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Temporary Immersion Culture: A Potential In Vitro Culture Method for the Clonal Propagation of Coconut

Zhihua Mu [1,2,*], Zhiying Li [3], Amirhossein Bazrafshan [1], Sundaravelpandian Kalaipandian [1,4,5], Eveline Yee Yan Kong [1,4], Julianne Biddle [1], Naga Prafulla Chandrika Nulu [1,*] and Steve Adkins [1,4]

1 School of Agriculture and Food Sustainability, The University of Queensland, Gatton 4343, Australia; a.bazrafshan@uq.edu.au (A.B.); s.kalaipandian@uq.edu.au (S.K.); e.kong@uq.edu.au (E.Y.Y.K.); julianne.biddle@uq.edu.au (J.B.); s.adkins@uq.edu.au (S.A.)
2 Hainan Seed Industry Laboratory, 7 Yumin Road, Yazhou, Sanya 572025, China
3 National Key Laboratory for Tropical Crop Breeding/Coconut Research Institute, Chinese Academy of Tropical Agricultural Sciences, Wenchang 571339, China; elaeis@catas.cn
4 Centre for Horticultural Science, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Indooroopilly 4068, Australia
5 Department of Bioengineering, Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha School of Engineering, Chennai 602105, Tamil Nadu, India
* Correspondence: 184457@hainanu.edu.cn (Z.M.); p.nulu@uq.edu.au (N.P.C.N.)

Abstract: As one of the most important members of the palm family, coconut (*Cocos nucifera* L.) currently faces a substantial gap between demand and production. Current plantings of this crop are aging, and these traditional varieties are susceptible to several devastating pests and diseases. Consequently, there is an urgent need to replant and expand coconut lands with new, genetically superior varieties. Such replanting cannot be met through the conventional method of seed nut planting, and tissue culture has emerged as a likely solution to address this problem. However, due to certain technical barriers, elevated costs, and a need for improved efficiency, the development of automated and highly efficient tissue culture techniques is yet to be developed. The present research explores the potential of an in vitro temporary immersion system (TIS) to improve the production of somatic embryogenic callus for plantlet regeneration. Results indicated that, in comparison to the conventional agar-based method used to produce coconut somatic embryogenic callus, the TIS method significantly enhanced embryogenic callus production. The optimal biomass of callus for inoculating the TIS was determined to be 0.2 g in each 900 mL vessel and the most favorable embryogenic developmental stage for employing TIS was the globular stage of embryo development. The most effective immersion time to give the highest yield of embryogenic callus was 5 min every 6 h. This foundational research demonstrates that a TIS step is likely to be important to rapidly produce, on a large scale, coconut plantlets to meet the escalating demand for materials for the replanting of coconut lands.

Keywords: somatic embryogenesis; bioreactor; somatic embryogenic callus; immersion time

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1. Introduction

The coconut palm (*Cocos nucifera* L.) is one of the most important palm species worldwide, being the sole member of the genus *Cocos* and a prominent member of the Arecaceae family. The palm is commonly known as the "Tree of Life" as every part of the palm is useful, and it is grown in over 90 countries [1,2]. Its diverse range of products, varying from daily necessities to luxurious indulgences, confirms its economic and cultural importance [3]. However, despite its expanding global markets and its contribution towards the economy of many Asia–Pacific countries [4], coconut production is declining due to palm aging, pests and disease, and the impact of natural disasters such as typhoons [5].

Replanting by traditional methods using seed nuts is inefficient due to the long breeding cycle of the palm required to produce them and the low rate of seed nut production. Thus, the industry needs a new innovative technique to surpass this traditional approach [6,7]. In the last two decades, coconut tissue culture technologies have advanced, enabling germplasm multiplication for research purposes in laboratories worldwide, but the large commercial-scale production of plantlets through tissue culture remains a challenge [8]. Somatic embryogenesis (SE) presents a promising avenue for the large-scale production of coconut plantlets, leveraging the inherent totipotency of plant cells to regenerate somatic embryos and ultimately produce large numbers of plantlets [9]. A two-step approach that maximizes somatic embryogenic callus and plantlet production, followed by successful acclimatization of plantlets ready for soil planting, appears to be promising [2]. Nevertheless, the current coconut tissue culture protocols are accompanied by several technical constraints. Firstly, the slow growth rates of callus cultures (taking up to 6 months) and the limited rate of somatic embryo formation have hampered the economic feasibility of the technique [9]. Finally, certain medium additives (e.g., activated charcoal) commonly used in coconut tissue culture pose challenges for the optimization of the concentration of other medium components [10,11]. In general, coconut SE employs a gelled medium system that requires frequent subculturing, which is costly and hinders attempts to automate the procedure [12]. Thus, it is crucial to develop a robust somatic embryogenic protocol capable of overcoming these current limitations and delivering a vast number of plantlets [11]. The limitations posed by a gelled medium system could be solved by the application of a temporary immersion system (TIS) [13]. The TIS technology involves periodically immersing somatic embryogenic precursor tissues in a liquid medium within a dedicated bioreactor system, followed by re-exposure to an air phase [14,15].

