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Effects of Four Different Capping Systems in the Micropropagation of Sugarcane (*Saccharum officinarum*) Variety B79-474

Irwin Puc[1], Javier Alegria[2], Dion Daniels[3], David Guerra[4], Stephen Williams[5]

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

Abstract: ***Aeration in a plant tissue culture vessel is an important concern because the tissue culture technology is inextricably bound-up with a requirement for sterility and prevention of dehydration. To improve the culture conditions for the micropropagation of sugarcane (Saccharum officinarum) variety B79-474 in the multiplication phase, different types of capping systems were designed and tested in this experiment. Four capping systems were used: Filter Disk (A), Plastic Wrap (B), Plastic Cap (C) and Cellophane (D). This research describes the culture vessels and culture systems, with an emphasis on different capping to improve the culture atmosphere and thus improve growth, multiplication coefficient and the quality of plantlets. The parameters used to determine better aeration and growth in the culture vessels were average height, number of dead leaves, and multiplication coefficient. The results showed that Cellophane had the highest average height and multiplication coefficient compared to the other capping systems. The results also indicated that Cellophane had the least number of dead leaves when sub-cultured after 28 days. Using capping system with cellophane for multiplication in vitrocan therefore lead to an increase in plant production while significantly reducing the number of dead leaves. The vigorous growth seen in the treatment with cellophane could have been seen in the growth and development of these plants in the acclimatization phase.***

Keywords: Sugarcane; micropropagation; capping system

1. Introduction

Sugarcane (*Saccharum officinarum*),being an important cash crop in Belize, an alternative technique for the highest possible yield and quality of this crop is essential to be determined. In Belize,sugar accounts for 60% of agricultural exports providing employment for more than 5,300 cane farmers both in the Orange Walk and Corozal Districts. In these Districts, currently an estimate of over 24,300hectares of sugarcane are in production (SIRDI, 2015).

Plant Biotechnology and Molecular Biology have developed opportunities in the field of agriculture. Methods have been developed for the propagation of varieties as well as more efficient regeneration through micropropagation (Ali *et al*., 2008). The focal advantage of micropropagation is the rapid multiplication of new varieties, improved plant health and its usefulness in germplasm storage. This is the most effective method for propagation as it produces plants that are similar to that of the mother plant and gives a higher multiplication rate. Micropropagation is currently the only realistic means of achieving rapid, large-scale production of disease-free quality planting material as seed canes of newly developed varieties in order to speed up the breeding and commercialization process in sugarcane (Behera& Sahoo, 2009).

Plant tissue culturetechniques have become especially important in the agricultural community and has effectively moved from the limitations of small laboratories and has taken its place among some of the more mainstream, broad-scale techniques employed by the agriculture industry (Hall, 1999).Plant tissue culture, many times referred to as micropropagation, can be broadly defined as a collection of methods used to grow large numbers of plant cells *in vitro*, in an aseptic and closely controlled environment. This technique is effective because almost all plant cells are totipotent, which means that each cell possesses the genetic information and cellular machinery necessary to generate an entire organism. Micropropagation therefore, can be used to produce a large number of plants that are genetically identical to a parent plant, as well as to one another (Raven *et al*., 1999).

Plant tissue culture vessels with their caps or closures make the boundaries between the internal microenvironment and the external environment of outside air. The physical properties of the vessels and caps or closures affect the microenvironment and growth of plantlets by the interface between inside and outside environments. The most important specifications for vessels are to provide uniform and adequate light quality, to isolate contamination of microorganisms and to allow gas exchange (Chen and Huang, 2005).

It is long believed that the growth of *in vitro* plants depend largely on the composition of the nutrient medium and thus efforts are mainly made to improve the nutrient composition of the growing medium (Zobayed, 2005). However, recent researches revealed that the growth and development of plants or explants produced *in vitro* can be seriously affected by the composition of the gaseous atmosphere (Kozai, 1991). The conventional protective conditions, such as the use of screw caps, aluminium foils, transparent films, polypropylene disc etc. under which plant materials are grown to prevent microbial contamination and to retard desiccation of the tissues and the nutrient medium can cause unintentional restriction of the exchange of gases between the vessel's atmosphere and the outside air (Buddendorf-Joosten and Woltering, 1994). Therefore, the gaseous environment *in vitro* is often abnormal when compared with the *ex vitro* environment.

