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A revolutionary temporary immersion bioreactor method for commercial-scale *Musa sp.* (Tanduk and Berangan) multiplication

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Abstract

Large-scale clonal banana production is required to supply current industry demand. Conventional tissue culture technique needs to be improvised to overcome the high-cost manufacturing and laborious work involved. Therefore, this study aimed to develop an efficient protocol in clonal banana production by using temporary immersion bioreactor (TIB) technology which uses intermittent immersion in liquid nutrients. This study evaluated the optimum protocol pathway for efficient *Musa acuminata* cv. Berangan and *Musa paradisiaca* cv. Tanduk propagation with the application of TIB system. *M. acuminata* and *M. paradisiaca* optimum initiation medium were developed in MS (Murashige and Skoog) media supplemented with 3 mg/L 6-Benzylaminopurine (BAP) and 0.2 mg/L Thiamine and 0.3 mg/L Thidiazuron (TDZ) respectively. The optimum proliferation medium in TIB developed in MS media supplemented with 3 mg/L BAP for both varieties. The highest number of shoots was achieved in TIB with 20 initial plantlets and a 15-minute immersion with a 6-hour gap along with a 14-day incubation period. Micropropagation of *Musa* sp. in Temporary Immersion Bioreactor System (RITA ®) has successfully shown significant growth as compared to MS media agar-conventional technique with reduced phenolic content released in both varieties. In conclusion, the production of *Musa acuminata* cv Berangan and *Musa paradisiaca* cv. Tanduk by using a temporary immersion bioreactor (TIB) system has huge potential to improve the scale production output with lower-cost plantlets.

1. Introduction

In the rapidly advancing field of biological sciences, plant tissue culture has emerged as one of the most promising applications of biotechnological tools, with significant implications for both current and future agricultural practices (Suman, 2017; Hasnain *et al*., 2022). Plant tissue culture technology plays a crucial role in the production of high-quality planting materials for selected novel high-quality varieties, thereby enhancing agricultural production (Datta *et al*., 2017). These techniques are now widely applied for purposes such as plant propagation, disease elimination, plant breeding, production of valuable secondary metabolites, and conservation of endangered plant species (Rahimi *et al*., 2023). In 2020, the global market for plant tissue culture was valued at $382.305 million. It is projected to reach $895.006 million by the year 2030, growing at a compound annual growth rate (CAGR) of 8.5% during the period from 2021 to 2030 (Kumar and Rani, 2022; Negi *et al*., 2022; Salunkhe *et al*., 2022).

Previous studies in *Musa* sp. mostly focused on somatic embryogenesis techniques for micropropagation. This technique plays a crucial role in the micropropagation of banana plants due to their parthenocarpic and triploid nature. It ensures the rapid and disease-free propagation of plants, maintaining their true-to-type characteristics (Manchanda *et al*., 2018; Justine *et al*., 2022). It is also beneficial for the mass production of new varieties or rejuvenation of existing ones (Datta *et al*., 2017; Manchanda *et al*., 2018). Moreover, micropropagation serves as an ideal method for conserving the gene pool of banana plants (Manchanda *et al*., 2018).

To meet the growing demand for clonal tissue culture banana plantlets, different tissue culture methods are necessary for large-scale clonal propagation of bananas (Manchanda *et al*., 2018). However, the widespread adoption of this technology at an industrial level has been limited due to certain drawbacks, such as high production costs. Besides that, the capital, labour,

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and energy-intensive nature of tissue culture techniques has also contributed to this cost inefficiency. Consequently, there is a need to prioritize the development of low-cost tissue culture technology in various sectors (Datta *et al*., 2017).

Previously, a lot of strategies were employed to simplify the operations involving *in vitro* propagation. These efforts aimed to reduce costs without compromising the quality of the resultant plantlets. These strategies include the development and utilization of cost-effective methods or improving process efficiency and optimizing resource utilization to make tissue culture more accessible and economically viable.

