Article

An Investigation into Using Temporary Immersion Bioreactors to Micropropagate *Moringa oleifera* Lam. Callus, Roots, and Shoots

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Abstract: *Moringa oleifera* Lam., a tree naturally grown in the tropics, is becoming increasingly popular as an industrial crop due to its multitude of useful attributes. Therefore, this study tested the effect of temporary immersion system (TIS) bioreactors for mass micropropagation of *Moringa oleifera* Lam. callus, roots, and shoots. TIS are tissue culture systems that make use of timers to periodically immerse and drain plant cultures in a liquid nutrient medium instead of using solidified media. In initial studies, *Moringa oleifera* seeds were germinated in vitro, and in vitro seedling leaflets were then used as explant material for callus production on the pre-culturing media. Two experiments were conducted to improve the protocol for TIS bioreactor production. The first experiment investigated the effect of 6-benzylaminopurine (BA) and kinetin, whereas experiment 2 was conducted to improve shooting production. For the first experiment, leaf material was cultured onto a solidified medium consisting of half-strength Murashige and Skoog (MS) basal salts and 0.5 ppm 1-naphthaleneacetic acid (NAA) to initiate callus production before splitting it between solidified media and bioreactors for shooting. The shooting media consisted of full-strength MS basal salts and different treatments of kinetin and BA. A significant increase in callus production was observed with the use of TIS bioreactors, compared to solidified media, whereas root production had a highly significant interaction effect between the media and the cytokinin treatments. With shoot proliferation in mind, experiment 2 was performed, where microcuttings from in vitro-grown seedlings were excised and cultured onto a solidified MS medium, consisting of a control (0 ppm BA) and two different concentrations of 6-benzylaminopurine (BA) (0.1 ppm BA and 0.2 ppm BA) in the pre-culturing phase. Microcuttings were again excised after two weeks and transferred to the shooting media containing 0.1 ppm BA in TIS bioreactors and semi-solidified medium. Results showed TIS bioreactors to be effective in increasing both the amount and length of shoots produced. Shoot and callus fresh weights were also higher in explants cultured in TIS bioreactors. The results of this study also suggest *M. oleifera* sensitivities to plant growth regulators (PGRs). In conclusion, this study successfully produced callus, roots, and shoots in both the solidified media and TIS bioreactors, emphasizing the prospect of using TIS bioreactors for mass micropropagation of *M. oleifera* callus, roots, and shoots.

Keywords: microcuttings; moringa; temporary immersion bioreactors; in vitro shooting; plant growth regulators

1. Introduction

Ancient communities were well-versed in the value of the *Moringa* species as a food and medicine source [1]. The study of moringa tissue culture began a few decades ago; some of the primary focuses included the use of tissue culture as a solution for food scarcity as well as for the rescue of endangered *Moringa* species, such as *M. arborea*, *M. borziana*, *M. longituba*, *M. rivae*, *M. ruspoliana*, and *M. stenopetala* [2–4]. Since *M. oleifera* is not a

threatened species and is the most well-known and widely used moringa, it is commonly used in tissue culture studies as a model species. Tissue culture is a cost-effective way to increase plant production with minimal use of source material. Pest and pathogen contamination can also be controlled while maintaining genetic integrity.

Stephenson and Fahey [3] found in vitro development of *M. oleifera* shoots was significantly improved when immature embryos were cultured on a membrane raft with a liquid medium compared to those planted onto a semi-solidified medium. Stephenson and Fahey [3] attribute this to the improved success of the membrane raft system which ensured contact of the tissue culture material with the liquid medium, enabling better nutrient uptake as well as enhanced dilution of phenolic compounds and stress metabolites. They also suggested that the improved aeration is advantageous for the non-aerial portions of the young plants, which tend to be sensitive to waterlogging. This improved system, known as TIS bioreactors, also proved to offer a multitude of other benefits compared to conventional tissue culture systems.

Since conventional tissue culture makes use of high amounts of small containers and requires the plant cultures to be very carefully placed into the solidified medium, the handling requirement is high, and planting and washing is time consuming. TIS bioreactors, with their larger volume and the possibility to automate, make handling and planting easier, thus reducing input costs [5]. Liquid nutrient media provide more uniform culturing conditions [6], culture immersion increases the uptake of nutrients and growth regulators, and the gaseous phase improves aeration and reduces conditions such as hyperhydricity or asphyxia, which are sometimes caused by other liquid culture techniques. Alternating immersion and gaseous phases also allow the explants to grow healthier or faster [7] and acclimatize better to outdoor conditions [8]. Designs such as Plantform TIBs (accessed on 1 February 2019 www.plantform.se), designed to reduce the carrying of equipment, are easier to install, and also enable stacking, which reduces storage space [9,10]. Mordocco et al. [11] mentioned that producers may use up to 20% of a sugarcane crop to replant the following season, significantly reducing harvest. Tissue culture provides a solution by decreasing the amount of material needed to replant, thereby minimizing the cost of production. This notion was supported by a study by Escalona et al. [12] who reported a 20% reduction in the cost of pineapple production using TIS compared to conventional methods. Hybrid hazelnut performed better in TIS in terms of the number of shoots and nodes produced, length of shoots, leaf area, and chlorophyll formation when compared to a solidified medium [13].

Besides improving the workability and input costs of a tissue culture system, TIS bioreactors provide several advantages to the plants or tissues grown in this system. Tissue culture methods hold the potential for the propagation of medicinal plant material due to the production of uniform plant material every season. TIS holds even more value in this regard [9], considering that the design is aimed at the commercialization of tissue culture, as well as the fact that it may improve secondary metabolite production in medicinal plants such as antioxidants, which improves the health benefits of crops, especially when oxygen supply is sufficient [14].

The trials discussed in this article were performed to improve the protocol of previous trials carried out by the same authors [15]. The uses of TIS bioreactors are not only to improve callus, shoot, and root production, but also to improve the production of secondary metabolites and beneficial compounds [14]. This study therefore aimed at developing an efficient production protocol for *M. oleifera*, but also to provide a platform for investigating secondary metabolite production in *M. oleifera*, making use of TIS bioreactors in the future.

