# OPTIMISING COCOA PROTOPLAST ISOLATION FOR EFFICIENT GENOME EDITING

**Norasekin, T.[1*], Monaliza, M. D.[1], Lea, J.[1], Ishak, Z.[2] and Mohd Zulhilmi, A. R.[1]**
[1]Malaysian Cocoa Board, Centre for Cocoa Biotechnology Research, Commercial Zone 1, Norowot Road, Kota Kinabalu Industrial Park (KKIP), 88460 Kota Kinabalu, Sabah, MALAYSIA
[2]Malaysian Cocoa Board, Cocoa Innovative and Technology Centre, Lot 12621, Nilai Industrial Area, 71800 Nilai, Negeri Sembilan, MALAYSIA
**Corresponding author: norasekin@koko.gov.my*

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**ABSTRACT -** *Protoplasts are incredibly useful in plant biotechnology due to their versatility. They have a wide range of applications, from fundamental biological research to the strategic development of breeding techniques that involve genome editing. Establishing an efficient protoplast isolation protocol is paramount in scientific endeavours involving protoplasts. This study has developed and optimised several potential methods to isolate the highly viable protoplast from diverse cocoa explants. Notably, young leaves sourced from mature cocoa trees exhibited the most favourable outcomes, yielding a substantial protoplast yield of 89.87 % and a viable protoplast density of 10.65 x $10^7$. The optimised isolation method provides valuable insights into developing an effective cocoa protoplast isolation protocol crucial for genome editing applications. Furthermore, these findings offer a tailored approach for cocoa researchers, emphasising the imperative nature of customised protocols to harness the full potential of protoplast-based genetic engineering in cocoa plants.*

***Keywords:*** Plant protoplast, biotechnology, genome editing, cocoa, highly viable, isolation

## INTRODUCTION

*Theobroma cacao* L., known as cocoa, is one of the world's most valuable commodities for cocoa-producing countries, including Ghana, Ivory Coast, Indonesia and Malaysia. The commodity significantly fosters economic growth and social development in the cocoa-producing country's agricultural sector. The total annual production of the commodity is approximately four million tonnes (Elwers & Lieberei, 2020) worldwide. In Malaysia, the cocoa industry contributed RM1.64 billion to GDP in 2020 (Malaysian Cocoa Board, n.d.). This tropical crop is cultivated primarily for its edible seeds, known as cocoa beans. These beans undergo processing to produce cocoa powder, cocoa butter, cocoa liquor, and chocolate. Although the demand for cocoa has increased by around three per cent since 2008 (WCF, 2014), supply is anticipated to continue falling short of meeting the growing demand. Additionally, cocoa output worldwide is declining annually due to various issues, including the presence of ageing tree stock, the proliferation of pests and diseases, production capacity, and climate change.

Therefore, many biotechnologists and plant breeders consider improving crop quality as an essential research topic in their respective fields. Implementing genome editing in cocoa cultivation appears to be a potential method for addressing the issue. However, with longer generation rates and the complex genetic makeup of the species, the process of genetic improvement of cocoa plants using traditional breeding methods is frequently slower and more complex (Bekele & Phillips-Mora, 2019). Alternatively, protoplast technology has emerged as a powerful tool in plant biotechnology. It provides a diverse platform for genetic manipulation and direct genetic modification through genome editing techniques such as CRISPR/Cas9 and somatic hybridisation (Yue *et al.,* 2021). Isolating protoplasts for further downstream studies is essential for improving the crop's sustainability. However, the success of such technologies hinges on the ability to isolate viable protoplasts efficiently, thus improving the plant regeneration system.

Cocoa protoplast isolation is notoriously challenging. To date, protoplast isolation techniques on this crop have been documented in very few studies (Collin *et al.,* 1988; Kanchanapoom & Charuvat, 1999). Therefore, this research attempted to optimise several potential methods to isolate the highly viable protoplast from diverse cocoa explants.

## MATERIALS AND METHODS

### *Plant material development*

This study used four types of explants to enhance and refine the isolating and culturing protoplast techniques. The primary samples, cacao flower buds and mature pods were collected from recommended clones grown at the Centre for Cocoa Biotechnology various areas in the centre (field, nursery and greenhouse) to guarantee top-notch quality and

uniformity. The secondary explants consisted of freshly harvested young cocoa leaves from seedlings and mature trees. The young leaves, with their light green colour and smooth texture, were carefully collected in the early morning to minimise transpiration, decrease metabolic activity, ensure stable environmental conditions, and preserve optimal hormone levels. This meticulous selection and precise timing are required to optimise the yield of the protoplasts generated during subsequent procedures.