Therefore, to combat this issue, this research aimed to explore the application of the temporary immersion bioreactor (TIB) technology for efficient clonal production. Efforts have been focused on the utilization of the RITA® temporary immersion bioreactor system to optimize tissue culture systems and reach production targets. The micropropagation industry is witnessing the emergence of a potentially innovative bioreactor technology which is the TIB (Uma *et al*., 2021; Uma *et al*., 2023). The cost-effective TIB technology represents one of the most promising solutions for the micropropagation industry. This innovative approach enables the partial automation of certain *in vitro* culture steps, thereby significantly enhancing the scalability of the process. As a result, it leads to improvements in both the biological and productive efficiency of clonal seedlings (Pożoga *et al*., 2023).

This TIB system offers several key advantages over conventional tissue culture methods (Sambolín*et al*., 2023). Firstly, it reduces the media cost substantially by minimizing the usage of gelling agents. Secondly, the nutrient media in the TIB system facilitates better nutrient absorption, resulting in improved growth of the plants and seedlings. Finally, the TIB technology helps to shorten the already brief production timelines typically associated with the tissue culture process (Zhang *et al*., 2018). In summary, this cost-effective TIB system represents a transformative solution for the micropropagation industry, enabling increased automation, scalability, and efficiency, while also reducing production costs and timelines. However, despite the success recorded with this system, it is important to identify the most efficient system or pathway in the micropropagation of plantlets.

Musa sp. is one of the most important fruit crops in the world. It ranks among the leading fruit crops cultivated in tropical and subtropical regions and is recognized as an economically efficient source of sustenance (Kumar *et al*., 2020). Banana production worldwide amounts to 155.2 million tons, covering an area of 5.6 million hectares, as per FAOSTAT 2018 data. Bananas are currently the most important fruit crop, accounting for over 39.40% of total fruit production. (Yadav *et al*., 2020). The banana is a versatile fruit that can be eaten fresh, fried, mashed, or used to flavor various dishes. Overall, bananas are highly valued for their flavor, nutritional value, and availability throughout the year. Malaysia is one of the origin countries of banana and plantain with about 50 types expected in the exportation business. Improvisation in tissue culture technique are poised to benefit to the banana micropropagation sector.

2. Materials and methods

2.1 Setting up of temporary immersion bioreactor system

The TIB system used was the RITA® system, with a container for plant culture and liquid media. Containers were linked by silicone tubes allowing medium flow. Air pumps produced water motion to aerate cultures at predetermined intervals. Immersion duration and frequency were pre-programmed and regulated by a timer. Air filters prevented contamination. The containers, medium reservoir, and tubes were autoclaved at 121°C and 1 kg/cm^2 pressure for 15 mins.

2.2 Plant materials

Suckers of *M. paradisiaca* and *M. acuminata* were chosen from superior and healthy mother plants acquired in Johor. The selected mother plants were aged between 6 and 7 months and had been cultivated in the field. The suckers were cut in half to a third of their original size, and all leaves and corm tissue were removed to create a 10 cm long block. The soil, dirt, and debris attached to the structure were removed and cleaned using tap water, leaving only the shoot apex portion remaining.

2.3 Tissue culture media preparation

Tissue culture media were prepared according to Dhanalaksmi and Stephan (2014) with some modifications. Murashige and Skoog (1962) created a vitamin-supplemented medium for the initiation of sterile shoots for micropropagation stage studies. Approximately 4.4 g/L of Murashige and Skoog (MS) medium, including vitamin powder, was diluted in half the volume of distilled water used in the final volume. An optimized plant growth hormone stock solution was supplemented with the media solution. The pH was adjusted to 5.8, which was necessary for plant development. After pH correction, a gelling agent was used. The medium was swirled constantly and cooked on a hot plate until the gelling agent was completely dissolved. The prepared medium was autoclaved for 25

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mins at 1.06 kg/cm^2 pressure and 121°C. The medium was placed into culture vials at a temperature of less than 50°C in a laminar airflow prior to solidification.

