International Journal of Scientific Engineering and Research (IJSER)
ISSN (Online): 2347-3878
Index Copernicus Value (2015): 56.67 | Impact Factor (2017): 5.156

Enhancing the Protocol for Efficient Sugarcane (*Saccharumofficinarum*) Micropropagation using the BioMINT Temporary Immersion System in the Variety B79-474

Dion Daniels*, Cindy Itza, Jafet Pat, David Guerra, Stephen Williams

Faculty of Science & Technology, University of Belize, Hummingbird Avenue, Belmopan, Cayo District, Belize C.A

Abstract: ***Sugarcane (Saccharum spp.) is a cultivated crop in Belize which is used as food source and helps generate income especially for rural areas. The variety B79-474 is the most dominant due to its robustness. In this study, the BioMINT temporary immersion system (TIS) was used to enhance the protocol for efficient sugarcane micropropagation. Sugarcane plants were micropropagated using the BioMINT TIS to determine the appropriate subculture interval for the variety B79-474. The subculture intervals studied were 30, 45 and 60 days. After the time interval, parameters such as average plant height, survival rate, dry weight and multiplication coefficient were used to determine the appropriate subculture interval. The best results from the subculture intervals proved to be 45 days based on the parameters evaluated. Different volumes of culture media were also tested. These included 300 mL, 400 mL and 500 mL. In the parameters evaluated and most crucial in the multiplication phase, the best result proved to be that with 400 mL of culture media in each BioMINT unit.***

Keywords: Sugarcane, Micropropagation, Temporary Immersion System, BioMINT

1. Introduction

Sugarcane (*Saccharumofficinarum*) is one of the world's leading cash crops, and it has such great importance that the discovery and domestication of this type of grass has led to the production of sugar and derivative products which have enabled the change of many cultures. Refined sugar is produced from both the cane sugar and beet sugar. The first country to have produce sugar commercially was China and it was then expanded to Western Europe and now it is commercially produced in the tropics and subtropics (Blackburn, 2004). Currently, Brazil is one of the largest sugarcane producers where the Southeastern region contributes 70% of the national sugarcane production (Cheavegatii-Gianotto *et al*., 2011). Therefore, this shows that production of sugar has become an important consumer product and it's of economic interest. In Belize, agriculture has contributed 14% to the gross national income over the past years. Under the agriculture sector there are three sub-sectors in which one of them includes the production of sugar and it is estimated that the cultivation of sugarcane utilizes approximately 60,000 acres (FAO, 2011).

The production of sugar has become important for economic purposes and consumption, but the yield of sugarcane has been declining gradually because of segregation, susceptibility to diseases, insects and environmental factors such as climate changes. However, the use of biotechnology and molecular biology has benefited the agriculture sector because tissue culture has been used as a technique to propagate sugarcane. Several methods have been developed and the more efficient method for plant regeneration is through micropropagation. Through micropropagation there is rapid multiplication of explants in a short period of time and can produced plants free from pathogens (Ali *et al*., 2008).

A temporary immersion system (TIS) is an automated system for the micropropagation *in vitro* of plants (Quiala*et al*., 2014). A bioreactor is an apparatus used in TIS where liquid media is used. Plants are temporarily immersed in liquid media for a specific time interval, which helpsto control contamination, allow adequate nutrients and oxygen supply. The first bioreactor was used by Takayama and Misawa in 1981 and from there many types of bioreactors have been developed (Watt, 2012).

The BioMINT unit is a mid-sized (1.2 L) reactor that operates on the principle of temporary immersion. It is built of polypropylene and is translucent, autoclavable, and reusable. It consists of two vessels, one for the plant tissues and the other one for the liquid culture media coupled together through a perforated adaptor piece that permits the flow of the liquid media from one vessel to the other. The structural simplicity and the modular and independent nature of the bioreactors simplify their operation and reduce the amount of hand labor required for transfers, thereby reducing the cost of the whole micropropagation process(Robert*et al*., 2006).