The use of TIS technology has been shown to be a promising approach for enhancing SE and subsequent plantlet regeneration in several other palm species including date palm (*Phoenix dactylifera* L.), oil palm (*Elaeis guineensis* L.), peach palm (*Bactris gasipaes* Kunth.) and sago palm (*Cycas revoluta* Thunb.). Othmani (2009) achieved efficient SE of date palm using a TIS with a medium immersion period of 5 min in each 8-h period [16]. Fki (2011) demonstrated that TIS improves date palm culture nutrient uptake and respiration when using an immersion time of 15 min in every 24-h period. Both treatments resulted in a higher shoot multiplication rate [17]. Ibraheem (2013) applied a TIS to date palm culture, improving acclimatization success with no abnormal plantlets being produced [18]. Marbun (2015) and Gomes (2016) successfully used a TIS for oil palm resulting in an increased number of somatic embryos and shoots [19,20]. Steinmacher et al. (2011) [21] and Heringer et al. (2014) [22] developed a TIS protocol for promoting somatic embryo development and plantlet production in peach palm. Their approach utilized nylon mesh baskets for holding the somatic embryos and an immersion period of 3 min every 6 h. Sumaryono and Kasi (2009) employed a TIS for sago palm SE and achieved a 40% survival rate for acclimatized in vitro plantlets [23]. The application of TIS technology has not been explored for coconut. The findings from the various palm species to date provide valuable insights for the application of TIS to improve coconut SE.

In this study, we report for the first time the employment and standardization of a TIS approach for coconut SE, to enhance both the coconut somatic embryogenic callus multiplication and the formation of plantlets. We examine the most appropriate developmental stage of the somatic embryogenic process to apply the TIS, to identify the optimal inoculum biomass for the TIS system and to ascertain the most effective duration for the liquid and air immersion phases. Experiments were conducted with somatic embryogenic tissues as these were most responsive in other experiments on other palm species. The liquid/air immersion phases, the stage for application of TIS, and the biological biomass of somatic embryogenic callus were tested as they were found to be the most crucial factors in determining culture success for other palm species.

2. Materials and Methods

2.1. Plant Materials and Media Composition

In experiment 1, 100 dehusked coconut fruits, sourced from an overseas supplier, were purchased from a local supermarket. Each fruit was split open with a machete and the endosperm plug containing the embryo was extracted using a sterile cork borer. Twenty plugs were placed into a glass container and carefully washed with a mild water–detergent solution followed by washing for 5 min under tap water. In a laminar airflow (LAF) hood, embryos were isolated, and their surface was sterilized with sodium hypochlorite (2%; *v*/*v*, water solution) for 5 min, followed by a triple wash in sterile water. The embryos were then placed individually into 30 mL culture vessels containing 5 mL of a solidified germination medium (GM; consisting of Y3 macro- and micro-nutrients [24], 30 g L^{-1} sucrose [Sigma-Aldrich, St. Louis, MO, USA], 3 g L^{-1} Gelzan [Sigma-Aldrich], and 2.5 g L^{-1} activated charcoal [Sigma-Aldrich] [1,2]) for 15 to 25 days under darkened conditions at 27 ± 2 °C. When the plumules were ca. 2 mm long they were dissected, and in lots of five, they were placed on a callus induction medium (CIM) consisting of the GM medium supplemented with 600 µM 2,4-dichlorophenoxyacetic acid (2,4-D [2]) in 9 cm diameter plastic Petri dishes. Incubation was continued in the dark at 27 ± 2 °C and after two 6-week subcultures onto media of the same constitution, the callus reached the globular somatic embryo stage of development and was ready for experimentation. Further 2 monthly subcultures were required to obtain cultures at the germinating and coleoptilar stages of embryos development. For the subculture, the same basal medium was used with a lower 2,4-D concentration (6 µM). After weighing on a decontaminated analytical balance placed in the LAF hood, the callus at the various stages of development was placed into the TIS containing a somatic embryogenic maturation medium (SEMM) consisting of GM medium without Gelzan but with 5 µM 6-benzylaminopurine (BAP).