2.4 In vitro regeneration of Musa acuminata in temporary immersion bioreactor

Shoots initiated during the shoot induction phase were used as explants for *in vitro* regeneration in TIB. The growth of the regenerated plantlets was evaluated based on their morphological characteristics and the number of shoots which were measured after 4 weeks of culture. During acclimation, the plantlets' survival percentage and *ex vitro* development were also assessed.

The study involved the evaluation of the TIB system for the propagation of *M. acuminata* cv. Berangan and *M. paradisiaca* cv. Tanduk. The initiation medium for each variety was developed using a specific optimal propagation medium in the TIB system. The number of shoots, number of initial plantlets, immersion duration, immersion frequency, and incubation period were analyzed to determine the most efficient protocol for clonal banana production.

2.5 Collecting and analysing data

Data from the number of shoots in both conventional and TIB methods were analysed with SPSS software and presented as means of standard errors. A one-way analysis of variance (ANOVA) was employed to identify statistically significant deviations with $P < 0.05$.

3. Results and discussion

3.1 Initiation of sterile plantlets

The results indicate that the optimum shoot initiation medium for the banana species *M. acuminata* as shown in Figure 1 and *M. paradisiaca* as shown in Figure 2 was MS media supplemented with 3 mg/L BAP. The optimal shoot initiation medium for *M. paradisiaca* was MS media supplemented with 0.2 mg/L of thiamine and 0.3 mg/L thidiazuron (TDZ).

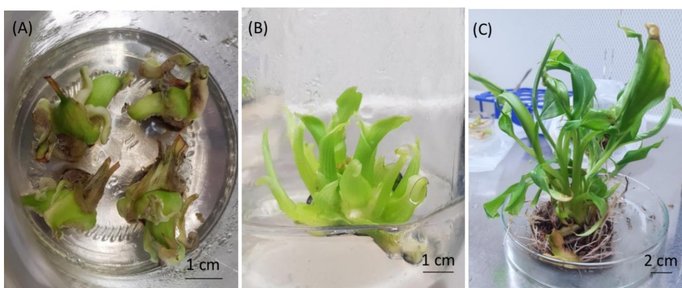

Figure 1. Initiation of *Musa acuminata cv.* Berangan sterile plantlets (A) A month culture (B) Two (2) months culture (C) Three (3) months culture.

These findings suggest that different *Musa* sp. have different requirements for plant growth regulators to achieve the best shoot proliferation from explants during *in vitro* regeneration. The specific type and concentration of cytokinin needed to be optimized for each *Musa* species, highlighting the importance of species-specific medium composition for successful micropropagation of these important crop plants and for developing efficient protocols for the *in vitro* propagation of banana and plantain varieties.

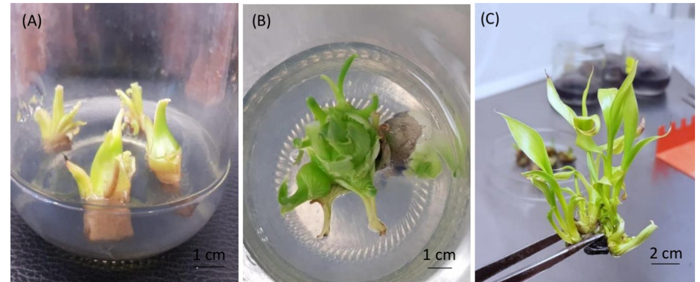

Figure 2. Initiation of *Musa paradisiaca* cv. Tanduk sterile plantlets (A) A month culture (B) Two (2) months culture (C) Three (3) months culture.

Baye *et al*. (2019) concluded in their study that the *in vitro* regeneration capacity and growth patterns can vary significantly based on the genetic makeup of the cultivar, the choice of explant material, and the plant growth regulator (PGR) supplementation in the culture medium. A single universal protocol may not be suitable or effective for the *in vitro* growth and regeneration of all varieties. Cultivar-specific optimization of the *in vitro* culture conditions, particularly the PGR components, is necessary to account for the genotypic and explant-specific differences in the plant's *in vitro* response.

