Temporary immersion bioreactors for clonal production of *Moringa oleifera* tissues

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Abstract

***Moringa oleifera* has excellent value as a medicinal plant due to the various secondary metabolites produced in all its tissues. These secondary metabolites are biologically active and give Moringa the properties that make it so useful against many ailments. The presence of such a variety of biologically active compounds gives Moringa the potential for discoveries and development of new drugs. One method to produce these metabolites for extraction is producing Moringa tissues in tissue culture. Tissue culture provides a controlled environment, and along with clonal reproduction, allows reduced variance between different batches. In this study, clonal proliferation of Moringa by using leaf material was investigated. Conventional solidified medium methods were compared to the production in temporary immersion bioreactor systems. Moringa seeds were germinated in vitro, and the seedling leaflets were used as explant material. The first method tested which involved planting leaf material directly onto media or into bioreactors for shoot production was unsuccessful due to high mortality rates of leaf material in the bioreactors. Another method proved more effective and involved first planting all leaf material onto a solidified medium to initiate some callus production before splitting these up between solidified media and bioreactors for shooting. The initiation media consisted of half-strength Murashige and Skoog (MS) basal salts and 0.5 ppm 1-Naphthaleneacetic acid (NAA). The shooting media consisted of full-strength MS basal salts and different treatments of Kinetin and 6-Benzylaminopurine (BA). It was found that there was a difference in the production of differentiated tissue between solidified media and bioreactors, whereas the bioreactors produced more substantial amounts of tissue (wet mass) compared to the solidified media.**

Keywords: Horseradish tree, tissue culture, micropropagation, plant growth regulators, differentiated growth

INTRODUCTION

Moringa oleifera, otherwise known as the horseradish tree, is well known for its medicinal properties and has long been used in ayurvedic, unani and allopathic systems of medicine (Mughal et al., 1999). This tree's medicinal value originates from the various biologically active compounds present within its tissues (Kasolo et al., 2010; Leone et al., 2015). Researchers hope to develop new drugs from the tree for use against various ailments or diseases such as diabetes or even cancer. Developing an improved in vitro production system for this tree may assist in the production of high amounts of clonal material for the year-round extraction of valuable compounds. Tissue culture methods have been proven useful for the mass production of pure clones and sterilised material under controlled environments (Hartmann et al., 2014). Bioreactor systems are types of tissue culture methods that developed in the process of designing more intensive culture systems. Bioreactors make use of a liquid nutrient medium which has the same nutrient and hormonal composition as solidified media but lacks a solidifying agent. They consist of a culture vessel which

accommodates cultured cells or explants and a control block which controls cultivation conditions (Georgiev et al., 2014). Many different types of bioreactors with different methods of agitation of the media (Hartmann et al., 2014) exist, which can be used effectively for different micropropagation systems. One main class of bioreactor system is the temporary immersion system (TIS) which developed alongside the commercialisation of micropropagation. TIS makes use of a liquid nutrient medium to immerse explants for set periods before draining again to prevent permanent immersion of explants which might lead to abnormalities or loss of material due to asphyxia or hyperhydricity (Etienne and Berthouly, 2002; Georgiev et al., 2014). Timers connected to air pumps control these immersion periods, and each plant species will have its optimal setting within a system. It is thought that these temporary immersion systems provide a more natural in vitro environment (Niemenak et al., 2008) which will, in turn, improve the health of explants, survival rates as well as the amount of biomass or plantlets or embryo production (Mordocco et al., 2009) or even biologically active compound production (Ptak et al., 2013). The improved health of explants in TIS is also thought to be caused by better contact with nutrient media and thus better nutrient uptake compared to solidified media, better gaseous exchange with the environment (Jeong et al., 2006), as well as reduced agitation, compared to other bioreactor systems (Georgiev et al., 2014). Bioreactors have already been adapted for the study of secondary metabolites in other crops (Jeong et al., 2006; Baque et al., 2012; Georgiev et al., 2014), but so far no sources could be found discussing TIS for *M. oleifera*. The *M. oleifera* tree is highly variable due to its heterogeneous nature (Jun-jie et al., 2017) and response to differences in seasonality and location (Leone et al., 2015), but by controlling as many factors as possible via an in vitro system will reduce variability in the quality of any product produced.

