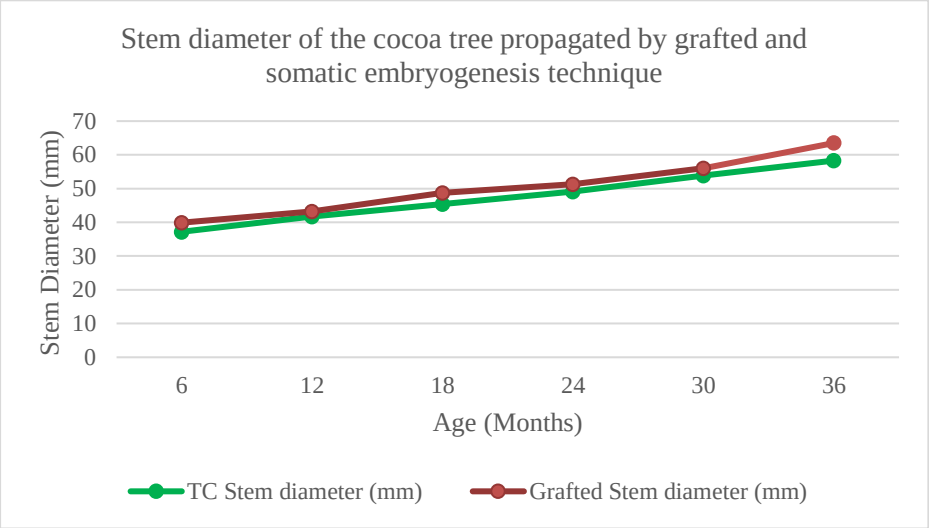


Figure 3: Stem height from the soil to the first jorquette of cocoa plant generated by grafted and somatic embryogenesis technique.



The diameter of the stems also steadily expanded almost at a linear rate, going from 37.2 mm at six months after planting to 58.3 mm at 36 months for cocoa trees propagated by somatic embryogenesis (Figure 4). However, the stem diameters of the grafted tree slightly higher grew at 39.9 mm to 63.5 mm from six to 36 months almost similar with the measurement of non-SE cocoa tree during the collecting data period. As mentioned in other studies on the field performance of cocoa tissue culture trees, the stem diameter in this study seems comparable and in line with the finding by Maximova *et al*., (2008) and Entuni *et al*., (2018).

Figure 4: Stem diameter of cocoa plant generated by grafted and somatic embryogenesis technique.



A cocoa tree's overall physiological and developmental stage is usually determined by the time of first fruit was produced (Sondahl *et al*., 1993). Thus, another developmental process evaluated in this study was the onset of first flowering that occurred in SE cocoa tree. In comparison to the other non-SE propagated cocoa trees, the SE cocoa tree began flowering and bearing fruit comparatively earlier. The SE cocoa tree started producing flowers at twelve months after transplanting, earlier than traditionally propagated cocoa trees (grafted or seed propagated cocoa trees) which is usually around 18 to 26 months (Wood and Lass, 1985). Even though younger cocoa trees often do not support fruit development due to cherelle wilts (Maximova *et al*., 2008), a total of 13 matured cocoa pods were harvested, after five months of the first fruiting of the SE cocoa tree.

A variety of morphological assessment have been used to differentiate the variation of SE and traditionally propagated cocoa trees, nevertheless these methods can be affected by the environment, agriculture inputs and practices. Thus, suggesting a visual morphological evaluation may not be sufficient to verify genetic fidelity and true-to-type SE-regenerated plants with the source of explant. Molecular tools are more reliable than phenotypic observations for evaluating variations as they are not affected by external influences (Chandra and **Thoyajaksha, 2018). SNP genotyping was used in this study as the molecular markers are readily available in the laboratory.** Both the grafted cocoa tree and SE cocoa tree were subjected to SNP genotyping using the proprietary cocoa core SNP panel. The raw data obtained through SNP genotyping was organized in Microsoft Excel and the samples fingerprint profiles were compared and matched. Based on the detected loci profile, no differences were observed between the grafted cocoa tree and the SE cocoa tree, indicating clonal fidelity between the individuals (Table 1).

Table 1: DNA fingerprints based on multilocus matching of SNPs between the grafted cocoa tree and SE cocoa tree.

Genotype	Cocoa SNP 1		Cocoa SNP 2		Cocoa SNP 3		Cocoa SNP 4		Cocoa SNP 5		Cocoa SNP 6		Cocoa SNP 7		Cocoa SNP 8		Cocoa SNP 9		Cocoa SNP 10	
Grafted cocoa tree	G	A	G	T	G	G	G	G	C	T	G	A	G	T	T	T	C	C	T	T
SE cocoa tree	G	A	G	T	G	G	G	G	C	T	G	A	G	T	T	T	C	C	T	T

CONCLUSION

This study has yielded important data regarding the morphological and genetic traits of cocoa trees propagated via somatic embryogenesis (SE), with specific findings that have are important for cocoa commercial propagation. Morphologically, the SE-derived cocoa trees in this study were comparable to trees propagated through conventional propagation methods, thereby validating the efficacy of the SE propagation protocol in maintaining essential morphological characteristics crucial for large-scale planting materials production.

Among the noteworthy outcomes was the observation that SE cocoa trees displayed enhanced vigour and reduced time to flowering and fruiting. These attributes may contribute to increased yields and more efficient production cycles, offering potential economic advantages for cocoa producers.

Genotyping through a cocoa core single nucleotide polymorphism (SNP) panel verified the genetic consistency of the SE-derived trees with their respective source clones. However, it is important to note that this does not exclude the possibility of nuclear genome modifications not captured by the SNP panel. These changes could potentially account for the observed phenotypic variations, such as increased vigour, and deserve further investigation.

These results not only confirm the genetic stability of SE-derived cocoa trees but also suggest that tissue culture techniques could be a viable alternative to traditional propagation methods. Consequently, this study paves the way for future research into the benefits and limitations of SE as a cocoa propagation method, thereby enriching our collective understanding and potentially advancing cultivation practices to the benefit of both producers and consumers.

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