The objective of this research is to determine the impact of the culture vessel capping system on plant growth in the multiplication and acclimatization phases of the sugarcane variety B79-474.

2. Materials and Methods

The research work was carried out at the Micropropagation Lab in Central Farm,University of Belize (UB), Cayo District, Belize, Central America. Vitroplants was used in the multiplication phase *in vitro* and these same plants were evaluated in the acclimatization phase *ex vitro*.

Culture media

Before the culture medium was prepared, glass culture vessels of 0.67Lvolumetric capacity were properly sterilized with 1% sodium hypochlorite solution. The culture vessels were immersed in the solution for approximately 2 minutes and then left to dry while the culture medium was being prepared.

The multiplication culture medium was composed of MS Salts (Murashige and Skoog, 1962) and supplemented with MS vitamins (100%), Myoinosotol at 100 mg/L, sucrose at 30 g/L, kinetin at 1.0 mg/L, 6-BAP at 0.6 mg/L, IAA at 0.65 mg/L and Cultar (paclobutrazol) at 0.05%.The culture medium was adjusted to a pH of 5.8 with 1N hydrochloric acid (HCl) and/or1N sodium hydroxide(NaOH) prior to sterilization. The culture medium was gelled with 6 g/L of agar. Fifty (50) mL of the multiplication media were put in each culture vessel. The vessels were immediately closed with the different capping systems as described in the experiment below. They were then sterilized in a vertical autoclave at 121°C for 20 minutesat a pressure of 1 bar. The culture media used for the rooting phase were MS salts (100%), 100 mg/L of Myoinositol, 20 g/L of sucrose, and 50 mg/L of ascorbic acid. The pH of the culture media was adjusted to 5.8 and it was gelled with 6 g/L of agar.

Different capping systems used in the multiplication phase

The selection of plastic cap type in combination with the plastic wrap (Figure 1B) limits gaseous exchange between the internal and external environment of the vessel affecting plant growth and subculture intervals. If gaseous exchange could be further enhanced, plant growth rates and multiplication coefficient, as well as shorter subculture intervals could improve. Therefore, this experiment was designed to improve plants' growth and development by modifying the culture vessel capping system to permit greater gaseous exchange *in vitro* without increasing contamination rates. Culture vessel capping system studied in this research include the following as seen in Figure 1.

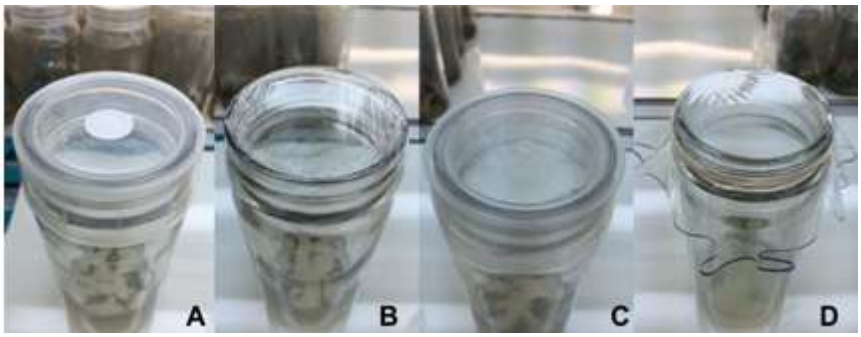

Figure 1: Four different capping types: Filter Disk (A), Plastic Wrap (B), Plastic Cap (C) and Cellophane (D)