Fresh leaves, unopened flower buds, and mature pods underwent rigorous sterilisation to ensure aseptic conditions throughout the experiment. The samples were initially washed with tap water to eliminate surface contamination. They were then immersed in 70% ethanol for 15 minutes to sanitise their external surfaces. After ethanol treatment, a two-step method involving a 10 % bleach and 0.1 % SDS solution, followed by a 1 % bleach and 0.1 % SDS solution, was implemented for 20 minutes each. A thorough rinse with sterile distilled water was conducted three times after these procedures to eliminate residual chemicals. These meticulously executed processes generated callus and suspension cultures, laying the foundation for future investigations.

Sterilised flower buds and pods were dissected aseptically using forceps and a scalpel to extract staminodes and seeds. Staminodes from flower buds and zygotic embryos from seeds were cultured on fresh induction solid media for at least one month to induce callus (Siti Norhana *et al.,* 2016). The induced callus were regular subcultures until a pale yellowish and friable callus was consistently observed. After that, the friable callus was employed to initiate cell suspension. Multiple suspension media were used, with treatment parameters tuned to established plant growth regulator recommendations and validated parameters reported in various recognised agricultural journals (Norasekin *et al.,* 2020; Guillou *et al.,* 2018).

***Protoplast isolation***

Dry sterilized leaves were directly chopped under aseptic conditions on a glass petri dish using forceps and a scalpel. For fresh cocoa leaves, two different methods were used to obtain the protoplast: mechanical and enzymatic, while protoplast from the callus was only isolated using the enzymatic method.

In the mechanical method, the excised leaves were thoroughly washed with a phosphate buffer and homogenized gently using a mortar and pestle. This meticulous process resulted in a crude protoplast suspension, which was then centrifuged at 1000 rpm for 10 minutes to isolate intact protoplasts. After centrifugation, 100 mL of the supernatant containing the intact protoplasts was carefully pipetted into new Eppendorf tubes, while the pellet, consisting of cellular debris, was discarded. To assess protoplast viability, 20 µL of Trypan blue stain was added to 20 µL of the protoplast suspension. The solution was gently mixed and placed onto a microscope slide or haemocytometer, covered with a coverslip, and observed under a microscope to assess the viability and density of the isolated protoplasts.

In the enzymatic method, freshly cut cocoa leaves (1 g) were immersed in 13 % mannitol for 1 hour for pre-plasmolysis. The treated leaves, or callus tissues, were then transferred into sterile 15 mL centrifuge tubes containing cell-wall digestion enzyme solution. The cells were thoroughly immersed in an enzyme solution and incubated in a dark, thermostatically regulated shake at 100 rpm. The incubation lasted between 2 to 10 hours, depending on the requirements of the enzyme and methods used, as listed in Table 1. After incubation, the protoplast suspension was filtered through a 58-micron Nylon mesh to remove cellular debris. The filtrate was then transferred into 1 mL aliquots in centrifuge tubes using sterile pipette tips and centrifuged at 1000 rpm and 25 °C for 5 minutes to collect the protoplasts. The supernatant and debris were discarded to purify the protoplasts further. The pelleted protoplasts were resuspended in a protoplast washing solution (CPW) and a 21 % sucrose solution. They were centrifuged again at 1000 rpm and 25 °C for 5 minutes, resulting in a cleaner, more concentrated protoplast population, which was then resuspended in a 1 mL buffer solution.

***Assessment of protoplast viability and density calculation***

A haemocytometer and staining-based method assessed the viability of freshly isolated protoplasts. Protoplast suspensions were prepared, ensuring proper dispersion. For fluorescein diacetate (FDA) staining, a 20 µL volume of the dye was added to the 20 µL of the protoplast suspension, followed by gentle mixing and incubation in the dark for 10-20 minutes at room temperature. The solution was mixed gently to ensure proper staining. A microscope slide and the haemocytometer were prepared with 20 µL of the stained protoplast suspension. Under a fluorescence microscope, viable protoplasts exhibited green fluorescence. Viability was determined by counting the number of viable and non-viable protoplasts in multiple fields of view (Larkin, 1976). The percentage of viability was calculated using the following formula:

$$Viability\ (\%) = \frac{(Number\ of\ Viable\ Protoplasts)}{(Total\ Number\ of\ Protoplasts)}\ x\ 100$$