In experiments 2 and 3, Malayan Yellow Dwarf fruit were harvested from the National Tropical Palm Germplasm Nursery of China and the Coconut Grand Park in Wenchang, Hainan, China. From these fruit endosperm plugs, embryos were extracted and treated as described in experiment 1 to produce somatic embryogenic callus at the globular stage of development.

2.2. Experimental System

The TIS used was a RITA® system developed by the Centre de coopération internationale en recherche agronomique pour le développement (CIRAD), Montpelier, France (Figure 1A). Nine 1 L RITA® vessels and an aquarium pump (generic aquarium product; 1 W) comprised the TIS set up, following the design by Teisson [25]. Within the RITA® system, the chambers were interconnected using silicone hoses linked to the aquarium pump and connected to an electronic timer. Sterilized air, passing through a 0.22 µm bacterial filter (Millipak, Burlington, VT, USA), was provided to the flasks every 6 h. This action pumped the liquid culture medium into the top portion of the vessels, immersing the callus for 1 min (unless otherwise described) until the aquarium pump was turned off, allowing the medium to return to the bottom of the vessel. For all TIS experiments, a light intensity of ca. 50 µmol m^{-2} s^{-1} (cool-white light from light-emitting diodes; LED), a 16/8-h (day/night) photoperiod, and a temperature of 27 ± 2 °C were used. The liquid medium used in this study was derived from the CIM medium, which consisted of Y3 macro- and micro-nutrients [24], 30 g L^{-1} sucrose [Sigma-Aldrich], and 6 µM 2,4-D.

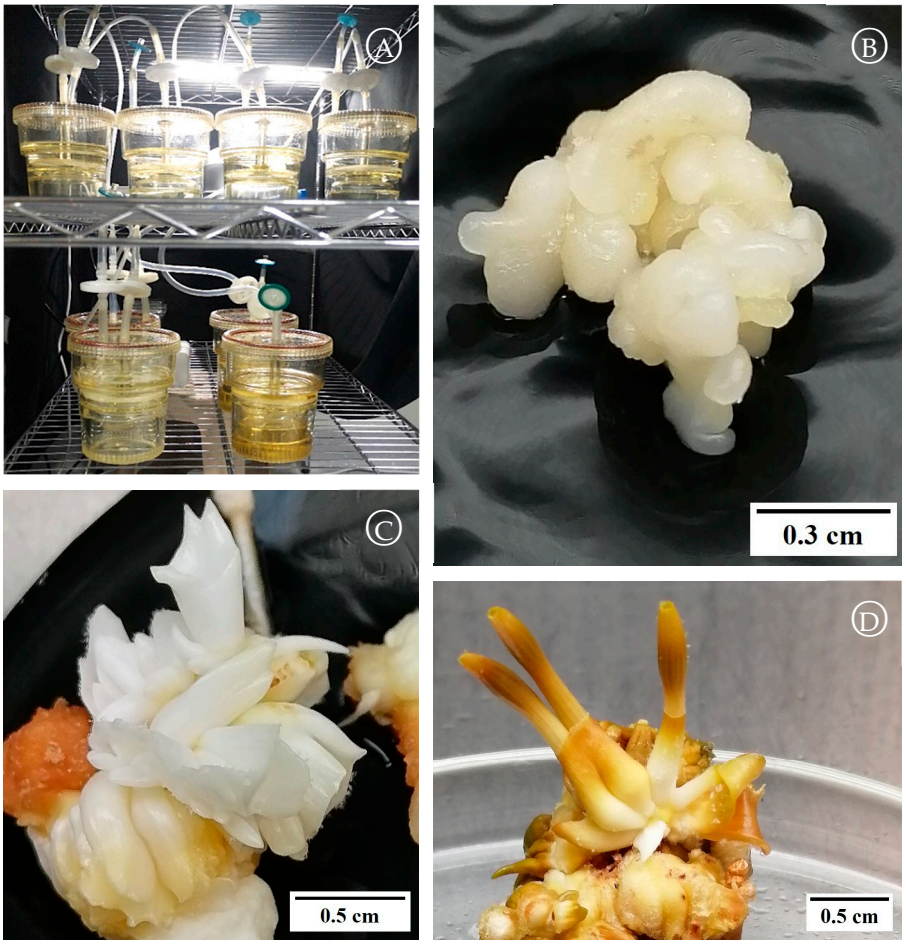

Figure 1. The temporary immersion system installed on a shelf in an incubator providing conditions of 27 ± 2 °C and 50 µmol m^{-2} s^{-1} cool-white LED light (**A**). The three categories of somatic embryogenic material employed in the TIS investigation including callus exhibiting somatic embryogenic structures at the globular stage of development (**B**), mature somatic embryos in the early stages of germination (**C**), and clusters of germinated somatic embryos (**D**).