2. Materials and Methods

A previous study by Coetser et al. [15] provided a protocol for *M. oleifera* production in TIS; however, several challenges were experienced, resulting in the follow-up experiments of this study. In initial experiments [15], sterilization of seed and the growing of seedlings in vitro were described, from which leaf material was taken to produce callus tissue on a

pre-culturing medium before the callus was transferred to either a solidified medium or to a TIS medium for shooting. This methodology had to be refined due to the failure of the method developed by Jun-jie et al. [16], which involved the direct planting of leaf material onto a shooting medium consisting of BA, KT, and NAA [15].

2.1. Growth Room and In Vitro Conditions

The difference between TIS and solidified media was tested in the shooting phase, but in the pre-culturing phase, only solidified media were used. All cultures were raised in a culture room at the tissue culture laboratory on the Experimental Farm of the University of Pretoria. The culture room was supplied with cool, white light (with a luminous flux of 2850 lm) with a photoperiod of 12 h, and temperatures were set at $\pm 26\,°C$.

All nutrient media consisted of MS basal salts (either full-strength or half-strength), 30 g L^{-1} sucrose, and solidified media had 8 g L^{-1} agar and plant growth regulators, depending on the treatments. Media were adjusted to a pH of 5.8 before autoclaving at 121 °C and pressure of 1.2 kgf·cm^{-2} for 20 min. Plantform (www.plantform.se) temporary immersion system (TIS) bioreactors were compared with conventional semi-solidified media. The bioreactors each contained 500 mL nutrient media of the same constituents as the semi-solid media, except for the solidifying agent, agar.

2.2. Seed Preparation

M. oleifera PKM-1 seeds from India were dehisced and washed in 98% ethanol for 30 s, then washed for ten minutes in a 10% JIK solution (a household sodium hypochlorite) and then rinsed three times in sterilized water for ten minutes. Seeds were then placed and grown in vitro in Pyrex test tubes on an autoclaved solidified nutrient medium consisting of half-strength MS basal salts. Each Pyrex test tube contained 10 mL of this nutrient medium. These seedlings were grown for two weeks before they were used to make microcuttings as explants in the following stages, instead of using the leaflets as described by Coetser et al. [15].

2.3. Experiment 1
2.3.1. Pre-Culturing Phase

A pre-culturing medium was prepared before the leaf material was transplanted to the shooting media in either the conventional tissue culture system or TIS. The pre-culturing medium consisted of half-strength Murashige and Skoog (MS) [17] and 0.5 ppm 1-naphthaleneacetic acid (NAA). These leaflets were then left to grow for three weeks before they were cultured on the shooting medium.

2.3.2. Shooting Phase

During the shooting phase, the callused cultures were divided between solidified medium and temporary immersion bioreactors, as well as three plant growth regulator (PGR) treatments of BA: 0 mg L^{-1}, 0.5 mg L^{-1}, 1.0 mg L^{-1}; and two treatments of kinetin: 0.25 mg L^{-1}, 0.5 mg L^{-1}.

2.4. Experiment 2

In experiment 1, cultures that produced more callus material and roots were obtained. Root production was attributed to the use of auxins during the pre-culturing phase. Therefore, in experiment 2, the pre-culturing phase was adjusted by testing different levels of the cytokinin BA instead of auxins, while the BA levels in the shooting media were held constant. Additionally, microshoots as explant material were also used instead of leaf material.

2.4.1. Pre-Culturing Phase

Before the shooting phase, a pre-culturing step was introduced to allow the microcuttings to induce callus growth as well as to reduce the risk of contamination. This improved

survival rates within the bioreactors. Seedlings were cut below the first axillary buds, and apical buds and leaves were removed. The microcuttings were planted on a solidified medium with half-strength MS basal salts in test tubes, each containing 5 mL of nutrient medium for two weeks before transplanting to initiate shooting with different levels of BA. The different levels of BA were: 0 ppm (referred to as T0), 0.1 ppm (T0.1), and 0.2 ppm (T0.2). The purpose of the different levels of BA was to test the effect of the pre-culturing phase on shoot growth in later stages.

2.4.2. Shooting Phase

During the shooting phase, the microcuttings were transplanted to either a solidified medium treatment or to TIS liquid medium. The shooting medium consisted of full-strength MS basal salts and 0.1 ppm BA to encourage shoot growth. For the solidified treatment, shoots were planted individually into GA-7 vessels, each containing 40 mL shooting medium. The shoots planted in TIS were planted in groups of ten within one TIS vessel, containing 500 mL shooting medium each. The experiment was set up in a complete randomized design. Each treatment consisted of groups of ten cultures, repeated three times with each treatment consisting of 30 cultures. An ANOVA analysis and Tukey's studentized range test were conducted to determine the significance of the results.

3. Results and Discussion

In previous studies, the protocol by Jun-jie et al. [16] was tested to see whether TIS influenced the shoot production of moringa compared to the solidified medium. By using this protocol, it was found that leaf explants planted directly into the TIS bioreactors did not survive [15]. Low survival rates were also observed by Latawa et al. [13] who reported that nodal explants of hybrid hazelnut underwent necrosis after two weeks when planted directly into a liquid culture, though explants on solidified media had much better survival rates. Mordocco et al. [11] also found that sugarcane explants struggled to produce shoots when planted directly into TIS and that shooting was greatly improved by pre-culturing explants on a solidified medium for two weeks beforehand. Thus, a pre-culture phase is essential before transfer to TIS. This pre-culturing step is also useful to eliminate contaminated explants before transplanting to bioreactors since bioreactors house many cultures in the same vessel; therefore, the risk of losses due to contamination is high.

It was found that the pre-culturing phase, which only made use of auxins, prevented the cultures from producing shoots in the later stage, which only involved cytokinins. It was therefore pertinent to test the effect of different levels of cytokinins during a pre-culture phase on shoot production, without the use of auxins, as well as the use of microcuttings instead of leaf material as the explant source. Mathur et al. [18] noticed that *M. oleifera* responded quickly to auxins by producing roots at low levels, which corresponds to our results in the initial experiment. They managed to produce shoots through callus material with the use of cytokinins alone, which indicates that moringa may not require auxins during the shooting phases in tissue culture.