3.2 Optimization of propagation medium treatment in RITA ®, a temporary immersion bioreactor system and comparison with control (solid medium)

The optimum proliferation medium for both *M. acuminata* (Figure 3) and *M. paradisiaca* (Figure 4) varieties in the TIB system was MS media supplemented with 3 mg/L of BAP. However, when the BAP concentration was increased to 5 mg/L in the MS media, the results showed a decrease in the number of shoots produced. This suggests that while a higher concentration of the shoot-inducing cytokinin which is 5 mg/L BAP treatment should be able to further enhance shoot proliferation compared to the 3 mg/L BAP level, there appears to be a limit to which increasing the cytokinin concentration becomes detrimental.

The percentage of shoot initiation decreased when the concentration of BAP was increased. Similar results were reported by Ishag *et al.* (2009) using shoot tip and cotyledonary node explants of the *Omdurman* cultivar which discussed that with a higher concentration of cytokinin, the percentage and shoot frequencies decreased. These results also aligned with Mukta (2014)

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Figure 3. (A) 1 mg/L BAP (B) 2 mg/L BAP (C) 3 mg/L BAP (D) 4 mg/L BAP (E) 5 mg/L BAP of *Musa acuminata* cv. Berangan.

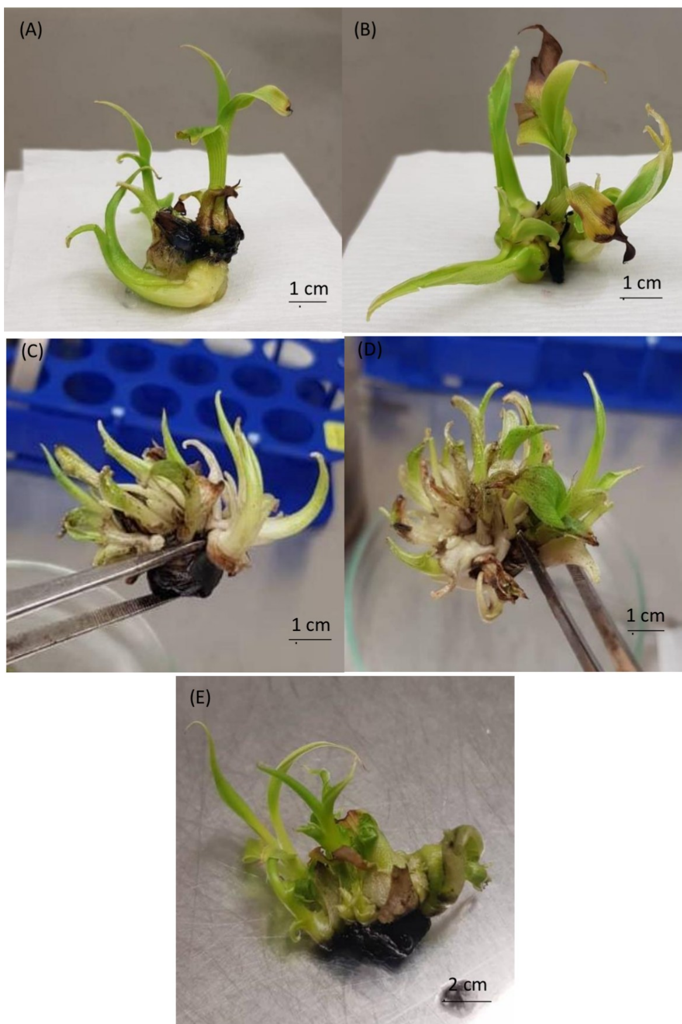

Figure 4. (A) 1 mg/L BAP (B) 2 mg/L BAP (C) 3 mg/L BAP (D) 4mg/L BAP (E) 5 mg/L BAP of *Musa paradisiaca* cv. Tanduk.

who reported that the regeneration percentage of three tomato cultivars decreased from 96% to 19% as the concentration of BAP was increased from 1 mg/L to 7 mg/L.