For the Filter Disk (A),a hole of approximately 1 to 2mm in diameter was made with a 2-inch nail at the center of the plastic cover, then the disk was placed on top. The caps with the filter disk were then wrapped separately in aluminium foil and autoclaved. The second treatment was the Plastic Wrap (B).Only one layer was added at the top of the culture vessel, followed by 2 or 3 layers around the edges of the culture vessel to ensure that it was sealed properly to prevent contamination. The third treatment was the Plastic Cap (C), which is the current and standard capping system used by Central Farm Micropropagation laboratory. After the plastic cap was put on, 2 to 3 layers of plastic wrap tape were added at the side of the cap to ensure that it was properly sealed to prevent contamination. The last treatment was using Cellophane (D).These were cut into circles as seen in Figure 2, each having a diameter of 17 cm to ensure that it would properly cover the culture vessel when placed over it. While autoclaving, each cellophane cover was placed between two magazine sheets. This prevented the cellophane covers to be distorted during the process. After placing the cellophane between the magazine sheets, they were placed inside a plastic bag and properly sealed with tape. A piece of autoclave tape was also added to serve as an indicator to ensure that it was autoclaved properly. The cellophane was fastened to the mouth of the culture vessel with rubber bands, which were also sterilized prior to use. This process was done by submerging the rubber bands in a 1% sodium hypochlorite solution for 10 minutes, followed by spraying them with 95% ethanol and placing them in a plastic bag. The rubber bands were then autoclaved. A total of 20 culture vessels per treatment were prepared. Ten explants were placed in each culture vessel.Data were collected over two consecutive subcultures, hence data presented is an average of these two subcultures. Percent contamination was recorded at both subcultures. At the conclusion of the multiplication phase, the vitroplants were passed to the rooting phase *in vitro* where they had the same capping system as the previous phase. The cultures remained in the rooting phase for 28 days before being transferred to the acclimatization phase.

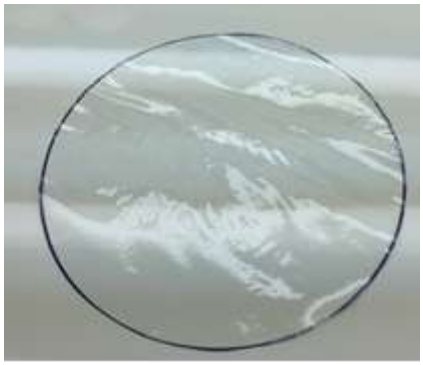

Figure 2: Circular cellophane cover having a diameter of 17 cm

Acclimatization phase

At the conclusion of the rooting phase *in vitro*, plants were placed in the hardening facility (which comprised of a greenhouse with relative humidity of approximately 90%).Relative humidity was measured by Fisher Scientific™ Traceable Relative humidity/temperature meter. The soil substrate for the sugarcane plantlets was prepared before planting. The substrate was a mixture of organic citrus compost with rice hull in a ratio of 5:1. The sugarcane plantlets were washed with water to remove the nutrient media. The roots of the vitroplants were soaked for 10 minutes in a fungicide solution (RIDOMIL® GOLD MZ 68WP) at 1%. Subsequently, vitroplants were then planted in polystyrene trays. The planting was done by creating a hole that is 2 cm deep and 1 cm in circumference in each orifice of the seedling trays. Plants were then planted in each seedling trays (containing 50 orifices) according to the four treatments studied in the multiplication phase. During the first two weeks, these plants were shaded with saran netting of 90% shade. An intermittent irrigation system activated every hour for one minute was used. After the two weeks period, the illumination was increased to 50%. Irrigation was increased to two minutes every hour. The plants spend another two weeks under these conditions. Finally the plants were then placed in an open area exposed to the sun and environment for one week. The survival of these plants is indicator of how successful they have made it through the acclimatization phase and is ready to be transplanted into the field. The survival rate was evaluated two weeks after being transplanted to the substrate.

The experimental design was a completely randomized block design with four replicates testing the response of the four treatments tested in the multiplication phase. Each treatment consisted of 200 sugarcane plantlets. The plots were all shielded with guard plants of a total of 250 that were merely use to protect the effect of variability of the natural environment. Parameters evaluated were plant height, number of leaves and survival rate.