3.1. Experiment 1

The development of a new protocol which involved a pre-culturing phase using a solidified MS-based medium with added NAA before transplanting to TIS, resulted in survival of the material in both the conventional and the TIS bioreactors. The "pre-cultured" explants were used for the shooting phase which involved different plant hormone treatments of BA and kinetin (KT). The treatments of T0 (no added cytokinins), T1 (0.5 ppm BA), T2 (1.0 ppm BA), T3 (0.25 ppm KT), and T4 (0.5 ppm KT) were applied to the material in both the conventional tissue cultures and TIS. The results are presented in Figures 1 and 2 and in Table 1. It was expected that the pre-culturing step would allow callus formation, from which adventitious shoots could be induced with the use of cytokinins in the shooting phase. Unexpectedly, the cultures from the pre-cultured test tubes did not produce any shoot material during the shooting phase, but only produced more callus tissue as well as

roots. Photos from the results of different treatments can be found in Table 1. Analyses were still performed to determine the effect of TIS in comparison to solidified media on the material produced. An ANOVA analysis of the results indicated that there was a highly significant increase (at $p < 0.01$) in callus production with the use of TIS compared to solidified media (Figure 1), but that root production had a highly significant interaction effect between the media and the cytokinin treatments, as illustrated in Figure 2. Callus production increased with the use of TIS, though with the Tukey's test it was determined that only the treatments of T0, which contained no added cytokinins, and T4, which contained 0.5 ppm KT, showed a significant increase, while the other treatments were not necessarily significant (Figure 1) when compared to the conventional tissue culture system (solid). The difference between the cytokinin treatments with solidified media was not significant, but more substantial differences were achieved with TIS.
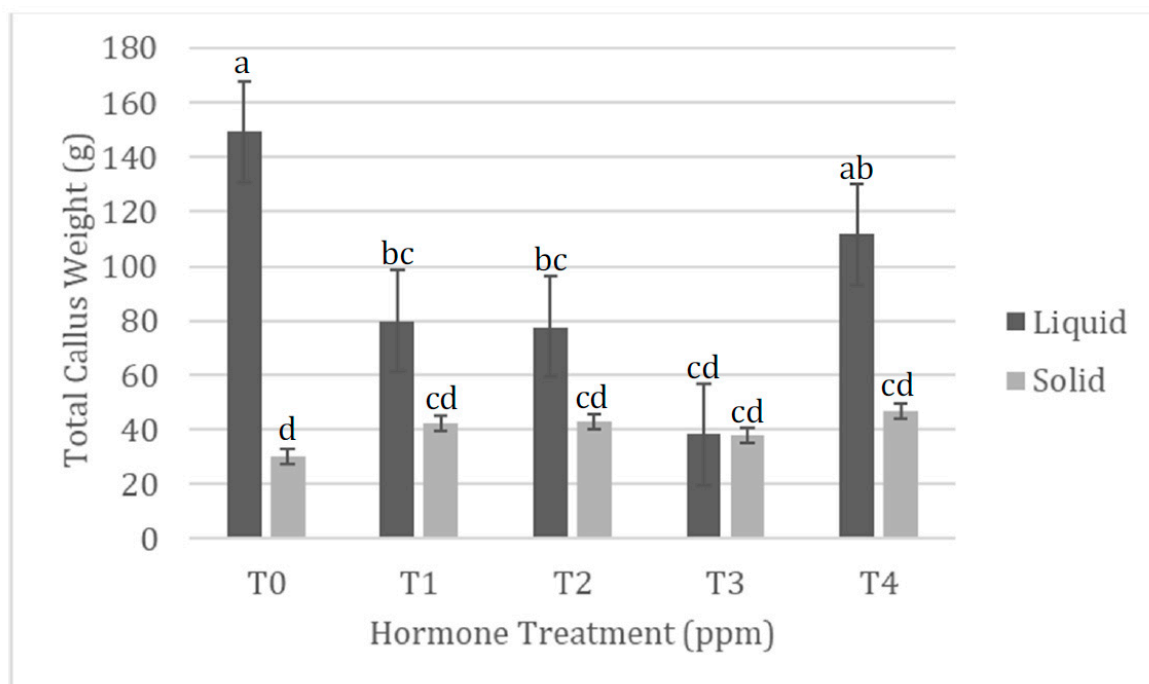
Figure 1. Total callus weight (g) in wet mass of callus material per treatment (30 explants) between different cytokinin treatments and tissue culture methods after a six-week growing period. Columns with a different letter (a–d) are significantly different at $p < 0.05$ according to the Tukey studentized test. T0: no added cytokinins, T1: 0.5 ppm 6-benzylaminopurine (BA), T2: 1.0 ppm BA, T3: 0.25 ppm kinetin (KT), and T4: 0.5 ppm KT. The liquid treatment refers to cultures grown in TIS bioreactors and the solidified treatment to those grown on a solidified medium.

Results on root growth were much more variable. Root production was only significantly improved with TIS when no cytokinins were added (Figure 2, T0). The BA treatments produced significantly fewer roots compared to the other treatments, though TIS seemed to produce slightly more roots, but not at a significant level. The kinetin treatments surprisingly produced more roots on solidified media compared to TIS, although only significant for kinetin at 0.5 ppm when compared to TIS.