In Belize, the most dominant and commonly used sugarcane variety is B79-474 because it's robust (Canto, 2015). The micropropagation laboratory at the University of Belize has been producing this variety for the sugar industry; however, via the conventional method using semisolid culture media. The laboratory has been introduced to the BioMINT system to improve production efficiencies.

The objective of this research is to determine the appropriate subculture interval and the volume of culture media used in the BioMINT TIS in the multiplication phase *in vitro* of the sugarcane variety B79-474.

2. Materials and Methods

This research was carried out at the Micropropagation Laboratory located at the University of Belize (UB) Central Farm Campus, Cayo District, Belize, Central America. Sugarcane (*Saccharum officinarum*) variety B79-474 vitroplants in the multiplication phase *in vitro* were used for this experiment.

Culture media

BioMINT units were sterilized in a 5% Clorox solution for 3 minutes and thereafter left to dry. The liquid culture medium was composed of Murashige and Skoog (1962) (MS) salt at 100%, 100mg/L of myoinositol, 30 g/L of sucrose and 50 mg/L of ascorbic acid. The pH of the culture media was adjusted to 5.8 with 1N hydrochloric acid (HCl) and/or 1N sodium hydroxide (NaOH) prior to sterilization. Twelve glass culture vessels of 0.67Lvolumetric capacity were used and each contained 300 mL of liquid media. The liquid media was sterilized in a vertical autoclaveat a temperature of 121^{o}C for 25 minutes ata pressure of 1 bar.

Subculture intervals

This BioMINT experiment included 4 replicates of the subculture interval to determine which is the most appropriate for the sugarcane variety B79-474. The subculture intervalsstudied were 30, 45 and 60 days. In this experiment there were a total of 12 BioMINT units. The BioMINTunits were set automatically to immerse the explants in the liquid culture media for 4 minutes every hour, to be in a horizontal position (180^{o} angle) during immersion and to be approximately 34^{o} angle when explants are not immersed. In addition, the growth room condition was set to 27^{o}C and a photoperiod of 16 hours light and 8 hours darkness. The constant variables for the 4 replicates were the volume of the liquid media, which was 300mL and the number of explants, which were 75 per each BioMINT unit. The arrangements of the 12BioMINT units were randomly placedin the BioMINT system.

A measurement line was drawn with a pencil on the laminar flowand this was used to measure the explants before inoculating in the BioMINT unit. Each explant was cut to 3 cm in length before placing in the BioMINT. Each explant was cleaned by removing any roots and dead leaves. Seventy-five explants were placed in each unit. The open end of each culture vessel with the liquid culture media was flamed before pouring it into the BioMINT unit. The explants were then inoculated into the liquid culture medium. After inoculation, the units were covered and plastic wrap was used to seal them. Thereafter, each unit was labeled and placed in the BioMINT Temporary Immersion System in the growth room. Observations were done every week to observe possible contamination.

After the end of each subculture interval, the other parameters evaluated were plant height, survival rate, multiplication coefficient and dry weight.To calculate the dry weight, plants were dried in a laboratory oven at a temperature of 38-45^{o}C for 48 hours. Thereafter the plants were weighed.

Volume of culture media

In this experiment, vitroplants in the multiplication phase were used with the same characteristics as the previous experiment. The culture media with the same components were used and the sterilization of the culture media and the BioMINT units were the same as described previously. The objective of this experiment was to test different volumes of culture media in the BioMINT units. The volumes tested were: 300 mL, 400 mL and 500 mL. Seventy-five explants were placed in each unit and evaluations were done using the best result from the previous experiment. The parameters evaluated were contamination, plant height, multiplication coefficient, survival rate and dry weight. Four replicates were used for each treatment.

Statistical Analysis

The data collected from both experiments were analyzed using statistical computer software known as Statistical Package for Social Science (SPSS) and MegaStats. Analysis of Variance (ANOVA) and Tukey's HSD test were used, which determined if there were statistical differences between treatments in each of the parameters evaluated.

