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# MASS PROPAGATION OF HIGH-YIELDING COCOA CLONES: A REVIEW

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**ABSTRACT -** Theobroma cacao *L. is the most important commodity, ranking in third after oil palm and rubber in Malaysia. In addition to providing the raw material for the chocolate industry and source of income for cocoa farmers, this commodity also has a number of non-food uses in the pharmaceutical and cosmetic industries. Plant regeneration via somatic embryogenesis as an alternative for mass propagation of elite cocoa clones has been well studied for decades. However, results showed that it is genotype-specific as there is a significant variation of efficiency when applied to a wide variety of cocoa genotypes. In Cocoa Biotechnology Research Centre, MCB, the research aims to develop protocols for mass propagation and plant regeneration of cocoa superior clones to improve planting materials through investigation of various combinations and formulation of cultured media. To date, 28 of MCB's recommended clones responded well to four different induction media. Thus, an overview of the mass propagation of high-yielding cocoa clones in Malaysia using tissue culture techniques is presented in this article.*

***Keywords:*** *Theobroma cacao*, somatic embryogenesis, tissue culture, genotype-specific.

## INTRODUCTION

*Theobroma cacao* L., also known as the cocoa tree, is an economically valuable agricultural commodity for millions of people worldwide. In Malaysia, cocoa is the third-ranking of the commodity after oil palm and rubber. Cocoa trees mainly reproduced via vegetative propagation methods such as by seeds, grafting or rooted cuttings. While seed planting is easy, the plant's agronomic performance profoundly varies due to its heterozygosity and complex self-incompatibility system (Maximova and Guiltinan, 2013).

The last few years, plant regeneration via somatic embryogenesis has become an alternative method for mass propagation of elite cacao clones for research and production in most of the cocoa production country. According to Maximova *et al.*, (2002), the main advantages of this method include the possibility of the rapidly generating asexually propagated uniform plants of high genetic value and the clonal production of orthotropic plants with normal dimorphic architecture and taproot formation. Other than that, the resultants clones also are true-to-type or genetically identical to their parental donor cells.

However, due to the high heterozygosity nature of cocoa clones, most of the published methods did not apply to all cocoa clones recommended by Malaysian Cocoa Board and the degree of response to culture media treatments also varied. For a successful culture, the plant tissue needs to have an aseptic environment, proper sterilization prior to culture, appropriate and suitable nutrition in culture media while taking necessary steps in the browning and somaclonal variation. Regardless of medium types or plant species, the multiplication of embryogenic cells is a pivotal step to evolve from a small research scale (petri dishes) to large volume, therefore increasing the number of plantlets. Hardening in the greenhouse and acclimatization to the field condition is the final problem and bottleneck to grow *in vitro* plants.

This study involved 52 Malaysian cocoa clones recommended by MCB from Class I, II, III and IV. These clones were previously classified based on their adaptability to a wide range of Malaysian agro-climatic conditions, yield

potential, pod and bean characteristics, tolerant level to major pest and disease and possession of high butter content and good flavour (MCB, 2012).

The research aimed to develop protocols for mass propagation and plant regeneration of cocoa superior clones to improve planting materials through investigation of various combinations and formulation of culture media. This paper will review the technique, process and the protocols used.

## MATERIALS AND METHODS

Figure 1 provides an overview of the cocoa mass propagation process performed in Malaysian Cocoa Board.

***Preparation of culture media***
A semi-solid culture media consisted of macro elements, micro elements, amino acids, vitamins, carbon source, iron source, plant growth regulators (PGRs) and gelling agent in double distilled water. There are several different types of media used for induction, expression and germination.

***Establishment of aseptic culture***
The starting material for the process is cacao staminodes and petals from 52 commercial and recommended clones by MCB. Samples were obtained from the healthy mother plants of bud grafted, field and glasshouse-grown clonal plants at Centre for Cocoa Biotechnology Research, Kota Kinabalu Industrial Park, Sabah, Malaysia.

Unopened flower buds were washed with tap water and surface-sterilized as described in Siti Norhana *et al.,* (2016). Following dissection, staminodes and petals were extracted and cultured on to the callus induction media containing PGRs.

***Induction of embryogenic calli and somatic embryos (SE)***
Explants are cultured onto the sterilized induction media (Nestle, Nestle modified, PennState, PennState modified, MCB, MCBa, MCBb and MCBc) containing various combinations of PGR. Petri dishes were incubated for 2-3 weeks (depending on the media used) in the dark at 25 ± 2 ºC before transferred onto a secondary calli induction media for another two weeks. Calli were then subcultured onto the differentiation media with periodic subculture at an interval of 3-4 weeks until primary SEs were observed or before suspension culture was established.