The viability and density of the protoplast were counted at least three times for more accurate measurement by calculating the average density of the protoplast. Under a fluorescence microscope, protoplast density is calculated using the following formula:

$$Protoplast\ Density\ \left(\frac{cells}{mL}\right) = \frac{(Number\ of\ Protoplasts\ Counted\ x\ dilution\ factor)}{(Volume\ of\ a\ square\ of\ haemocytometer\ (mL)}$$

Table 1: Various percentage combinations of enzymes used for protoplast isolated from calli and leaves from seedling and mature trees.

| Solution No | Xylanase (%) | Macerozyme R10 (%) | Incubation Time (Hr) |
|---|---|---|---|
| 1 | 2 | 0.5 | 2 |
| 2 | 1.5 | 0.5 | 2 |
| 3 | 2 | 0.5 | 4 |
| 4 | 1.5 | 0.5 | 4 |
| 5 | 2 | 0.5 | 6 |
| 6 | 1.5 | 0.5 | 6 |
| 7 | 2 | 0.5 | 8 |
| 8 | 1.5 | 0.5 | 8 |
| 9 | 2 | 0.5 | 10 |
| 10 | 1.5 | 0.5 | 10 |

**RESULTS AND DISCUSSIONS**

A specially formulated medium for *Theobroma cacao* has been developed, allowing for the efficient dedifferentiation and callus development of explant cells within 2-4 weeks. Various morphological types of calli were observed, such as fluffy, yellow, and friable calli. The friable and embryogenic callus employed to initiate cell suspension was aged 6 to 8 weeks. After approximately three weeks in a liquid medium, the fresh weight of the culture increased, showing that the culture grew during the gentle agitation, creating concentrated suspension.

The mechanical disruption approach of protoplast isolation produced significantly less viable protoplasts than expected. Trypan Blue staining viability studies showed a substantially lower viability percentage than anticipated. As demonstrated by the uptake of Trypan Blue dye, a significant fraction of the separated protoplasts was found to have impaired membrane integrity (Figure 1). Based on the figure, the protoplasts in the green circles, which were not stained with Trypan blue, indicated viable protoplasts. In contrast, the protoplasts in the red circles, which showed stained protoplasts, indicated non-viable protoplasts.

Mechanical disruption, which involves the application of physical forces that can cause cell ruptures and damages, might be responsible for the lower cell protoplast viability than anticipated. While mechanical approaches are generally effective for protoplast isolation, the decrease in viability in this study highlights the need for further optimization and refinement of the protocol to minimize cell damage during the protoplast isolation procedures. This would include adjustments to homogenization parameters, buffer compositions, and incubation conditions to enhance protoplast viability. These refinements are crucial for ensuring the success of downstream experiments and applications in plant tissue culture and biotechnology.

Promising results were obtained for protoplast viability and density, notably for young leaves collected from mature trees, using the enzymatic approach employed for protoplast isolation. Viability assessments conducted using the FDA staining method indicated a remarkably high percentage of

*Figure 1: Isolated protoplast through mechanical approach. The red circles show non-viable protoplasts, while. the green circles show viable protoplasts.*

viable protoplasts extracted from mature trees in all treatments, as shown in Table 2. The results shown that solution four (4), containing 1.5 % xylanase + 0.5 % Macerozyme R10 and incubated for 4 hours, gave the highest percentage of viable protoplast, recorded at 89.87 %. The density of the viable protoplast was also recorded at 10.65 x $10^7$. This outcome indicated the effectiveness of the enzymatic method in producing healthy and intact protoplasts from young leaves of mature trees. These results are in accordance to the results reported by Thompson *et al*., (1987), whereby it was noted that the best result of protoplast isolation was obtained from young leaves and the protoplast remained viable and metabolically stable for up to 40h. Furthermore, slicing the tissue into smaller strips before enzyme treatment increased the surface area and contributed to the release of more protoplasts (Reed & Bargmann 2021).

On the other hand, the viability of protoplasts from callus and leaves of seedlings was notably lower than that observed in mature leaves in all treatments. The highest protoplast viability isolated from these explants was achieved with solution number five (5), containing 2 % xylanase + 0.5 % Macerozyme R10 and incubated for 6 hours. The viability was recorded at 56.86 % for callus and 65.71 % for leaves collected from cocoa seedlings. The protoplasts from these tissues exhibited compromised membrane integrity compared to the protoplast isolated from the leaves of mature trees, as indicated by the uptake of the stain (Figure 2).