Increasing the BAP concentration above an optimal level becomes detrimental to the shoot propagation and overall plant regeneration in plant propagation. This showed the importance of the optimal plant growth hormone concentration range for efficient *in vitro* propagation, and exceeding this range can lead to decreasing numbers in the shoot initiation and proliferation rates. Higher cytokinin concentrations do not necessarily translate to improved regeneration outcomes for these banana species.

3.3 Optimum initial number of shoots subcultured in RITA ®

The optimum initial culture in the RITA ® system had been developed in the initial culture of 20 shoots. The initial culture of 25 shoots showed limited nutrient activities in morphology, growth and high phenolic content released. Figures 5 and 6 show the condition of both varieties in *M. acuminata* cv. Berangan and *M. paradisiaca* cv. Tanduk respectively.

3.4 Comparison of number of shoots in control medium (solid media) and in temporary immersion bioreactor (RITA ®)

As compared to the control, the research findings in Figure 7 show that the TIB system, with its intermittent immersion in liquid nutrients, significantly improved clonal banana production with a significant difference P <0.05 in both *M. acuminata* cv Berangan (Figure 8) and *M. paradisiaca* cv. Tanduk (Figure 9). The optimal protocol involved the use of 20 initial plantlets, 15-minute immersion with a 6-hour gap, and a 14-day incubation period. This protocol resulted in the highest number of shoots, indicating successful propagation of the desired banana varieties.

TIB RITA® method demonstrates a two-fold increase in shoot propagation period compared to the semi-solid media. The RITA® method can harvest the plantlets in half the time required for the semi-solid media approach as shown in Figure 7.

Shoot growth and rooting were shown to be efficient in explants cultivated in RITA ® with 200 to 250 mL of culture medium per run. This is in line with the report by Uma *et al*. (2021), which stated that shoot multiplication was 2.7 times higher in TIB than in semisolid culture, which is ideal for large-scale production of planting material for commercial uses.

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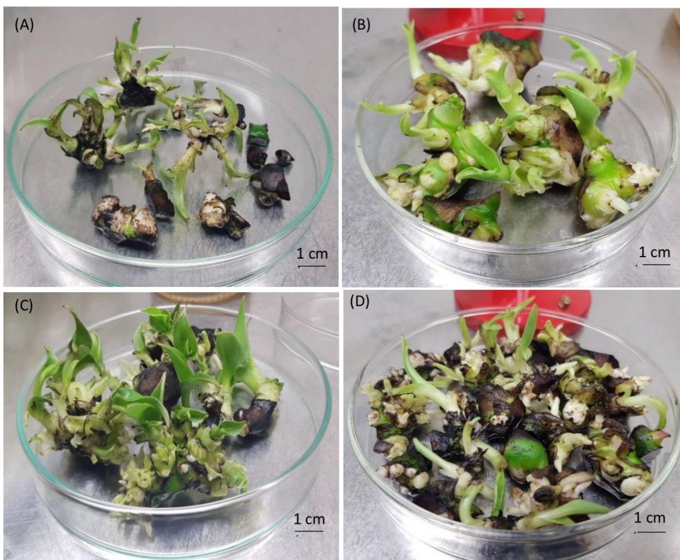

Figure 5. The morphology and condition of *Musa auminata* cv. Berangan plantlets in a Temporary Immersion Bioreactor (TIB) system, RITA ® during the subculture stage, with the initial number of shoots observed (A) 8 shoots (B) 15 shoots (C) 20 shoots and (D) 25 shoots.