***Somatic embryogenesis from cell suspension culture***
Two formulations of solid media (Nestle and PennState) and suspension culture (NIM3liq and CC21liq) were used in this study. The method for suspension culture was explained in Norasekin *et al.,* (2017). Cultures with normal and good proliferation of SEs were agitated continuously until adequate culture establishment was reached before SEs undergo maturation, germination and conversion to plantlets.

***Maturation, germination and conversion into plantlets***
White-opaque cotyledonary SEs, 5-10 mm in length, were isolated and cultured on maturation media supplemented with different concentration and combination of PGR. The dishes were placed in the dark at a constant temperature of 26 ± 2 ºC. After approximately 4 weeks, mature SEs were transferred onto a jar containing germination media (CMC6, CMC7, NG8, PEC, RD, NAEM, and NAGM). The embryos were kept under light (16/8h photoperiod), with a constant temperature of 26 ± 2 ºC.

***Plantlet regeneration***
Two months old cacao plantlets with at least two normal leaves and developed root systems were later acclimated to *ex vitro* and allowed to grow in the greenhouse under natural conditions. The plantlets were grown in 10 x 18 cm polybags filled with black soil or Jiffy 7® and placed randomly inside the chamber before transferred to 12 x 23 cm polybags after three months. The seedlings were fertilized with 2 gram NPK green fertilizer monthly. Misting was done solely for the first three months before starting watering from month four onwards as described in Nik Aziz *et al.,* (2016).

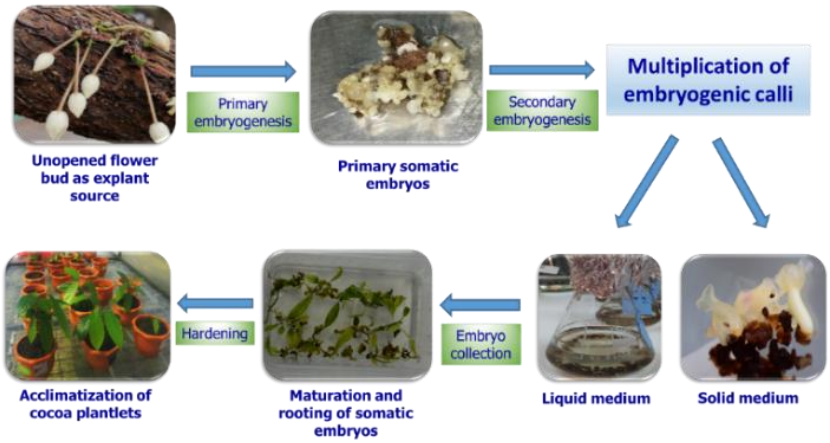


*Figure 1: The process of producing cacao plantlets using somatic embryogenesis.*

## RESULTS AND DISCUSSION

An appropriate combination of PGR used in the induction media results in dedifferentiation of explants' cells to form calli within 2-4 weeks. Several morphological variations were observed in calli formation such as fluffy, compact white and/or yellow calli and friable calli with an occasional mixture of both.

A total of 52 cocoa clones were tested using eight different induction media, but only 28 clones responded well to four media (Nestle, Nestle modified, PennState and MCB's). Primary SEs emerged consistently at a higher mean percentage ranged 8.33% to 65.33% (Table 1) (full data not shown) in between four to eight weeks after culture initiation. These embryos were formed on the surface of the calli and appeared globular in structure before classified into three morphological categories; (a) normal embryos, which are usually in a globular, heart and torpedo shape; (b) fasciated embryos, which consist of two or more fused embryos with multiple cotyledons and (c) abnormal embryo-like structure which is formed in groups, missing apical meristem development and lateral symmetry.

All primary SEs were subjected to cyclic embryogenesis to induce the production of secondary SEs, either using solid or liquid media. For solid technique, only two media were used, and the secondary SEs produced ranged between 9.33 to 87.5% (Table 2) (full data not shown). Time taken to produce secondary SEs is consistent at around 5-13 weeks. As reported in other plants (Yang *et al.*, 2013), the secondary SEs produced are more uniform, well-developed and offers the advantages of a high multiplication rate, independence from explants source effects, repeatability and can be maintained for a prolonged period by repeated cycles of secondary embryogenesis.

At the same time, two formulations of media, namely, NIM3liq and CC21liq, used for suspension culture. This technique produced up to 71% of secondary SEs in 12 weeks (Table 2). Even though SEs produced were more uniform and consisted of a mixture of embryos from all developmental stages, i.e. globular, heart and torpedo stage, only 12 cocoa clones responded to these liquid media. Another major issue related to this liquid technique is hyperhydricity of the cocoa cultured embryo. It is a physiological disorder that results in morphological and physiological alteration of plants, often giving them a 'glassy' appearance, due to apoplastic water accumulation, which will lead to the abnormal growth and necrosis.