Before focusing on the secondary metabolite production of the horseradish tree with different in vitro methods, the effect on growth of its tissues needs to be studied. The study used as a starting point for the trials in this study was done by Jun-jie et al. (2017). Where researchers tested different sources of explants like cotyledonary nodes or hypocotyl explants. For many plants, especially those plants challenging to regenerate, such as conifers, these explant sources are useful (Hartmann et al., 2014). However, these sources of explants are not abundant, and Jun-jie et al. (2017) found that these resources reported relatively low regeneration frequencies. It was decided to develop an efficient protocol using leaf material which is both abundant as well as having little genetic variation for adventitious shoot formation. They proceeded to test different methods and managed to develop a protocol that had a shoot bud induction frequency of 93.33% with an average shoot production of 4.40 per explant, compared to 1.65 for other reported results. The method as described by (Jun-jie et al., 2017) found most effective was followed and adjusted (the solidifying agent removed) for TIS in order to compare the effect on the growth of explants.

The scope of this study includes a series of trials conducted in order to determine whether temporary immersion bioreactors (TIB) have the potential to improve in vitro proliferation of *Moringa oleifera* tissues compared to conventional semi-solidified media.

MATERIALS AND METHODS

Seeds of *M. oleifera* variety PKM-1 were used. Seedlings for all trials were grown in vitro to ease the sterilisation process of young material which can be sensitive to sterilisation processes. Therefore, it was not necessary to sterilise any seedling material when planting it onto any medium; however, all planting was done under a laminar flow cabinet. Seeds were first dehisced before sterilising under a laminar flow cabinet since no method could thus far be found that sterilised the husk. These seeds were washed with 100% ethanol for 30 seconds, then with 10% JIK (a household sodium hypochlorite solution) for ten minutes and finally rinsed in sterilised water three times for 10 minutes each time. The sterilised seeds were planted onto a semi-solidified medium in Pyrex test tubes. The seedling medium

consisted of half-strength Murashige and Skoog (MS) basal salts, 30g L^{-1} sucrose, 4.5 g L^{-1} agar and adjusted to a pH of 5.8. Each test tube contained 10mL of the growing medium and was autoclaved at 121°C at a pressure of 1.2 kgf cm^{-2}, for 20 minutes. All seedlings and treatments were grown under a cool white fluorescent light with a photoperiod of 12 hours for about two weeks until leaflets opened up.

Jun-jie et al. (2017) proposed a protocol for producing Moringa shoots directly from leaf cultures. The researchers showed success with one of their treatments which consisted of planting leaf segments directly onto a solidified medium with MS basal salts, 0.8 mg L^{-1} 6-benzyl adenine (BA), 0.2 mg L^{-1} kinetin (KT), 0.05 mg L^{-1} α-naphthalene acetic acid (NAA) and solidified with 4.5 g L^{-1} agar (Jun-jie et al., 2017). With our first trial, we compared their growing media protocol in a similar medium, by only lacking the solidifier agar, in temporary immersion bioreactors. Leaflets from the sterilised seedlings were planted either individually in GA 7 vessels containing 40 mL of the solidified medium or planted in groups of ten in temporary immersion bioreactors each containing 500 mL liquid nutrient medium of the same constituents except for the agar. The immersion period for the TIB was set for every two hours for two minutes. These plantlets were grown for six weeks before results were taken.

The second trial was done in order to try and improve the abovementioned protocol. Seedlings were cut under the laminar flow cabinet and the leaflets planted individually into test tubes, each containing 5mL of a solidified medium. This medium consisted of half-strength MS basal salts, 0.5 mg L^{-1} NAA, 30g L^{-1} sucrose, solidified with 4.5g L^{-1} agar and adjusted to a pH of 5.8. These leaflets were then left to grow under the same conditions as mentioned for the seedlings for three weeks before transfer to different treatments.