It is interesting to note how the T0 solidified treatments, both for callus and root production, did very poorly, while the T0 in TIS treatments did the best overall.
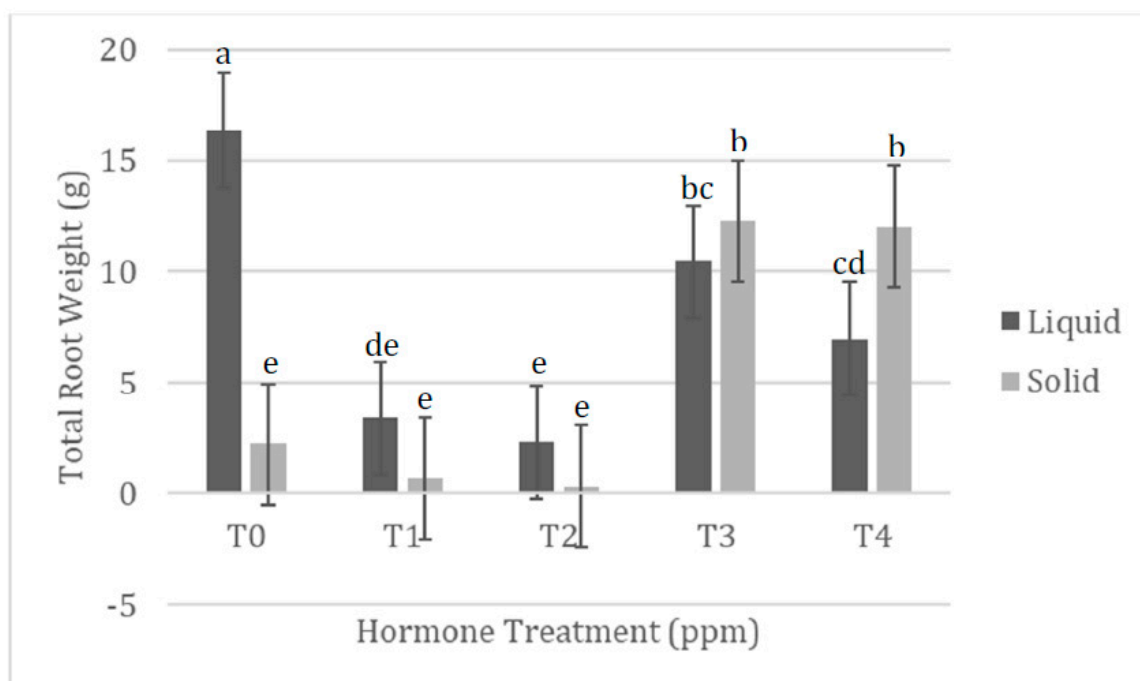
Figure 2. Total root weight (g) of root material per treatment (total of 30 cultures) between different cytokinin treatments and tissue culture methods. Columns with different letters (a–e) are significantly different at $p < 0.05$ according to the Tukey studentized test. T0: no added cytokinins, T1: 0.5 ppm BA, T2: 1.0 ppm BA, T3: 0.25 ppm KT, and T4: 0.5 ppm KT. The liquid treatment refers to cultures grown in TIS bioreactors and the solidified treatment to those grown on a solidified medium.

Table 1. Summary of treatment outcomes describing the effect of each treatment, as well as a photograph representing the material for each treatment.
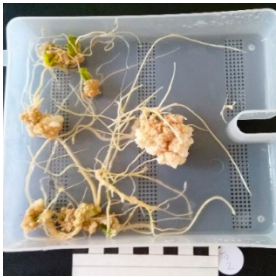
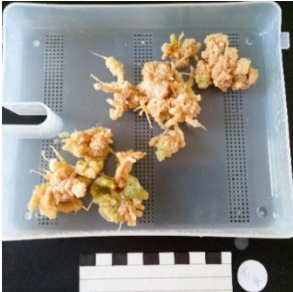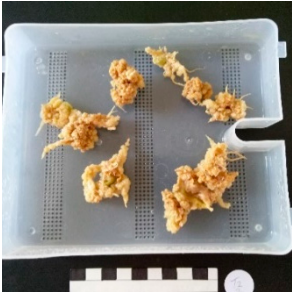| Treatment	Effect	Photo
0.5 ppm NAA pre-culture No added cytokinins Solid media	Production of long fine roots and callus tissue. Green tissues are relics of the leaf tissue used as explants. Less tissue in terms of wet mass produced than TIS.	
0.5 ppm NAA pre-culture No added cytokinins TIS	Production of long fine roots and callus tissue. Green tissues are relics of the leaf tissue used as explants. More tissue in terms of wet mass produced than solidified medium treatment.	

Table 1. *Cont.*

Treatment	Effect	Photo
0.5 ppm NAA pre-culture 0.5 ppm BA Solid media	Mostly production of callus. Production of some fine, but short roots. Slightly less callus wet mass production than TIS treatment. Similar results to 1.0 ppm BA treatment on solidified media.	
0.5 ppm NAA pre-culture 0.5 ppm BA TIS	Mostly production of callus. Production of some fine, but short roots. Slightly more callus wet mass production than solidified treatment. Similar results to 1.0 ppm BA treatment on solidified media.	
0.5 ppm NAA pre-culture 1.0 ppm BA Solid media	Mostly production of callus. Production of some fine, but short roots. Slightly less callus wet mass production than TIS treatment. Similar results to 0.5 ppm BA treatment on solidified media.	
0.5 ppm NAA pre-culture 1.0 ppm BA TIS	Mostly production of callus. Production of some fine, but short roots. Slightly more callus wet mass production than solidified treatment. Similar results to 0.5 ppm BA treatment on solidified media.	
0.5 ppm NAA pre-culture 0.25 ppm KT Solid media	Production of callus and long fine roots. Callus wet mass production similar to TIS treatment and 0.5 ppm KT treatment. Root wet mass slightly more than TIS treatment, but similar to 0.5 ppm KT treatment.	

Table 1. *Cont.*

| Treatment	Effect	Photo
0.5 ppm NAA pre-culture 0.25 ppm KT TIS	Production of callus and long fine roots. Callus wet mass production similar to solidified treatment, but less than 0.5 ppm KT treatment. Root wet mass slightly less than solidified treatment, and slightly more than 0.5 ppm KT treatment.	
0.5 ppm NAA pre-culture 0.5 ppm KT Solid media	Production of callus and a few fine roots. Callus wet mass production less than TIS treatment, but similar to 0.25 ppm KT treatment. Root wet mass significantly more than TIS treatment, but similar to 0.25 ppm KT treatment.	
0.5 ppm NAA pre-culture 0.5 ppm KT TIS	Production of callus and some fine roots. Callus wet mass production significantly more than solidified treatment and 0.25 ppm KT treatment. Root wet mass significantly less than solidified treatment, and slightly less than 0.25 ppm KT treatment.	
0 ppm BA pre-culture 0.1 ppm BA Solid media	Production of shoots and leaf material with wet mass significantly lower than TIS treatment, but similar to (slightly more than) 0.1 ppm and 0.2 ppm BA treatments. Callus wet mass production significantly less than TIS, but similar to other BA treatments.	
0 ppm BA pre-culture 0.1 ppm BA TIS	Production of shoots and leaf material with wet mass significantly higher than solidified treatment, but similar to (slightly more than) 0.1 ppm and 0.2 ppm BA treatments. Callus wet mass production significantly more than solidified treatment, but similar to (slightly more than) other BA treatments.	