Contamination and browning of the explants and culture media has been found to limit the establishment of cocoa primary cultures. Other than the right nutritional semi solid media consists of macro and micro elements, amino acids, carbon and iron source, vitamins and gelling agent; manipulations of auxin and cytokinin hormones (both in combinations and concentrations) were found to be the most critical factors in determining the

pathway of the cocoa cells and increased the frequency of callusing explants. Addition of PGRs into the media is the preferred way to induce morphogenetic responses and to enrich the metabolite synthesis particularly plant bioactive molecules (Chinappan R. S., 2018).

From this study, PennState protocol showed highly efficient to induce primary SEs for the most QH clones, while Nestle and Nestle modified is suitable for non-QH clones. However, most of the primary SEs responded well only to the Nestle modified media to induce secondary SEs. While most resulting embryos were normal morphology, some abnormalities were also observed where embryos were fused at the cotyledons or hypocotyls or failed to show bipolar organization. Tan *et al*., (2003) reported that normally these abnormal embryos do not germinate into plantlets.

Masseret *et al*., (2009) also reported that the most of cocoa clones which are reactive to primary SEs will respond positively to produce secondary SEs. However, it is also possible to have certain clones that react well to primary SEs yet respond laboriously to secondary SEs. These results proved that the response between primary and secondary SEs is clone or genotype-dependent.

Seven types of media (with or without phytohormones) were tested for embryo conversion into plantlets. Four weeks after culture initiation, somatic embryos showed size and length increment for all media. All embryos were transferred on to germination media. After six weeks in germination media, different morphology of embryos converted into plantlets was observed including stem length and size, number of leaves and root system development. Three types of plantlets form were recorded - normal (with at least two leaves and a root system), mix (with only a leaf and a root system) and abnormal (no leaf but has a root system) as described in Norasekin *et al*., (2016). The results showed that PEC is the best conversion media with 48% converted embryos followed by NG8 (40%) and NAGM (38%). However, the percentage of converted plantlets is still low. It might be due to the fact that cocoa genotypes will respond differently to every stage in the tissue culture process that may be attributed to their genetic makeup (da Silva *et al*., 2008; Maximova *et al*., 2002; Maximova *et al*., 2014; Quainoo and Dwomon, 2012). The average of SEs converted into plantlets was shown in Table 3.

Plantlet age two months perform well during acclimatization if compared to the other plantlet. The result in this study is similar to the result reported by Palee *et al*., (2012) from their research on the influence of different plantlet age on acclimatization success of *Stemona curtisii*. Apart from that, two months old plantlets showed more vigour and are less prone to develop hiperhydricity compared to plantlets that are kept longer in culture media. The plantlets were also able to produce a new flush within day seven of acclimatization. Plantlets planted on black soil also resulted in 50% survival while only 28.3% survived when Jiffy 7® peat pellets were used. Death frequency of plantlets planted in Jiffy 7® increased with average temperature increment and consequent drop in humidity (RH).

The extreme weather during the preparatory test cultivation will increase water loss through inadequate stomatal structure and insufficient epicuticular wax coverage on leaf surface. Usually plant photosynthesis and transpiration rate are reduced when facing temperature increase in the environment (Zhang *et al*., 2010 and Lipiec *et al*., 2013). However, the use of the chamber, as mentioned in Nik Aziz *et al*., (2016), has enabled *ex vitro* rooting and increased cocoa plantlets survival up to 39.1% by providing more ambient environment to prepare the plantlets against drastic environmental changes.

Table 1: The most two responsive clones to produce primary SEs for each media

| Clones | Induction media | % of primary somatic embryos |
|---|---|---|
| MCBC 1 | Nestle | 18.33 |
| KKM 22 | Nestle | 15 |
| PBC 123 | Nestle Modified | 43.33 |
| PBC 112 | Nestle Modified | 38.33 |
| QH 240 | PennState | 80 |
| QH 1003 | PennState | 65.33 |
| PBC 140 | MCB | 13.93 |
| PBC 154 | MCB | 11.26 |

Table 2: The most two responsive clones to produce secondary SEs for each media

| Clones | Induction media | Media Type | % of secondary somatic embryos |
|---|---|---|---|
| MCBC 9 | Nestle Modified | Solid | 87.5 |
| PBC 123 | Nestle Modified | Solid | 80 |
| MCBC 2 | PennState | Solid | 80 |
| QH 1003 | PennState | Solid | 80 |
| QH 1003 | CC21liq | Liquid | 69.2 |
| MCBC 7 | CC21liq | Liquid | 71.4 |
| PBC 123 | NIM3liq | Liquid | 70 |
| MCBC 2 | NIM3liq | Liquid | 60 |

Table 3: Average of somatic embryos converted into plantlets

| Conversion media | PGRs | % of SEs converted into plantlets |
|---|---|---|
| CMC6 | 2,4-D, BA | 26.67 |
| CMC7 | NAA, BA | 13.33 |
| NG8 | NAA, 2-iP | 40 |
| PEC | No | 48 |
| RD | No | 33 |
| NAEM | No | 33 |
| NAGM | No | 38 |