Statistical Analysis

A Randomized complete block design was used for the experiment *in vitro*. This setup consisted of 4 replicates. Each replicate contained the 4 treatments (different caps being tested), and each treatment had 20 culture vessels. The 4 treatments were randomly placed in each replicate. Guard plants were placed all around the setup to prevent some treatment of being exposed to morenatural light. The parameters evaluated were height of the plants, multiplication coefficient and number of dead leaves. The data collected were then analysed using a statistical computer software: Statistical Package for Social Science (SPSS) and MegaStats. An analysis of variance (ANOVA) was conducted for each variable being height of the plants, multiplication coefficient and number of dead leaves to see the statistical differences or similarities between them.

3. Results and Discussions

An analysis was conducted to test the equality of the variances which allowed us to make inferences about the difference in the mean heights, multiplication coefficient and number of dead leaves of the vitroplants within the four treatments.

Different capping systems used in the multiplication phase

It's extremely important to find a balance in terms of gaseous exchange between the internal and external environments of the culture vessel. That balance should be to facilitate carbon dioxide in the culture vessel to allow photosynthesis to be at its optimum, while preventing contamination of the cultures. Table1 illustrates the different treatments (capping systems) along with the plant height values. As for the average height of the vitroplants, treatment D being cellophane presented the best results with significant differences when compared to the other treatments. The p-value is considered statistically significant when it is less than 0.05 (*alpha*), indicating that there is a significant difference within the treatment. Treatment D showed a significantly higher value (3.065 cm) than the other treatments, which stands superioramong the remaining three treatments. It can thus be inferred that this capping system is most adequate for obtaining optimum plant height. The worse capping system proved to be Plastic Wrap(B) with an average plant height of 2.838 cm and Plastic Cap (C) with an average plant height of 2.840 cm. Both treatments were significantly inferior to treatments A and D. This result is attributed to the porosity found on the cellophane that permits the gaseous exchange between the internal and external environments of the culture vessel. According to Posada Perez *et al*. (2015), vitroplants grown in mixotrophic conditions showed better development in *ex vitro* conditions, hence facilitating the gaseous exchange and allowing CO_2 into the culture vessel certainly enhances photosynthesis.

Table1: Height of the vitroplants for the different treatments

Treatments	Plant Height (cm)
Filter Disk (A)	2.898 b
Plastic Wrap (B)	2.838 c
Plastic Cap (C)	2.840 c
Cellophane (D)	3.065 a

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

The results for the multiplication coefficient in the different treatments (capping system) are presented in table 2. Treatment D being cellophane presented the best results having significant differences among the other treatments. This treatment had significantly higher value (7.7) than the other treatments, which stands superior compared to the other three treatments. This result is attributed to cellophane providing the best gaseous exchange which allowed

optimum growth and multiplication of the vitroplants. The results also showed the possible potential for increasing mass production as it had the highest multiplication coefficient. This means that more vitroplants can be produced in the same lab space hence increase the overall productivity. The least effective capping system was observed to be Plastic Cap(C) with a multiplication coefficient value of 3.3 (lowest), which also had a significant difference between the other capping systems.

Table 2: Multiplication coefficient for the different treatments

Treatments	Multiplication Coefficient
Filter Disk (A)	5.8 b
Plastic Wrap (B)	5.5 b
Plastic Cap (C)	3.3 c
Cellophane (D)	7.7 a

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

Cellophane had the least number of dead leaves with significant difference with Plastic Wrap (B) and Plastic Cap (C), but without significant difference with the Filter Disk (A) (Table 3).Cellophane showed the lowest value of 4.4.This result could also explain why this treatment (Cellophane) had the highest multiplication coefficient. Since Cellophane had proven to have the least number of dead leaves, this allows the plantlets to multiply more efficiently reducing the chances of blackening and increasing the multiplication coefficient. Blackening is a problem in this variety of sugarcane. This greatly affects the plantlets because the dark crust would reduce absorption of nutrients from the culture media, causing death and leading to lower multiplication coefficient. The least effective capping system proved to be Plastic Wrap (B), having a value of 9.1 in terms of the number of dead leaves.