Table 1. *Cont.*

| Treatment	Effect	Photo
0.1 ppm BA pre-culture 0.1 ppm BA Solid media	Production of shoots and leaf material with wet mass lower than TIS treatment, but similar to 0 ppm and 0.2 ppm BA treatments. Callus wet mass production significantly less than TIS, but similar to other BA treatments.	
0.1 ppm BA pre-culture 0.1 ppm BA TIS	Production of shoots and leaf material with wet mass higher than solidified treatment, but similar to 0 ppm and 0.2 ppm BA treatments. Callus wet mass production significantly more than solidified treatment, but similar to other BA treatments.	
0.2 ppm BA pre-culture 0.1 ppm BA Solid media	Production of some shoots and leaf material with wet mass lower than TIS treatment, but similar to (slightly lower than) 0 ppm and 0.1 ppm BA treatments. Callus wet mass production less than TIS, but similar to other BA treatments.	
0.2 ppm BA pre-culture 0.1 ppm BA TIS	Production of some shoots and leaf material with wet mass higher than solidified treatment, but similar to (slightly less than) 0 ppm and 0.1 ppm BA treatments. Callus wet mass production more than solidified treatment, but similar to other BA treatments.	

3.2. Experiment 2

The differences between treatments based on the number of shoots produced, the average length of shoots, and the average wet weight of shoots and callus tissue are described and discussed below in Figures 3–6.