2.3. Experiment 1: Identification of the Somatic Embryo Developmental Stage for TIS

This study was conducted at the University of Queensland (UQ) Gatton Campus, Australia. Three types of explants were employed in this study: control and stage 1 (S1, Figure 1B) consisting of clusters of somatic embryogenic callus at the globular stage of development (3 months after initiation), stage 2 (S2, Figure 1C) consisting of clusters of germinating somatic embryos (8 months after initiation), and stage 3 (S3, Figure 1D) consisting of clusters of coleoptilar-stage somatic embryos (10 months after initiation). Each treatment involved 0.5 g fresh weight of callus and was run for 6 weeks under constant conditions (27 ± 2 °C; 50 µmol m^{-2} s^{-1} cool-white LED light). After 6 weeks, the performance of the cultures was evaluated by determining the culture fresh weight index (FWI), the number of somatic embryos produced (SEN), and an estimate of culture quality as assessed by a survival rate (SR). For the control treatment, 0.5 g of SE callus at the globular embryo stage of development (6 months after initiation) was cultured on an SEMM solid medium (SEMM with 3 g L-1 Gelzan) in 30 mL culture tubes. These cultures were run for 6 weeks before being assessed for FWI, SEN and SR. The experiment was a randomized design, and three replicates (5 explants in each) were used for each treatment, including the control.

2.4. Experiment 2: Identification of the Inoculation Biomass for the TIS

This study was conducted at the Coconut Research Institute, Chinese Academy of Tropical Agricultural Sciences (CATAS), Hainan, China. Somatic embryogenic callus clusters were generated as described in Section 2.1. Freshly formed somatic embryogenic callus at the globular stage of development (6 months after initiation) and at four different biological densities (viz. 2.0, 1.0, 0.5, and 0.2 g of embryogenic callus per explant) were inoculated into 12 TIS vessels. The TIS treatment was applied for 6 weeks to all cultures under constant conditions (27 ± 2 °C; 50 µmol m^{-2} s^{-1} cool-white LED light). The performance of the cultures was evaluated by determining the FWI, SEN and SR. The experiment was a randomized design, with three replicates (5 explants in each) of treatments at the globular stage of embryo development.

2.5. Experiment 3: Identification of the Immersion Duration for the TIS

This study was conducted at CATAS. Freshly formed somatic embryogenic callus (0.2 g) at the globular stage of development (6 months after initiation), was inoculated into 12 TIS vessels. In this experiment, two immersion times of 3 or 5 min were applied at intervals of 3, 6, 12, and 24 h. The TIS treatment was applied for 6 weeks to all TIS under constant conditions (27 ± 2 °C; 50 µmol m^{-2} s^{-1} cool-white LED light). The performance of the cultures was evaluated by determining the FWI, SEN, SR and vitrification rate (VR). The experiment was a randomized design, with three replicates (5 explants in each) for each of the two immersion times and four periods and using embryos at the globular stage of development.

2.6. Measurements and Statistical Analysis

The FWI was obtained by dividing the fresh weight determined at the end of the experiment by the fresh weight determined at the start of the experiment. The weighing process was conducted in the LAF hood using a decontaminated four decimal place analytical balance. The culture material was removed from the TIS vessels, washed with sterile water and placed on filter paper to air dry before being weighed. The SEN was the difference between the number of SEs found at the end of the experiment and the number of SEs present at the beginning of the experiment and was determined under a decontaminated microscope (60× magnification). The SR was determined by calculating the percentage of each culture not showing signs of necrosis, a method previously described by Mu (2024) [2]. In experiment 3, the rate of vitrification was determined by calculating the percentage of each callus showing signs of vitrification. All datasets were statistically analyzed using an Analysis of Variance (ANOVA), with $p < 0.05$ considered as the threshold for significant difference. The mean comparisons were calculated based on the Student's *t*-test.