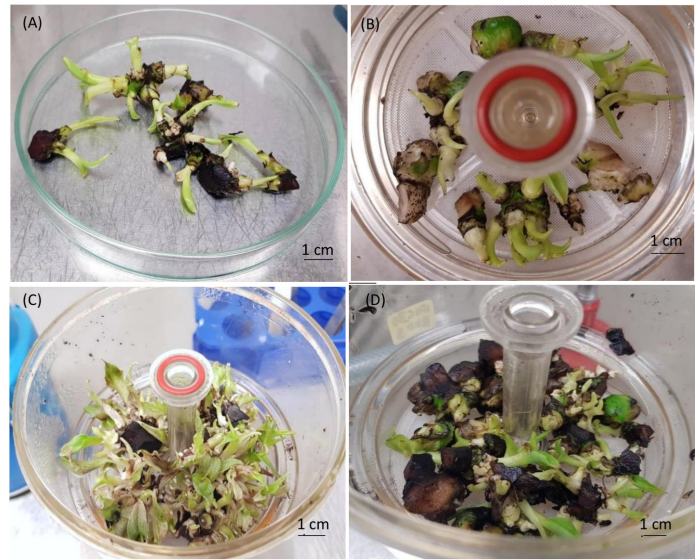

Figure 6. The morphology and condition of *Musa paradisiaca* cv. Tanduk plantlets in a Temporary Immersion Bioreactor (TIB) system, RITA ® during the subculture stage, with the initial number of shoots observed (A) 8 shoots (B) 15 shoots (C) 20 shoots and (D) 25 shoots.

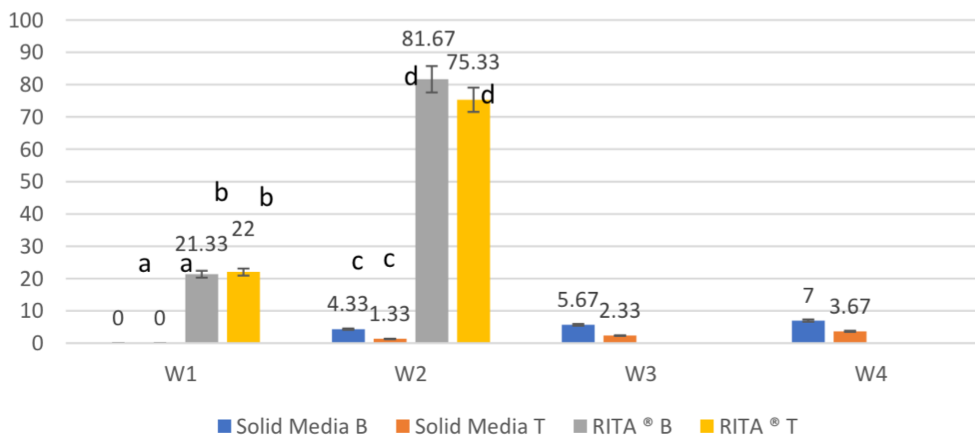

Figure 7. The above figure compares the number of shoots in Solid Media (control) and in Temporary Immersion Bioreactor (RITA ®) for *Musa* sp cv. Berangan (B) and *Musa* sp. Tanduk (T) across four weeks: W1, W2, W3 and W4. Each group's error bar letters indicate that there is no significant difference within the same media type, but significant differences exist between different media types. In week 1 and week 2, Solid Media B and Solid Media T is significantly different from RITA ® B and RITA ® T, respectively. In W3 and W4, the shoots for RITA ® B and RITA ® T has been harvested, while shoots in solid media B and solid media T can only be harvested in W4. Bars with different notations are statistically significant different between groups.

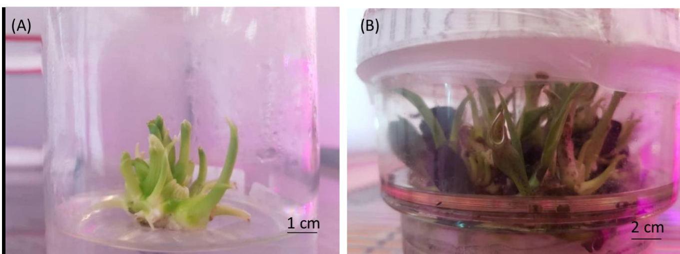

Figure 8. *Musa acuminata* cv. Berangan in (A) Controlled agar MS media and (B) Temporary Immersion Bioreactor (TIB) method with the utilization of liquid media.