## CONCLUSIONS

Despite the numerous mass propagation methods available for *Theobroma cacao*, most of them are genotype-dependent. The current article combines the study of cocoa mass propagation and tissue culture performed by researchers in the Malaysian Cocoa Board, (MCB). The main objective of the study is to develop protocols for mass propagation and plant regeneration of cocoa superior clones to improve planting materials. In the Cocoa Biotechnology Research Centre, MCB, a protocol for the induction of embryogenic calli for 28 cocoa clones was successfully obtained. However, to date, the large-scale production of cocoa plantlets by tissue culture is still limited due to several factors such as low conversion rate of plantlets and somaclonal variation. Though many techniques are developed to date to improve yield and economy of tissue culture, more research should be carried out for further development.

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## REFERENCES

da Silva, T.É.R., Cidade, L.C., Alvim, F.C., de Mattos Cascardo, J.C. and Costa, M.G.C. (2008). Somatic embryogenesis and plant regeneration in elite clones of *Theobroma cacao*. Pesquisa Agropecuária Brasileira. 43(10): 1433-1436.

Lipiec, J., Doussan, C., Nosalewicz, A. and Kondracka, K. (2013). Effect of drought and heat stresses on plant growth and yield: a review**. *Int. Agrophys*. 27: 463-477.

Malaysia Cocoa Clones (2012). Malaysian Cocoa Board.

Masseret, B., Gianforcaro, M., Bouquet, J.F., Brulard, E. and Florin, B. (2009). Somatic embryogenesis applied to the creation of a cacao collection. Malaysian Cocoa J. 5: 1-10.

Masseret, B. (2009). Implementation of somatic embryogenesis as a propagation technique for cacao. Bali, Indonesia: Nestle, R&D Center Tours.

Maximova, N.S., Florez, S., Shen, X., Niemenak, N., Zhang, Y., Curtis, W. and Guiltinan, M.J. (2014). Genome-wide analysis reveals divergent patterns of gene expression during zygotic and somatic embryo maturation of *Theobroma cacao* L., the chocolate tree. BioMed Central Plant Biology 14: 185.

Maximova, S. N., Alemanno, L., Young, A., Ferrière, N., Traoré, A. and Guiltinan, M. J. (2002). Efficiency, genotypic variability, and cellular origin of primary and secondary somatic embryogenesis of *Theobroma cacao* L. In Vitro Cell. Dev. Biol. Plant. 38:252-259.

Nik Aziz, N. I., Jasli, M., Misak, E., Abdul Asui, N. and Kasran, R. (2016). Improvements of *Theobroma cacao* acclimatization to *ex vitro* environment. Malaysian Cocoa Journal Vol. 9(1): 11-18.

Norasekin, T. and Ducos, J. P. (2016). The effect of ascorbic acid in the maturation of cocoa somatic embryos. Malaysian Cocoa Journal Vol. 9(1): 5-10.

Norasekin, T., Siti Norhana, M. A. and Kasran, R. (2017). Induction and propagation of somatic embryos from cell suspension cultures of *Theobroma cacao* L. Malaysian Annual Scientist Workshop 2017. Kota Kinabalu.

Palee, J., Dheeranupattana, S., Jatisatienr, A., Wangkarn, S., Mungkornasawakul, P., Pyne, S., Ung, A. and Sastraruji, T. (2012). Influence of plantlet age and different soilless culture on acclimatization of *Stemona curtisii* Hook.f. Asian Journal of Plant Sciences, 11(6): 294-299.

Quainoo, A.K. and Dwomon, I.B. (2012). The effect of abscisic acid in the conversion of cocoa somatic embryos into plantlets. Frontiers in Science 2(2): 6-10.

Siti Norhana, M.A., Haikal Eman, A., Ahmad Kamil, M.J. and Kasran, R. (2016). Assessment of somatic embryogenesis potential of elite Malaysian *Theobroma*

*cacao* clones. Malaysian Cocoa Journal Vol. 9(1): 1-4.

Tan, C.L. and Furtek, D.B. (2003). Development of an in vitro regeneration system for *Theobroma cacao* from mature tissues. Plant Sci. 164: 407-412.

Yang, J.L., Wu, S.Q. and Li, C.H. (2013). High efficiency secondary somatic embryogenesis in *Hovenia dulcis* Thunb. through solid and liquid cultures. The Sientific World Journal. Vol. 2013. Article ID 718754.

Zhang B., Liu W., Chang S.X., and Anyia A.O. (2010). Water-deficit and high temperature affected water use efficiency and arabinoxylan concentration in spring wheat. *J. Cereal Sci*. **52**: 263-269.