3. Results

3.1. The Impact of the Stage of the Explants on Their Growth in TIS

To determine the most appropriate stage of somatic embryo development to use in the TIS, a study was conducted with somatic embryogenic callus at three different stages of development (control, S1, S2 and S3) with each treatment maintained for 6 weeks and compared by FWI, SEN, and SR (Figure 2). Somatic embryogenic callus at all three stages of development showed a significant increase in FWI when compared to the control (C) treatment, using somatic embryogenic callus at the globular stage of development but cultured on a gelled medium (Figure 2). Within the TIS treatments, the somatic embryogenic callus at the globular stage of development (S1) exhibited a significantly greater gain (49%) in fresh weight when compared to the other two stages of development. Somatic embryogenic callus at the S1 stage of development showed a significantly higher SEN when compared to C (Figure 2B). No significant differences were observed in the SR for all somatic embryogenic callus development stages and when compared to the gelled medium control (Figure 2C).

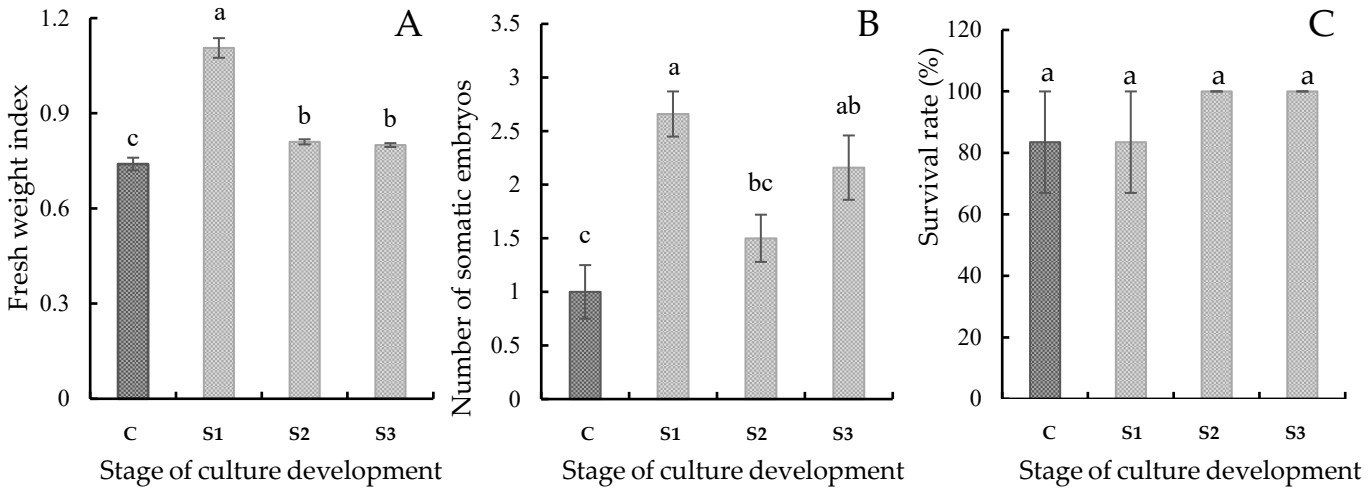

Figure 2. A comparison of somatic embryogenic callus performance when introduced into the temporary immersion system at one of three different developmental stages (S1: consisting of clusters of somatic embryogenic callus at the globular stage of development; S2: consisting of clusters of germinating somatic embryos; S3: consisting of clusters of coleoptilar-stage SEs; and C: a control, consisting of somatic embryogenic callus at the globular embryo stage of development on a solid medium). This comparison was based on three metrics determined after 6 weeks of incubation: (**A**) the fresh weight gain of the cultured materials, (**B**) the increase in the number of somatic structures generated, and (**C**) the survival rate as assessed by the percentage of non-necrotic tissue per culture. Bars represent two standard errors of the mean. Significant differences within each dataset are denoted by different letters positioned above the bars.

3.2. The Impact of Culture Biomass on Plant Growth in TIS

To determine the most appropriate biomass of somatic embryogenic callus to apply in the TIS, a study was conducted with somatic embryogenic callus inoculated at four biomass levels for 6 weeks and compared by FWI, SEN and SR (Figure 3). Within the TIS treatments, the somatic embryogenic callus at 0.2 g and 0.5 g inoculation (T4, T3) exhibited a significantly greater gain in FWI when compared to the other two inoculation densities (Figure 3A). T3 and T4 inoculation biomass also showed a significantly higher SEN when compared to T1 and T2 (Figure 3B). The T4 (0.2 g) inoculation produced a significantly higher SEN than all the other treatments (Figure 3B). T4 also gave a significantly higher SR than C, indicating that this inoculated biomass showed little necrosis (Figure 3C).