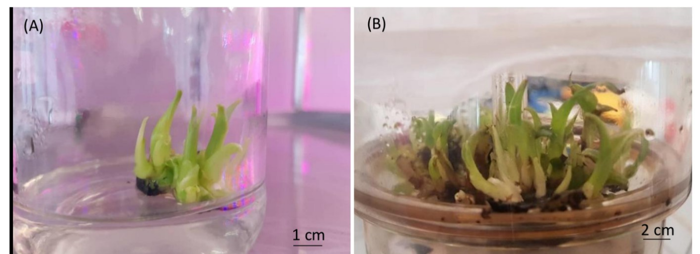

Figure 9. *Musa paradisiaca* cv. Tanduk. in (A) Controlled agar MS media and (B) Temporary Immersion Bioreactor (TIB) method with the utilization of liquid media.

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The rooted plantlets were moved to several stages of growth conditions in order to acclimate them to the *ex vitro* environment. The plants then exhibited rapid growth during this acclimatization period (Figure 10). Plantlets grown with the RITA® technique are also hardier and better acclimate, resulting in higher yields of hardened-off plants.

Figure 10. (A) Plantlets washed to prepare for the acclimatization stage, (B) acclimatization of plantlets in nursery. (C) Plantlets that are ready to be transferred in the soil and (D)Transferred plantlets of both varieties, *Musa acuminata* cv. Berangan and *Musa paradisiaca* cv. Tanduk.

This rapid growth and propagation period is parallel to the findings reported by Alister *et al*. (2005) report on RITA ® application, multiplication cycles last 14 to 18 days, compared to 25–28 days in a semi-solid system. Plants in the RITA® system have less callus on their leaves and stem bases, and cold-tolerant plants have better rooting competency than plants in the semi-solid system. *Ex vitro* rooting of RITA® generated plantlets is significantly better than semi-solid medium plants.

In bioreactor systems, plant tissues are propagated while completely submerged in a liquid growth medium. However, this condition can lead to physiological disorders and material loss in certain plant species, due to the low oxygen content and high water retention capacity causing asphyxiation and hyperhydricity of the plant tissues (Kevers *et al*., 2004; Aziz *et al*., 2021). However, The TIB method, which floods plant tissues with a liquid culture medium at intermittent intervals instead of continuous submersion, helps overcome the physiological issues of low oxygen and high water retention caused by full bioreactor immersion, thereby improving the efficiency of plant propagation in these systems (Welander *et al*., 2014; Mosqueda *et al*., 2017; Aziz *et al*., 2021). The temporary immersion system (TIS) bioreactor has emerged as an efficient and cost-effective method for the *in vitro* propagation of many types of plant species (Hwang *et al*., 2022). Temporary Immersion Systems (TIS) are considered among the best options for commercial-scale micropropagation. While successes have been demonstrated with TIS, it remains necessary to identify and establish the most suitable system configurations for specific plant species and applications (Bello-Bello *et al*., 2019).

4. Conclusion

The application of the temporary immersion bioreactor (TIB) system shows promising results for commercial-scale clonal banana production. The study protocol presented in this research offers a more efficient and cost-effective approach to meet the industry's demand aligns with sustainable practices and supports the strategic planning of ABI and NIBM in ensuring food security and sustainable supply in the market-driven crop industry. The findings conclude that the TIB technology can be an effective strategy for rapid *in vitro* propagation methods for both banana varieties. It is important to use this study report as a reference for further developing efficient micropropagation protocols for all important crop plants, especially in Malaysia. This wide application of this method would help in reviving the agriculture sector as well as contribute to the solutions for food security issues. Future research should focus on further optimizing the TIB system considering their impact on plant growth and phenolic content. Additionally, studies on the long-term performance and stability of clonal banana plants produced using the TIB system would provide valuable insights for commercial implementation.

Conflict of interest

The authors declare no conflict of interest.

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