The variation in viability among different tissue types suggested that the enzymatic approach is particularly effective for young leaf tissues from mature trees but poses challenges for young leaves from seedlings and callus. The age of the source tissue can significantly impact protoplast yield, viability, and regeneration success (Kiełkowska & Adamus, 2012). The variation in protoplast viability among different tissue sources can also be attributed to the differences in cell wall composition and thickness (Keegstra, 2010). The high viability and density of protoplasts from young leaves of mature trees make them an ideal source material, as demonstrated in studies on *Elaeis guineensis* (Masani *et al*., 2014) and Magnolia (Shen *et al*., 2017).

Callus and seedling leaves have cells with different properties, such as higher cell division rates and potentially different cell wall compositions (Palin, 2011), which may contribute to the lower protoplast viability observed in our study. Furthermore, protoplast isolation from the cultured cells or callus faced high browning phenomenon. This condition is normal as cocoa-tree is naturally high in polyphenolic content. Therefore, it is necessary to isolate the protoplast at early stages (<2 months-old culture) to avoid the polyphenolic compound (Tsai & Kinsella, 1981). To improve viability for these tissue types, further investigation is needed to refine the enzymatic protocol, including adjusting enzyme concentration, digestion duration, and other parameters.

These findings underscore the crucial role of tissue-specific optimization in protoplast isolation procedures. Different tissue types may necessitate tailored protocols to achieve high viability. The enzymatic approach allows for the controlled isolation of protoplasts, a crucial step in various plant tissue culture and biotechnology applications. Suitable enzymatic digestion and purification ensure high-quality protoplasts suitable for further experiments.

Table 2: Percentage of protoplast viability isolated from callus and leaves of seedling and mature cocoa tree.

| Solution No | Type of Explants | Total Protoplast | Viable Cell of Protoplast | Viable protoplast (%) | Protoplast Density |
|---|---|---|---|---|---|
| 1 | Callus | 24 | 5 | 20.83 | $2.5 \times 10^6$ |
| | Leaves (Seedling) | 56 | 33 | 58.92 | $16.5 \times 10^6$ |
| | Leaves (Mature) | 102 | 86 | 84.31 | $43 \times 10^6$ |
| 2 | Callus | 28 | 8 | 28.58 | $4 \times 10^6$ |
| | Leaves (Seedling) | 64 | 31 | 48.44 | $15.5 \times 10^6$ |
| | Leaves (Mature) | 189 | 156 | 82.54 | $78 \times 10^6$ |
| 3 | Callus | 35 | 18 | 51.43 | $9 \times 10^6$ |
| | Leaves (Seedling) | 67 | 39 | 58.21 | $19.5 \times 10^6$ |
| | Leaves (Mature) | 121 | 108 | 85.26 | $54 \times 10^6$ |
| 4 | Callus | 39 | 19 | 48.72 | $9.5 \times 10^6$ |
| | Leaves (Seedling) | 68 | 33 | 48.53 | $16.5 \times 10^6$ |
| | **Leaves (Mature)** | **237** | **213** | **89.87** | **$10.65 \times 10^7$** |
| 5 | **Callus** | **51** | **29** | **56.86** | **$14.5 \times 10^6$** |
| | **Leaves (Seedling)** | **70** | **46** | **65.71** | **$23 \times 10^6$** |
| | Leaves (Mature) | 92 | 80 | 86.96 | $40 \times 10^6$ |
| 6 | Callus | 42 | 17 | 40.48 | $8.5 \times 10^6$ |
| | Leaves (Seedling) | 61 | 18 | 29.51 | $9 \times 10^6$ |
| | Leaves (Mature) | 57 | 36 | 63.16 | $18 \times 10^6$ |
| 7 | Callus | 26 | 8 | 30.77 | $4 \times 10^6$ |
| | Leaves (Seedling) | 44 | 22 | 50 | $11 \times 10^6$ |
| | Leaves (Mature) | 51 | 29 | 56.86 | $14.5 \times 10^6$ |
| 8 | Callus | 21 | 5 | 23.81 | $2.5 \times 10^6$ |
| | Leaves (Seedling) | 27 | 13 | 48.15 | $6.5 \times 10^6$ |
| | Leaves (Mature) | 40 | 26 | 65 | $13 \times 10^6$ |
| 9 | Callus | 18 | 3 | 16.67 | $1.5 \times 10^6$ |
| | Leaves (Seedling) | 19 | 9 | 47.37 | $4.5 \times 10^6$ |
| | Leaves (Mature) | 34 | 14 | 41.18 | $7 \times 10^6$ |
| 10 | Callus | 2 | 0 | 0 | 0 |
| | Leaves (Seedling) | 8 | 1 | 12.5 | $5 \times 10^5$ |
| | Leaves (Mature) | 15 | 6 | 40 | $3 \times 10^6$ |

Several factors also influence protoplast isolation and its regenerative capacity. One important factor is the length of the digestion time, which typically spans from 2 to 10 hours. Even so, it's important to find the right digestion time, because the digestion must be long enough to release more protoplasts, yet not too long as the cell viability diminishes due to cell damage or depletion of essential nutrients and growth regulators in the enzymolysis solution (Reed & Bargmann 2021). Other than that, temperature also plays a pivotal role in affecting enzyme activity, protoplast yield, viability, and regenerative capacity. It is important to maintain a temperature close to the growth conditions of the culture to minimize temperature shocks (Reed & Bargmann 2021). Specific temperature treatments might enhance regenerative capacity.