3. Results& Discussions

In using the BioMINT temporary immersion system as a means of micropropagating the sugarcane variety B79-474, it is important to determine what are the right parameters for optimal shoot multiplication. It is with this in mind that these parameters were studied.

Subculture intervals

With respect to the plant height, the treatment with 60 days showed the greatest height (4.33 cm) with significant differences with the other two treatments (Table 1). This could be attributed to the fact that the explants had a longer exposure time to the culture media, where they were absorbing nutrients. There were no significant differences between the treatments with 30 days and 45 days.

Although the treatment with 60 days had the highest plant height, that same treatment had the lowest survival percentages with 90%, which was significantly lower than the other two treatments. The vitroplants stayed in the culture media twice as long as the first treatment with only 30 days. Over an extended period of time the nutrients from the culture media become exhausted and could lead to the death of some of the explants. This could be the reason for this lower survival percentage seen in the treatment with 60 days (Table 1).

Table 1: Plant height and survival rate after the end of each culture period.

Treatments (Days in culture)	Plant Height (cm)	Survival (%)
30 days	3.91 b	95 b
45 days	3.91 b	98 a
60 days	4.33 a	90 c

Different letters between treatments differ statistically for p<0.05 according to Tukey's HSD

Dry weight is a real indicator of growth. In this experiment, the treatment with the highest dry weight was 45 days. This

shows that after 45 days of culture the explants had the highest increase in dry weight (27.70 g), which was significantly superior to the other treatments (Table 2). Having the vitroplants in cultures for a longer period of time (60 days) negatively affected the dry weight. This could be attributed to the competition of the shoots for nutrients, space and carbon dioxide, which led to a slower rate of cellular division and elongation.

Table 2: Dry weight and multiplication coefficient after different culture periods

Treatments (Days in culture)	Dry weight (g)	Multiplication coefficient
30 days	8.70 b	7.4 b
45 days	27.70 a	12.0 a
60 days	9.68 b	8.0 b

Different letters between treatments differ statistically for p<0.05 according to Tukey's HSD

The BioMINT temporary immersion system is used in the multiplication phase, where the objective is to produce the largest number of shoots possible. Hence, obtaining the highest multiplication coefficient in this phase is crucial. In this experiment, the treatment with 45 days of culture resulted in the highest multiplication coefficient (12), which was significantly higher than the other two treatments (Table 2). The multiplication coefficient in this same laboratory using conventional micropropagation with semisolid culture media of this sugarcane variety is 5.5. This means that the use of this BioMINT temporary immersion system more than double the multiplication coefficient of this sugarcane variety. This, therefore, allows the productive capacity of the laboratory to increase.

Volume of culture media

In the different volumes of culture media tested, three different parameters were evaluated as seen in table 3 below. In the case of plant height, the treatment with 400 mL of culture media had the highest value (4.29 cm) with significant difference between the other two treatments (Table 3). The average plant height was lower when the volume was increased to 500 mL. This is showing that there should be the adequate amount of culture media per explants. Having more culture media can affect the overall growth a development of the plants. In this experiment, no hyperhydricity of the vitroplants was observed.

Table 3: Parameters evaluated in different volumes of culture media

Treatments (Volume of culture media)	Plant Height (cm)	Multiplication coefficient	Dry weight (g)
300 mL	3.98 b	11.8 b	26.01 b
400 mL	4.29 a	13.8 a	29.77 a
500 mL	4.01 b	11.2 b	26.28 b

Different letters between treatments differ statistically for p<0.05 according to Tukey's HSD

In the case of the multiplication coefficient, the best results were also obtained with 400 mL of culture media with significant difference when compared to the other treatments. In this treatment, the multiplication coefficient was 13.8. This is significantly higher than the multiplication coefficient in the conventional micropropagation with semisolid culture media, which averages 5.5. Medeiros de Araújo Silva (2015) worked with temporary immersion in sugarcane using vitroplants obtained from somatic embryos where there was a tremendous increase in the multiplication coefficient.