3.3. The Impact of Immersion Time on Somatic Embryogenic Callus in TIS

To determine the most appropriate length of time to immerse the embryogenic callus in nutrient medium in the TIS, a study was conducted using callus at the globular stage of development and inoculated at a biomass of 0.2 g for 6 weeks, then assessing its performance by comparing FWI, SEN and SR (Figure 3). It was observed that the 3- and 5-min immersion times, applied every 3 h, and the 5-min immersion time, applied every 3 or 6 h were the best treatments, providing enhanced FWI compared to all other treatments. Whereas 5 min/6 h was similar to 5 min/3 h (Figure 4A). The 3- and 5-min immersion times, applied every 6 h, increased the SEN, compared to the 3- and 5-min immersion times applied every 3 h (Figure 4B). Both of these treatments also produced a high-quality callus (SR of ca. 90%), compared to the 24 h treatment which maintained a moderately low VR of ca. 60%.

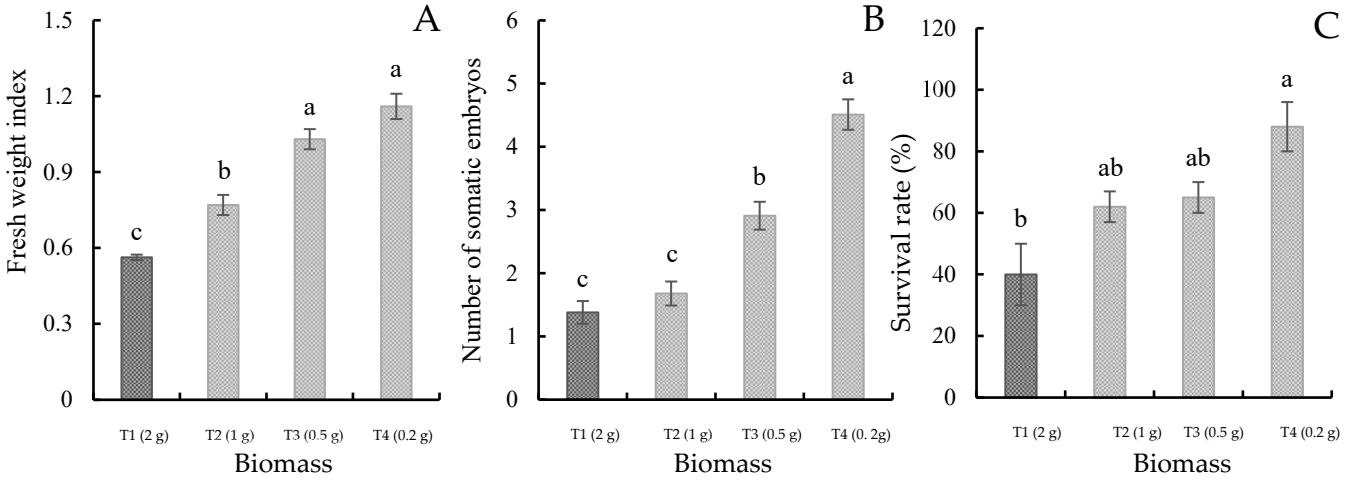

Figure 3. A comparison of somatic embryogenic callus performance when introduced into the temporary immersion system at one of four different biomasses (T1: biomass of 2 g; T2: a biomass of 1 g; T3: a biomass of 0.5 g; and T4: a biomass of 0.2 g). This comparison was based on three metrics determined after 6 weeks of incubation: (**A**) the fresh weight gain of the cultured materials, (**B**) the increase in the number of somatic structures generated, and (**C**) the survival rate as assessed by the percentage of non-necrotic tissue per culture. Bars represent two standard errors of the mean. Significant differences within each dataset are denoted by different letters positioned above the bars.