Dark environments, which are generally preferred by most tissues (Reed & Bargmann2021), play a crucial role in protoplast isolation. They effectively limit photosynthetic activity, thereby preventing the buildup of reactive oxygen species (ROS) that can damage cells and reduce protoplast yield. (Lai *et al.,* 2020; Pavese *et al.,* 2022). While protoplast yield and viability are important, it is

crucial to note that the conditions that yield the highest values may not be the most suitable for protoplast regeneration. This underscores the need for careful optimization to achieve successful regeneration in specific plant species and tissues. The precision required in this process highlights the potential implications for future research, as well as the need for a deeper understanding of the complex relationships between these variables and their impact on protoplast regeneration.

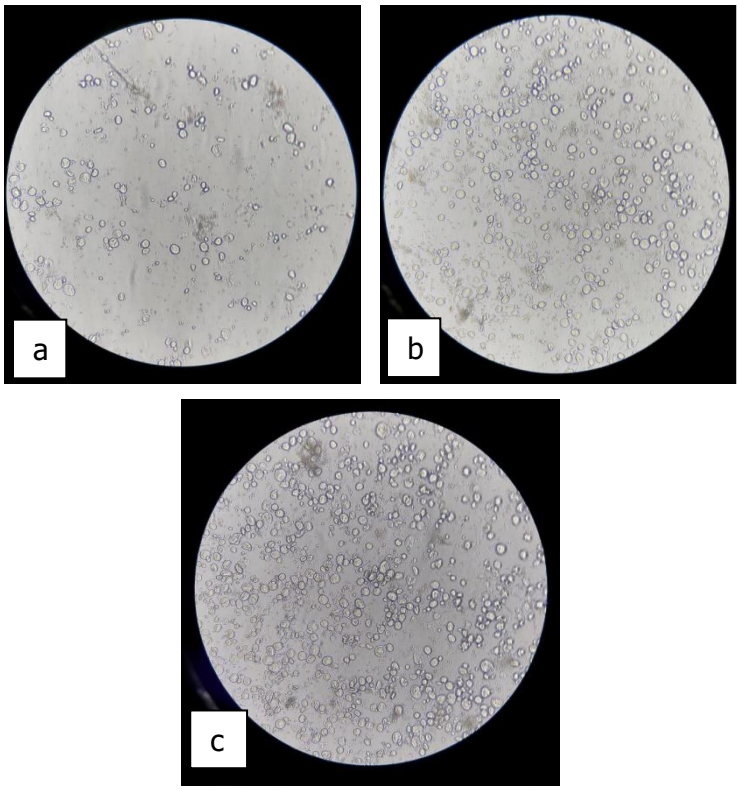


*Figure 2: Isolated protoplast from different explants, (a) callus; (b) fresh leaves of seedlings; (c) fresh leaves of mature tree.*

## CONCLUSIONS

In conclusion, this study employed mechanical and enzymatic methods to isolate protoplasts from various cocoa plant tissues, including callus, young leaves of mature trees and seedlings. Our assessments of protoplast viability and membrane integrity revealed that the enzymatic method was significantly more effective, yielding highly viable and intact protoplasts, especially from the leaves of mature trees compared to the mechanical method. These results underscore the efficiency and promise of the enzymatic approach for protoplast isolation in cocoa plants, making it an ideal method for various applications in plant biotechnology. Our findings provided valuable insights into the challenges and opportunities associated with protoplast isolation, particularly for advanced studies such as CRISPR and genetic engineering. The high viability and integrity of protoplasts obtained through enzymatic methods highlight the potential of protoplast-based technologies in addressing critical issues in food security, agriculture, and sustainable biotechnology. Protoplast technology offers transformative promise for climate-resilient agriculture, environmentally friendly biofactories, conservation of endangered plant species, and accelerated crop breeding. This study lays the groundwork for future research and applications, contributing to advancing plant biotechnology and agricultural innovation.

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