An ANOVA analysis indicated that there was a highly significant ($p < 0.01$) interaction effect between the BA treatments and the tissue culture method used (conventional solidified medium method or TIS method) on the number of shoots produced per culture. This interaction effect means that an increase in the number of shoots produced does not depend on the effect of BA concentrations nor on which method was used on its own, but rather on the combined effect of these two factors. Thus, the combination of a particular concentration of BA used in combination with one of these methods will make a significant difference.
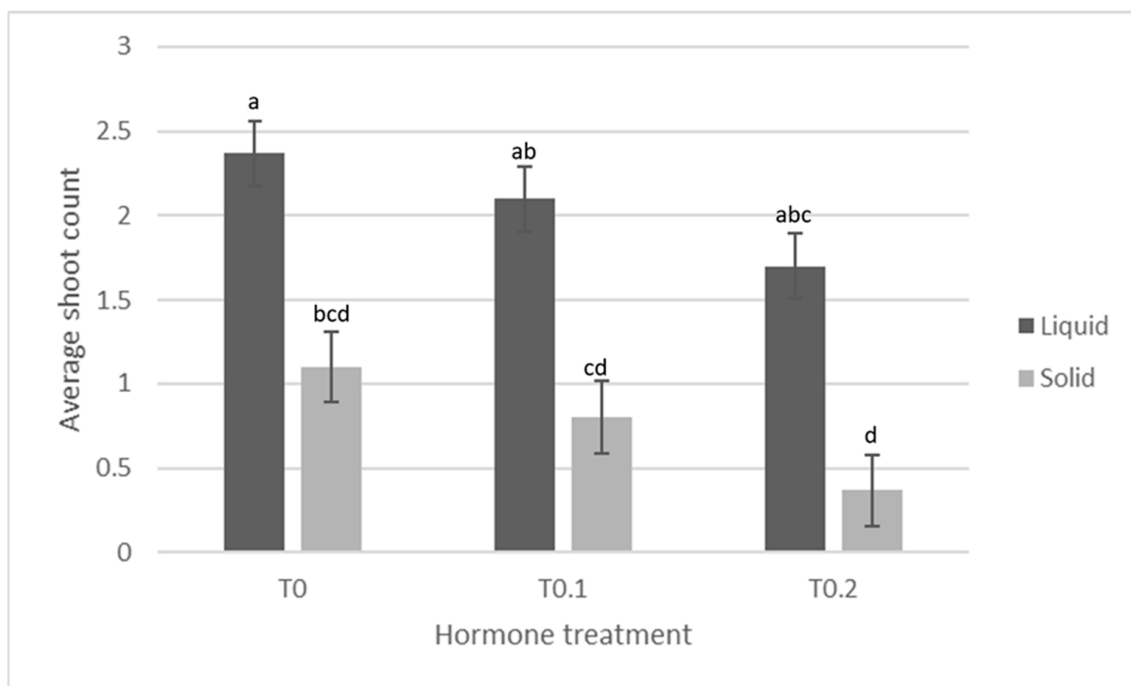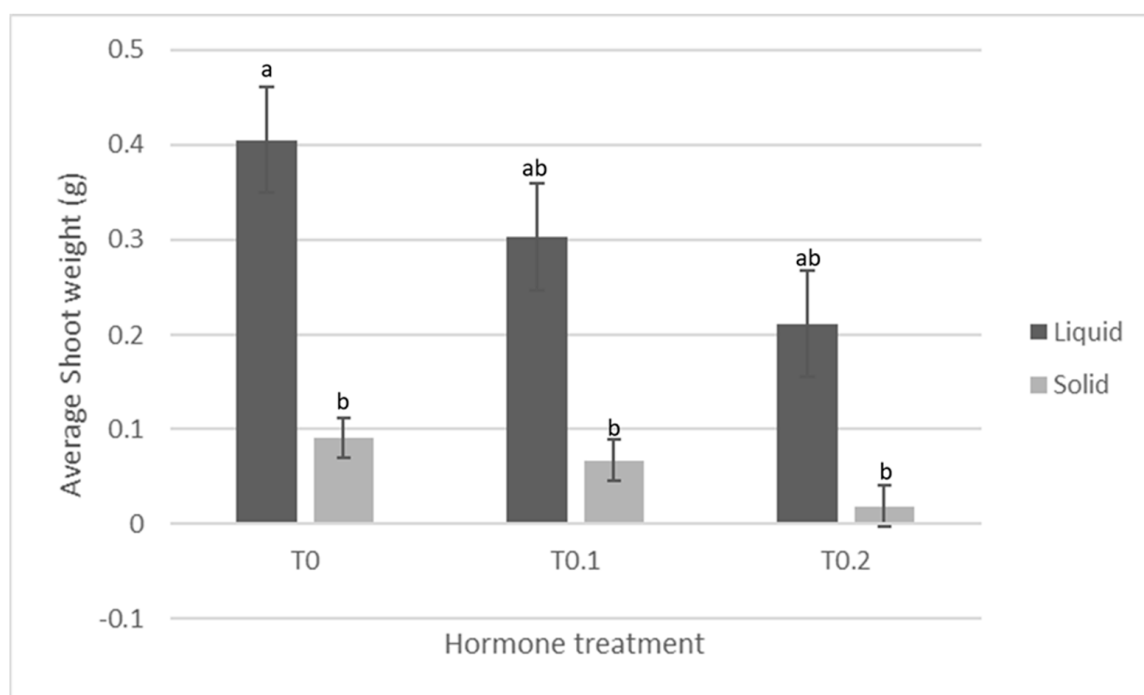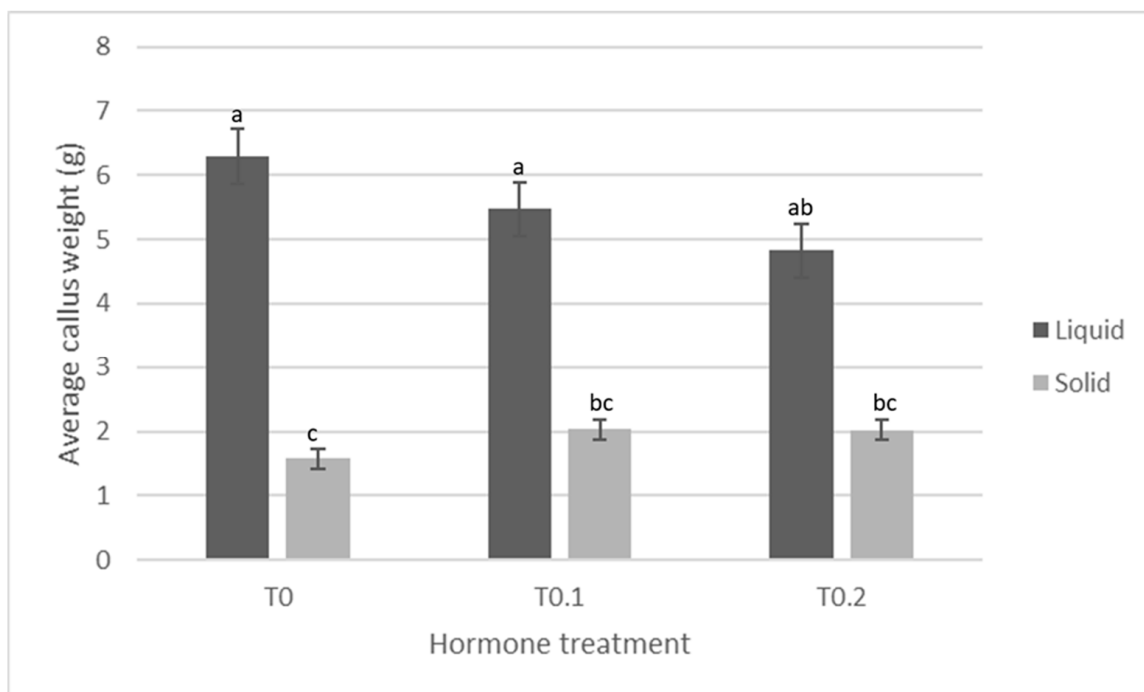
Figure 3. Average shoot count per explant across different treatments(T0 (BA at 0 ppm), T0.1 (BA at 0.1 ppm), and T0.2 (BA at 0.2 ppm)) after a six-week growth period in TIS bioreactors (liquid) and on solidified medium in G-7 vessels (solid). Columns with different letters (a–d) are significantly different at $p < 0.05$ according to the Tukey studentized test. Hormone treatment BA: 6-benzylaminopurine.
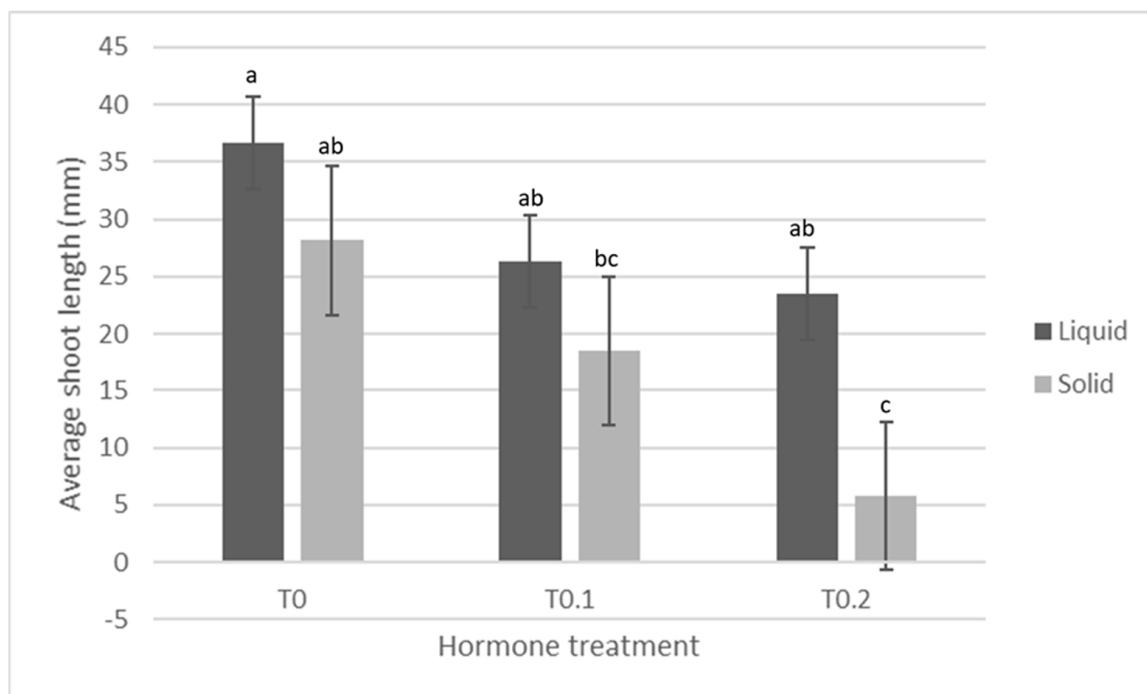
Figure 4. Average shoot length (mm) per explant across three different treatments (T0 (BA at 0 ppm), T0.1 (BA at 0.1 ppm), and T0.2 (BA at 0.2 ppm)) after a six-week growth period in TIS bioreactors (liquid) and on solidified medium in G-7 vessels (solid). Columns with different letters (a–c) are significantly different at $p < 0.05$ according to the Tukey studentized test. BA: 6-benzylaminopurine.