Table 3: Number of dead leaves for the different treatments

Treatments	Number of dead leaves
Filter Disk (A)	4.6 b
Plastic Wrap (B)	9.1 a
Plastic Cap (C)	8.4 a
Cellophane (D)	4.4 b

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

This experiment proved that cellophane is a better capping system than the conventional plastic caps because it resulted in the best results in all the parameters evaluated. In all cases, 0% contamination was recorded. No results of similar capping system experiments for this particular variety of sugarcane have been found in literature, which makes the results obtained in this research novel.

According to Zobayed *et al*. (2000), plantlets exhibit less water loss after transplantation. As a result, leaves do not wilt and grow fast even without any special *ex vitro* acclimatization conditions. In photoautotrophic micropropagation, humidity is controlled (< 90 %) and the CO_2 concentration is raised and ethylene cannot accumulate simply due to increased air exchange rate of the culture vessel. The results showed in this paper proved that improving the CO_2 concentration inside the culture vessel can certainly facilitate photoautotrophic micropropagation for sugarcane.

Acclimatization phase

The survival and overall growth and development of plants in the acclimatization phase is a huge part of a successful micropropagation process. It would be a complete failure to produce plants *in vitro* and not being able to convert these plants in the acclimatization phase. The first parameter evaluated in this phase was survival and this was done two weeks after being transplanted to the acclimatization phase. All treatments recorded a survival rate of 98%. Cultural practices that contributed to this high survival rate is that the plants were transplanted late in the evening when the ambient temperature was not too high. Another is the irrigation system that was in place to guarantee moisture and a constant flow of water throughout the plant. The mist irrigation also played a key role in maintaining a high relative humidity especially in the earlier days of the acclimatization phase.

Another parameter evaluated in the acclimatization phase was plant height. Plants from treatment D (cellophane) had the highest value of 14.2 cm, which was significantly higher than the other treatments (Table 4). This is a carryover effect from the multiplication phase because the plants from this same treatment showed the best overall growth and development. This is attributed to the facilitation of gaseous exchange by the cellophane. There were no significant differences among the other treatments.

Table 4: Plant height at the end of the acclimatization phase

Treatments	Plant Height (cm)
Filter Disk (A)	13.2 b
Plastic Wrap (B)	13.0 b
Plastic Cap (C)	13.1 b
Cellophane (D)	14.2 a

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

In the acclimatization phase, plants should fully adapt to being autotrophic and hence the number of leaves are important to increase the photosynthetic capacity of the plants. This was another parameter evaluated in the acclimatization phase. As seen in Table 5 below, treatment D (cellophane) had the highest number of leaves (5.4) with significant differences to the other treatments. Treatment B (plastic wrap) had the least number of leaves (2.9), and this was significantly inferior to all the other treatments.

Table 5: Number of photosynthetically active leaves at the end of the acclimatization phase

Treatments	No. of leaves
Filter Disk (A)	4.3 b
Plastic Wrap (B)	2.9c
Plastic Cap (C)	4.3 b
Cellophane (D)	5.4 a

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

Rangel-Estrada *et al*. (2016), in working with different sugarcane varieties in Mexico, obtained survival rates of between 95-98%. They stated that the key in getting high survival percentages is having the appropriate substrate,

humidity and temperature. Reyes Esquirol *et al*. (2014) also had excellent results in the acclimatization of sugarcane. They used a bio-stimulant (Fitomas-E) and obtained superior results in all the parameters evaluated when compared to the control.

4. Conclusion

It can be concluded that cellophane used as a capping system in the multiplication phase of the micropropagation of sugarcane (*Saccharum officinarum*) variety B79-474 had the best results in all the parameters evaluated (plant height, multiplication coefficient and number of dead leaves). Plants coming from the treatment with cellophane also had greater height and number of leaves in the acclimatization phase. It is therefore recommended that the conventional plastic caps currently used in the multiplication phase be replaced with cellophane in order to increase productivity and efficiency in the micropropagation of the sugarcane variety B79-474.

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