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}. The nutrient media consisted of full-strength MS basal salts, 30 g L^{-1} sucrose, solid media were solidified with 4.5 g L^{-1} agar and all media were adjusted to a pH of 5.8 and autoclaved before use. The solidified medium treatments were done in GA 7 vessels which contained 40 mL nutrient media each. The liquid medium treatments were done in TIB, each containing 500 mL nutrient media. Immersion periods were set for two minutes every two hours. All treatments were grown for six weeks before results were taken.

Trials were laid out in a complete randomised design, with three reps of each treatment consisting of groups of 10 individual explants. The solidified medium treatment groups contained ten explants planted individually in GA7 vessels, and the TIB treatment explants were planted in groups of 10 per TIB vessel. Results were subjected to ANOVA analysis and Tukey's studentised range test with $P< 0.05$.

RESULTS AND DISCUSSION

The results were unexpected, but some were understandable after some consideration. The solidified medium treatment in the first trial failed to produce anything other than callus material which appeared green in places (figure 1), and leaf explants planted directly into temporary immersion bioreactors did not survive.

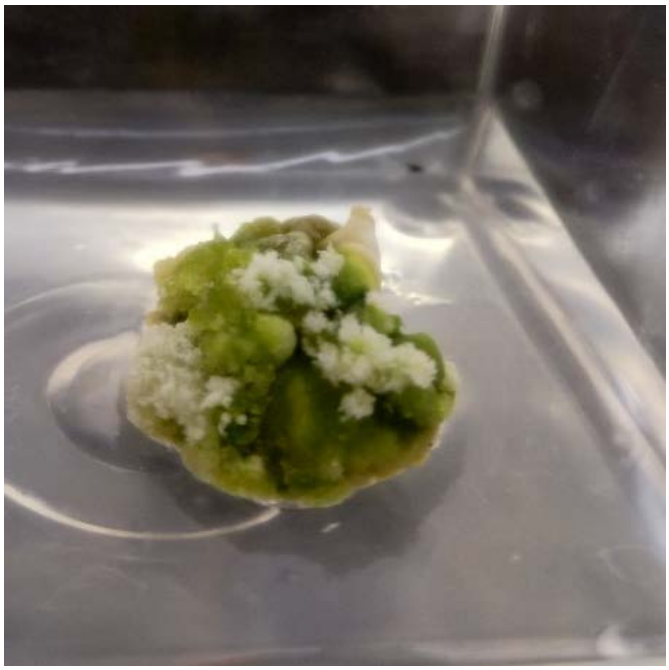

Figure 1. Callus material produced after six weeks on solidified medium following (Jun-jie et al., 2017) method

The second trial aimed to improve on this protocol for the production of *M. oleifera* shoots in vitro, using TIS by adding a step to first induce some callus production before moving explants to shooting treatments. It was later found that this is also a good idea in terms of reducing the risk of contamination in TIB. By pre-culturing explants on a solidified medium before transfer to TIB it ensured that any contaminated cultures could be detected and discarded before contaminating a whole vessel full of explants (Mordocco et al., 2009).

However, no shoots could be produced with this method either, but it did manage to produce roots and callus tissue. In the Materials and Methods section, it was mentioned that leaf material in the second trial was initiated to produce callus on solidified media containing NAA before transfer to shoot induction treatments. This was done after it was found that in preliminary trials, callus material could very quickly be produced with the use of auxins and that such cultures had a 100% survival rate in TIB when no contamination occurred. This production of callus tissue was also supported by Devendra et al. (2012) who managed to induce callus growth of zygotic embryo explants with NAA before maintenance of calli and somatic embryogenesis, and Khalafalla et al. (2011) who used 2,4-D for callus production from leaf material. The idea was that callus material could be used as a starting point for shooting since it is undifferentiated and could be induced to shoot with the application of cytokinins. The root production of the explants (Figure 2) can be explained by the "determination" of cells to produce a specific adventitious organ once an inductive signal has been received (Hartmann et al., 2014). Due to the auxin treatment, the cells of the explants might have been "determined" to produce roots despite treatment with cytokinins for shoot induction.