Figure 5. Average shoot fresh weight (g) per explant across three different treatments (T0 (BA at 0 ppm), T0.1 (BA at 0.1 ppm), and T0.2 (BA at 0.2 ppm)) after a six-week growth period in TIS bioreactors (liquid) and on solidified medium in G-7 vessels (solid). Columns with different letters (a,b) are significantly different at $p < 0.05$ according to the Tukey studentized test. BA: 6-benzylaminopurine.

Figure 6. Average callus fresh weight (g) per explant across three different treatments (T0 (BA at 0 ppm), T0.1 (BA at 0.1 ppm), and T0.2 (BA at 0.2 ppm after a six-week growth period in TIS bioreactors (liquid) and on solidified medium in G-7 vessels (solid). Columns with different letters (a–c) are significantly different at $p < 0.05$ according to the Tukey studentized test. BA: 6-benzylaminopurine.

In Figure 3, for each treatment of BA, the number of shoots produced in TIS bioreactors was significantly higher than those produced on solidified media (different letters indicate significant differences). These results correlate with those found by Quiala et al. [19], who managed to improve the shooting of teak in TIS. Quiala et al. [19] also found that an increase in BA concentrations caused the formation of more shoots, but also more hyperhydric shoots. High BA concentrations also led to a decrease in shoot length and diameter. However, in this study the number of shoots produced did not increase with the increase in BA concentrations; an apparent decrease in production can be seen in Figures 3–6, except for a slight increase in callus production on solidified media. Shahzad et al. [20] also found that callus tissue increased at the cut ends of the explants with an increase in cytokinin concentrations, which seemed to hinder shoot production. The reduced production with increased BA concentrations could be an indication that the BA concentrations were possibly higher than necessary for plants such as *M. oleifera* in this study.

There was, according to statistical analysis, no significant increase in the average shoot length of cultures across TIS treatments, although the highest average shoot length was obtained with no BA (T0) (Figure 4). This indicates that the combined effect of the use of TIS and BA concentration needs to be considered and both aspects of the tissue culture method and use of plant growth regulators need to be tested.

For shoot weight, there was a highly significant increase in weight using TIS for treatment T0 (Figure 5). TIS showed a higher average shoot weight for all treatments, although not significantly for T0.1 and T0.2.

For callus weight, there was a highly significant increase in weight using TIS for treatments T0 and T0.1 (Figure 6). The T0.2 treatment in TIS also outperformed the conventional tissue culture callus weight, although it was not statistically significant.

Table 1 summarizes different treatment outcomes by providing illustrations with observations over a six-week growing period from the initial and follow-up experiments.

Excess callus production could be a result of too much PGR in the nutrient media. These results contradict those found by Saini et al. [21] who managed to develop a protocol to produce moringa plantlets by first growing shoots on an MS medium containing BA and then inducing root growth with the combination of indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA). These researchers managed to produce multiple axillary shoots by including BA in the media, whilst in this study, the addition of BA did not improve shooting.

Steinitz et al. [4] successfully developed a microcloning system for, amongst others, *M. oleifera*, without the use of any PGRs. Their results correlate with the improved growth of cultures when a pre-culture phase is used without any added BA, as seen in Figures 3–6. As BA concentrations increased, the health of cultures seemed to decrease. This phenomenon was more pronounced in solidified medium treatments. Cultures grown on higher concentrations of BA struggled to support shoot growth and even resulted in the loss of leaves. This shoot growth reduction at higher concentrations of BA is supported by Quiala et al. [19], who found that higher levels of BA caused more anatomical defects and physiological disorders in teak cultures. The teak shoots grown in higher levels of BA showed lower lignification and development of xylem vessels and deformed leaf stomata. Quiala et al. [19] suggest that somehow BA affects the metabolism of phenolics, including lignins and their precursors, causing hypolignification, which is often associated with hyperhydricity.

Consistently in all experiments, TIS without any PGRs produced the highest average callus weight, root weight, shoot length, and shoot numbers (Figures 1–6). It may be possible that due to the natural presence of cytokinins in moringa tissues, the addition of cytokinins in the growing media may have inhibited the growth of shoots. Förster et al. [22] found that nodal cultures of *M. oleifera* showed increased callus production with increased BAP concentration in the growing media and shorter shoots, although with an increase in the number of shoots at the lower levels of BAP. The addition of lower levels of BAP did help with the multiplication of shoots in vitro, which was not the case in this study. Decreasing

the use of PGRs may also reduce the cost of production of moringa plantlets combined with TIS even further and make the possibility of expansion of tissue culture more economical. TIS without any PGRs is therefore recommended for *M. oleifera* production.

The overall increase in tissue growth in TIS may be attributed to increased nutrient uptake through the liquid nutrient medium. Cultures grown on solidified media may have limited access to these nutrients since the surface area in contact with the medium is limited, and no mixing of the media is present. However, since TIS provides cultures with a different growing environment to conventional methods, the availability of nutrients may not be the only explanation and could be explained by the numerous benefits associated with TIS such as more uniform culturing conditions [10], improved aeration, and the reduction in conditions such as hyperhydricity or asphyxia.

4. Conclusions

This study shows that TIS has the potential to increase the growth of *M. oleifera* tissues in vitro using leaflets and microcuttings. Consistently in all experiments, TIS outperformed material produced in conventional tissue culture conditions. TIS improved the number and length of shoots produced, and the weight of shoots. However, the experiments clearly showed the need for a pre-culturing phase before transfer to TIS. The study also showed that *M. oleifera* callus tissues did not respond favorably to the increase in PGRs in the growing media and production in TIS is recommended without the use of PGRs which is another benefit of the system as it simplifies the production protocol and reduces the cost of production.