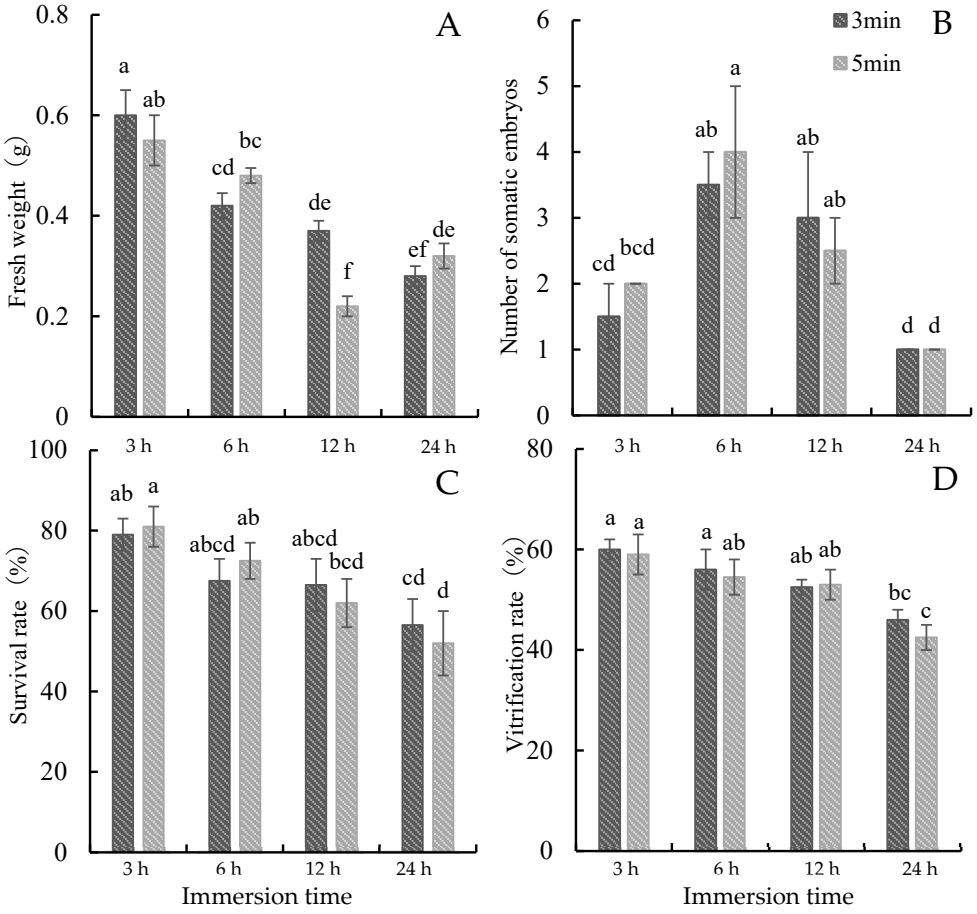

Figure 4. A comparison of somatic embryogenic callus performance at the globular stage of development and introduced into the RITA temporary immersion system at a biomass of 0.2 g. Two nutrient immersion times (3 or 5 min), applied at one of four different time intervals (3, 6, 12 or 24 h) were used.

This comparison was based on four features determined after 6 weeks of incubation: (**A**) the fresh weight of the cultured materials, (**B**) the number of somatic structures generated, (**C**) the percentage of non-necrotic tissue per culture, and (**D**) the percentage of culture vitrification. Bars represent two standard errors of the mean. Significant differences within each dataset are denoted by different letters positioned above the bars.

4. Discussion

The results have shown that the TIS offers several significant advantages over the traditional solid medium system for supporting coconut SE, particularly as it promotes the FWI and the SEN (Figure 1). Other past studies have shown TIS to be superior to other culture approaches as it enhances nutrient and oxygen availability [26], prevents tissue vitrification [27], improves tissue growth and multiplication rates [28] and stimulates overall tissue growth and development [2]. Further studies have also demonstrated that TIS promotes clonal propagation in several tropical species and palms, including pineapple (*Ananas comosus* (L.) Merr.) [29,30], plantain (*Musa* spp.) [31], papaya (*Carica papaya* L.) [32], sugarcane (*Saccharum officinarum* L.) [33,34], dragon fruit (*Hylocereus undatus* (Haw.) Britton & Rose) [35], potato (*Solanum tuberosum* L.) [36], chestnut (*Castanea dentata* (Marsh.) Borkh.) [37], sago palm [23], peach palm [21,22], oil palm [19,20], and date palm (*Phoenix dactylifera* L.) [16]. It is thought that the innovative design of the TIS, which provides a nutrient-rich culture phase followed by an oxygen-rich phase, provides superior culture growth and development, and lower necrosis and vitrification rates. Vitrification, or hyperhydricity, can be affected by the duration and frequency of immersion of the cultures in a TIS. Studies have shown that adjusting the parameters of immersion can control or even prevent hyperhydricity, which is important for improving plantlet production and quality during micropropagation. Further investigation into the vitrification response and the immersion times in TIS deserves further investigation.