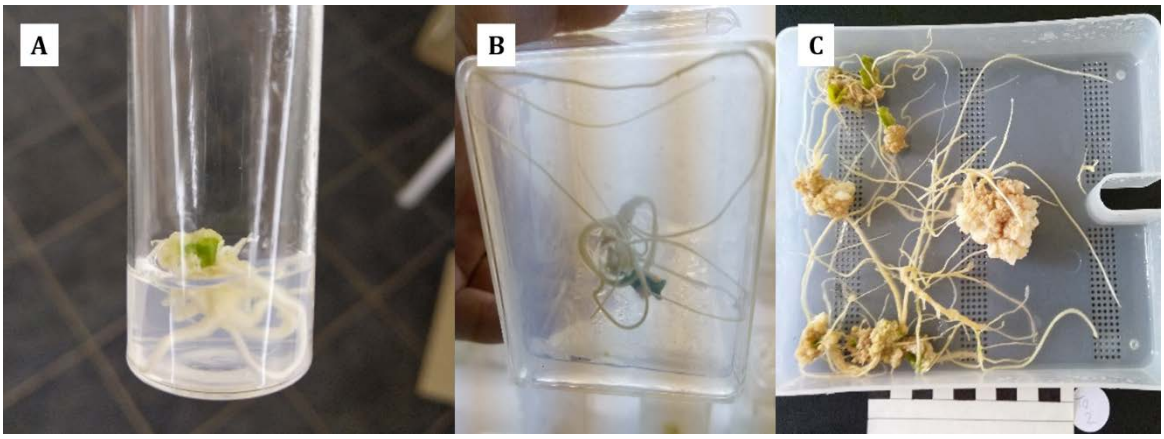

Figure 2.a) Root production of leaf explant in the initiation phase, b) root production after six weeks on solidified shooting medium, c) and TIB

Statistical analyses showed that there was a highly significant increase (at $P<0.01$) in the production of callus tissue (wet mass) in temporary immersion bioreactors compared to solidified medium methods; A total of 457g of callus tissue were harvested from TIS and 200g from solidified media. Thus, TIS was able to support more than double the amount of callus tissue than solidified media. On the other hand, a highly significant interaction effect (at $P<0.01$) was found for root production: Whether TIS methods improved root production depended on the PGR treatment. Both BA treatments (0.5 and 1.0 $mg.L^{-1}$) showed improved root growth when TIS methods were applied, with root wet mass totals of 3.4g and 2.3g, compared to solidified media with totals of 0.6g and 0.3g. In contrast, Kintetin treatments (0.25 and 0.5 $mg.L^{-1}$) improved root growth when combined with solidified media with root wet mass totals of 12.3g and 12.0g, compared to 10.4g and 7.0g for TIS. Overall, Kinetin treatments supported better root growth compared to BA treatments, and interestingly, TIS methods containing no added PGR showed the best root growth of all with a total wet mass of 16.3g, whilst its solidified medium counterpart supported root growth comparable to that of the BA treatments, 2.2g.

CONCLUSION

The use of temporary immersion systems increased the production of *M. oleifera* tissues. A significant increase in the production of callus material from leaf explants were found with the use of temporary immersion bioreactors. An increase in the production of root material when temporary immersion bioreactors were used either without any additional PGR or with BA used as treatment compared to when solidified media were used. However, Kinetin treatments supported better root growth overall compared to BA treatments, with the solidified media supporting better root growth. Of all treatments, the TIS treatment with no added PGR supported the best root growth. Future studies could include better PGR selection in the initiation phase to improve the production of shoot material. Reduction of callus material may also be beneficial since excess callus production uses up resources that could have been used to produce shoots. The effect of immersion periods in TIS could also be studied since plant growth react differently to this.

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