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References

1. Leone, A.; Spada, A.; Battezzati, A.; Schiraldi, A.; Aristil, J.; Bertoli, S. Cultivation, genetic, ethnopharmacology, phytochemistry and pharmacology of *Moringa oleifera* leaves: An overview. *Int. J. Mol. Sci.* **2015**, *16*, 12791–12835. [CrossRef] [PubMed]
2. Mughal, H.M.; Ali, G.; Srivastava, P.S.; Iqbal, M. Improvement of drumstick (*Moringa pterygosperma* Gaertn.)—A unique source of food and medicine through tissue culture. *Hamdard Med.* **1999**, *42*, 37–42.
3. Stephenson, K.K.; Fahey, J.W. Development of Tissue Culture Methods for the Rescue and Propagation of Endangered *Moringa* Spp. Germplasm. *Econ. Bot.* **2004**, *58*, S116–S124. [CrossRef]
4. Steinitz, B.; Tabib, Y.; Gaba, V.; Gefen, T.; Vaknin, Y. Vegetative micro-cloning to sustain biodiversity of threatened *Moringa* species. *In Vitro Cell. Dev. Biol. Plant* **2009**, *45*, 65–71. [CrossRef]
5. Lyam, P.T.; Musa, M.L.; Jamaleddine, Z.O.; Okere, U.A.; Odofin, W.T. The Potential of Temporary Immersion Bioreactors (TIBs) in Meeting Crop Production Demand in Nigeria. *J. Biol. Life Sci.* **2012**, *3*, 67–86. [CrossRef]
6. Debnath, S. Bioreactors and molecular analysis in berry crop micropropagation—A review. *Can. J. Plant Sci.* **2011**, *91*, 147–157. [CrossRef]
7. Businge, E.; Trifonova, A.; Schneider, C.; Rödel, P.; Egertsdotter, U. Evaluation of a new temporary immersion bioreactor system for micropropagation of cultivars of Eucalyptus, Birch and Fir. *Forests* **2017**, *8*, 196. [CrossRef]
8. Etienne, H.; Berthouly, M. Temporary immersion systems in plant micropropagation. *Plant Cell Tissue Organ Cult.* **2002**, *69*, 215–231. [CrossRef]

9.   Welander, M.; Persson, J.; Asp, H.; Zhu, L.H. Evaluation of a new vessel system based on temporary immersion system for micropropagation. *Sci. Hortic.* **2014**, *179*, 227–232. [CrossRef]

10. Welander, M.; Sayegh, A.; Hagwall, F.; Kuznetsova, T.; Holefors, A. Technical improvement of a new bioreactor for large scale micropropagation of several *Vaccinium* cultivars. *Acta Hortic.* **2017**, *1180*, 387–392. [CrossRef]

11. Mordocco, A.M.; Brumbley, J.A.; Lakshmanan, P. Development of a temporary immersion system (RITA®) for mass production of sugarcane (*Saccharum* spp. interspecific hybrids). *In Vitro Cell. Dev. Biol. Plant* **2009**, *45*, 450–457. [CrossRef]

12. Escalona, M.; Lorenzo, J.C.; Gonzalez, B.; Daquinta, M.; González, J.L.; Desjardins, Y.; Borroto, C.G. Pineapple (*Ananas comosus* L. Merr) micropropagation in temporary immersion systems. *Plant Cell Rep.* **1999**, *18*, 743–748. [CrossRef]

13. Latawa, J.; Shukla, M.R.; Saxena, P.K. An efficient temporary immersion system for micropropagation of hybrid hazelnut. *Botany* **2016**, *94*, 1–8. [CrossRef]

14. Jeong, C.-S.; Chakrabarty, D.; Hahn, E.-J.; Lee, H.-L.; Paek, K.-Y. Effects of oxygen, carbon dioxide and ethylene on growth and bioactive compound production in bioreactor culture of ginseng adventitious roots. *Biochem. Eng. J.* **2006**, *27*, 252–263. [CrossRef]

15. Coetser, E.; Du Toit, E.; Prinsloo, G. Temporary immersion bioreactors for clonal production of *Moringa oleifera* tissues. *Acta Hortic.* **2021**, *1306*, 13–18. [CrossRef]

16. Zhang, J.; Yang, Y.; Lin, M.; Li, S.; Tang, Y.; Chen, H.; Chen, X. An efficient micropropagation protocol for direct organogenesis from leaf explants of an economically valuable plant, drumstick (*Moringa oleifera* Lam.). *Ind. Crops Prod.* **2017**, *103*, 59–63.

17. Murashige, T.; Skoog, F. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiol. Plant.* **1962**, *15*, 473–497. [CrossRef]

18. Mathur, M.; Yadav, S.; Katariya, P.K.; Kamal, R. In vitro propagation and biosynthesis of steroidal sapogenins from various morphogenetic stages of *Moringa oleifera* Lam., and their antioxidant potential. *Acta Physiol. Plant.* **2014**, *36*, 1749–1762. [CrossRef]

19. Quiala, E.; Cañal, M.-J.; Meijón, M.; Rodriguez, R.; Chávez, M.; Valledor, L.; De Feria, M.; Barbón, R. Morphological and physiological responses of proliferating shoots of teak to temporary immersion and BA treatments. *Plant Cell Tissue Organ Cult.* **2012**, *109*, 223–234. [CrossRef]

20. Shahzad, U.; Jaskani, M.J.; Ahmad, S.; Awan, F.S. Optimisation of the micro-cloning system of threatened *Moringa oleifera* LAM. *Pak. J. Agric. Sci.* **2014**, *51*, 449–457.

21. Saini, R.K.; Shetty, N.P.; Giridhar, P.; Ravishankar, G.A. Rapid in vitro regeneration method for *Moringa oleifera* and performance evaluation of field grown nutritionally enriched tissue culture. *3 Biotech* **2012**, *2*, 187–192. [CrossRef]

22. Förster, N.; Mewis, I.; Ulrichs, C. *Moringa oleifera*—Establishment and multiplication of different ecotypes in vitro. *Gesunde Pflanz.* **2013**, *65*, 21–31. [CrossRef]