In a study examining which stage of culture development was best for introduction into the TIS, results indicated that somatic embryogenic callus at the globular stage of development responded best, providing better FWI growth and generating a greater SEN than somatic embryogenic callus introduced into the TIS at the germinating or coleoptilar stage of development (Figure 1). Using globular-stage embryogenic callus to inoculate TIS offers several advantages. It significantly enhances the consistency and synchronization of somatic embryo development, leading to higher-quality embryos with reduced abnormalities. TIS could provide a better nutrient absorption, which boosts the proliferation rate of globular-stage embryogenic callus and supports the robust growth of embryos. Many previous studies have also indicated that the introduction of callus at the early stages of somatic embryogenesis respond best to TIS [13–16,20–22].

In a study examining which biomass was best for inoculation into the TIS, results indicated that callus at the globular stage of development and inoculated at a rate of 0.2 g responded best, providing better FWI growth and generating a greater SEN than globular stage somatic embryogenic callus introduced into the TIS at higher inoculation rates (Figure 2). Previous research on other palm species [18–20,22] has also demonstrated that having excessive amounts of plant material in the TIS vessels can create a competitive environment, leading to limited nutrient and oxygen availability. An overly crowded TIS vessel can also result in surface wounds to the tissues, which are detrimental to the formation of somatic embryos and can lead to tissue necrosis, as observed in sugarcane cultures [38]. Additionally, high biological densities in the TIS can trigger the release of phenolic compounds, leading to tissue necrosis [39]. To address the challenges caused by high biological densities, some studies have introduced substances like ascorbic acid or citric acid into the medium to help reduce the rate of necrosis [38]. Shortening the subculture cycle and physically removing necrotic tissues have also proven to be effective in reducing overall explant necrosis when inoculated at high biological densities [16]. The application of a modified-SEMM liquid medium or shortening the time between subcultures may allow for a higher biomass to be used in the TIS than that observed in the present study. One

further possibility is to use coconut water as an important component of the SEMM liquid medium as has been suggested by another study [40].

In a study examining which immersion duration and frequency were best for culture growth and development in the TIS, results indicated that callus at the globular stage of development and inoculated at a rate of 0.2 g responded best when subjected to 5-min immersion time every 6 h. This treatment gave a good FWI and a superior SEN (Figure 3). This observation aligns with past studies where clusters of secondary somatic embryos, immersed for 3 min every 6 h, were used on peach palm [21], and about 0.8–1.0 g of somatic embryos at the torpedo-shape stage, immersed for 1 min every 6 h, on oil palm [20]. Minor differences in immersion duration (e.g., 3 as compared to 5 min) are unlikely to have a significant impact on TIS success. However, for date palm, the interval between immersion phases was found to improve the growth when reduced from 24 h to 3 h [22], and a similar finding was found for oil palm [19]. Notably, in the present study, the longest immersion interval suggests that plants immersed for 24 h resulted in the lowest FWI and SEN (Figure 3C,D). Vitrification is a physiological disorder caused by water accumulation in the callus and is known to occur when immersion intervals are prolonged [41]. However, the shorter immersion frequencies in the TIS can be used to mitigate the problem of vitrification, as stated in other crops [42].

The TIS's potential in coconut SE lies in its ability to optimize culture access to nutrient and oxygen supply, enhancing the growth and quality of the somatic embryos produced. Future directions for using TIS for coconut SE include refining immersion times and modifying the SEMM nutrient formulation. Automation should follow, and it will be important to streamline processes, increase efficiency, and enable scalable production to meet global demand for planting materials [43].

5. Conclusions

This study explored TIS as a method to enhance the production of somatic embryogenic callus and plantlets in coconut for the first time. In comparison to the standard solid-medium-based SE method typically used for coconut, TIS demonstrated advantages in achieving a higher FWI and an increased SEN. The optimal developmental stage for implementing TIS was found to be the globular stage of embryo development. Additionally, the study determined that using a biomass of 0.2 g in the TIS vessel gave the best yield. Furthermore, an immersion period of 5 min, applied every 6 h, was the optimal treatment for achieving the highest SEN. Future studies should focus on the steps of acclimatization to get the small plantlets from in vitro to ex vitro soil conditions. At the same time, the commercial sector and research institutes should start to scale up the method into a TIS bioreactor system to produce plantlets on a commercial scale.

Author Contributions: Conceptualization, S.A. and Z.M.; performed experiments, Z.M. and Z.L.; data collection, A.B and Z.L.; writing—original draft preparation, N.P.C.N., Z.M. and E.Y.Y.K.; writing—review and editing, Z.M., N.P.C.N., S.K., E.Y.Y.K., J.B., S.A., A.B. and Z.L.; research supervision, S.K., S.A. and J.B.; project administration, S.K., S.A. and J.B. All authors have read and agreed to the published version of the manuscript.

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