There is a correlation between the multiplication coefficient and the dry weight. The treatment with 400 mL that had the greatest multiplication coefficient and had the greatest dry weight with significant differences when compared to the other treatments. Etienne and Berthouly (2002); Berthouly and Etienne (2005) described the different types of temporary immersion system. They stated that temporary immersion generally improves plant material quality and vigor. A lot of study in temporary immersion systems focuses on the frequency and duration of the immersion; however, the amount of volume used is equally important.

4. Conclusion

Based on the results obtained in this research, using the BioMINT temporary immersion system for the *in vitro* multiplication of the sugarcane variety B79-474, the most appropriate volume of culture media is 400 mL and subculture every 45 days. These results were significantly superior to the other treatments evaluated.

References

[1] Ali, A., Naz, S., Siddiqui, F. A. and Iqbal, J. (2008). An Efficient Protocol for Large Scale Production of Sugarcane Through Micropropagation. *Pak. J. Bot., 40* (1), 139-149.

[2] Berthouly M. and Etienne H. (2005) Temporary immersion system: a new concept for use liquid medium in mass propagation. In: Hvoslef-Eide A.K., Preil W. (eds) Liquid Culture Systems for in vitro Plant Propagation. Springer, Dordrecht

[3] Blackburn, F. (2004). An Introduction to Sugarcane. In G. James (Ed.), *Sugar-cane* (Second Edition ed., pp. 1-19). UK: Blackwell Science Ltd.

[4] Canto G. (2015). The Emerging Roles of the Sugar Industry Research and Development Industry Management Information System (SIMIS). *The Belize Ag Report* , p. 7.

[5] Cheavegatti-Gianotto, A., de Abreu, H.M.C., Arruda, P.,Carlos J., Filho, B., Burnquist, W., Creste, S., di Ciero, L., Ferro, J., Figueira, A., de Sousa Filgueiras, T., Grossi-de-Sá,M., GuzzoE.,Hoffmann, H., Landell, M., Macedo N., Matsuoka, S., Reinach, F., Romano, E., da Silva, W., Filho, M., and Ulian E. (2011). Sugarcane (Saccharum X officinarum): A Reference Study for the Regulation of Genetically Modified Cultivars in Brazil. Tropical Plant Biology , 62-89.

[6] Etienne, H. &Berthouly, M. (2002). Temporary immersion systems in plant micropropagation. Plant Cell, Tissue and Organ Culture (2002) 69: 215.

[7] FAO (2011). Country Programming Framework for Belize: 2011-2015. Retrieved February 2017, from http://www.fao.org/3/a-bp543e.pdf

[8] Medeiros de Araújo Silva M., Cabral Medeiros E., Mota Lima G., Willadino L., Camara T. (2015). In vitro propagation in Temporary Immersion System of sugarcane plants variety 'RB 872552' derived from

somatic embryos. Biotecnología Vegetal Vol. 15, No. 3: 187 - 191

[9] Murashige T. Y. and Skoog F. (1962). A revised medium for the rapid growth and bioassays with tobacco tissue culture. Physiol Plant. 15(3): 473-497.

[10] Quiala E., Barbón R., Capote A., Pérez-Alonso N., Chávez M. and de Feria M. (2014). Scaling-up the biomass production of Cymbopogoncitratus L. Biotecnología Vegetal, 14, 67-71.

[11] Robert M.L., Herrera-Herrera J.L., Herrera-Herrera G., Herrera-Alamillo M.Á., Fuentes-Carrillo P. (2006) A New Temporary Immersion Bioreactor System for Micropropagation. In: Loyola-Vargas V.M., Vázquez-Flota F. (eds) Plant Cell Culture Protocols. Methods in Molecular Biology™, vol 318. Humana Press

[12] Watt, P. (2012). The status of temporary immersion system (TIS) technology for plant micropropagation. African Journal of Biotechnology, 11 (76), 14025-14035