Effects of Different Paclobutrazol-Cultar Concentrations on the Micropropagation of Sugarcane (Saccharum Officinarum) Variety CPCL99-4455

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Abstract

Sugarcane (Saccharum officinarum), being an important cash crop in Belize, accounts for 60% of agricultural exports providing employment for thousands of Belizeans. This research was carried out to determine the effects of different Paclobutrazol (PBZ-Cultar) concentrations used in the culture media on in vitro multiplication of sugarcane variety CPCL99-4455. Three PBZ-Cultar concentrations were tested and compared with the control. The parameters evaluated to determine the effects of PBZ-Cultar were average height, number of dead leaves and multiplication coefficient. The plants from this experiment were planted in the acclimatization phase to determine if the use of PBZ-Cultar had any effect of the growth and development of the plants ex vitro. The results revealed that the culture medium supplemented with 0.08% PBZ-Cultar concentration had the best results both in the multiplication phase as well as the acclimatization phase.

Introduction

Raw sugar is derived from both sugarcane and sugar beet. Brazil and India are the world's two largest sugar producers. Together, they have accounted for over half the world's sugarcane (Saccharum officinarum) production for the past 40 years (Taylor and Koo, 2012). Sugarcane is an important cash crop for Belize's economy. This crop is a highly placed commodity in consumer products as it serves as a major source of edible processed sugars (Ali et al., 2008). As a result, the sugar industry of Belize is of fundamental social and economic importance to the country. Other than providing support to livelihoods and to approximately 15% of Belizeans, it also contributes significantly to the national economy. Day by day increasing use of sugar and its relevant products have created a challenging situation for sugarcane researchers and growers (SIRDI, 2015).

However, the potential yield of many sugarcane varieties is gradually decreasing on a daily basis due to segregation, susceptibility to diseases, insects, and changes in the climatic environment (Ali et al., 2008). There are also very limited resources to increase rapid multiplication procedures which have long been a problem of concern in sugarcane breeding programs. In recent times, plant biotechnology and molecular biology have created exceptional opportunities and promises in the field of agriculture. The potential of plant tissue culture has attracted interest all over the world of agriculture. Specific methods have been generated for the propagation of genotypes and efficient regeneration of plants by means of micropropagation (Kyte and Kleyn, 1996).

In sugarcane, micropropagation is essential for rapid multiplication of elite genotypes/clones and for the quick spread of new varieties (Biradar et al., 2009). The concentration of growth regulators in the culture medium used in tissue culture must be optimized and may change according to the desired result. Most plant growth regulators (PGRs) used in the greenhouse or nursery are used to regulate shoot growth of containerized crops. These PGRs are referred to as growth retardants (Latimer & Scoggins, 2012). A typical growth retardant is Paclobutrazol (PBZ), which is used in plant growth media to minimize or limit plant growth rate and produce a plant size that is easier to manipulate while encouraging growth of new shoots. These PGRs control plant height by inhibiting the production of gibberellins which is the primary plant hormone responsible for cell elongation. Furthermore, the effects of these growth-retardants are primarily seen in stem, petiole, and flower stalk tissues. Lesser effects are seen in reductions of leaf expansion, resulting in thicker leaves with a darker green color (Latimer & Scoggins 2012).

PBZ has also been reported to increase leaf cuticle formation and thus improve plant survival in the acclimatization phase ex vitro. However, high concentrations of PBZ will deliver a plant that is unmanageable in vitro. PBZ has also been identified as a growth regulator that inhibits the stem growth and breaks the apical dominance; it is able to inhibit the generation of gibberellic acid. It also inhibits the internodal elongation and also improves the abilities of stress tolerance. This compound promotes the lateral bud growth and also enhances or inhibits photosynthesis in the plant depending on the concentration. PBZ is scientifically known to improve the respiration intensity of root, and slows down the respiration of aboveground parts of crops (Latimer & Scoggins, 2012).

The primary objective of this research is to determine the effects of Paclobutrazol (PBZ) – Cultar concentration on in vitro multiplication of sugarcane variety CPCL99-4455 in terms of height, number of dead leaves, as well as the multiplication coefficient and determine its effect if any, in the acclimatization phase.

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 CPCL99-4455 vitroplants in the multiplication phase in vitro were used for this experiment and these same plants were evaluated in the acclimatization phase ex vitro.

Culture media

Culture vessels of 0.67 L volumetric capacity were used for the experiment in vitro. Before placing the culture medium in it, these culture vessels were properly sterilized with 1% sodium hypochlorite solution for two minutes and allowed to dry. Each culture vessel had 50 mL of culture media. The culture media were prepared using the standard 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-Bencilaminopurine (6-BAP) at 0.6 mg/L, Indole-acetic acid (IAA) at 0.65 mg/L. Paclobutrazol (PBZ-cultar) was added at the different concentrations outline below. The culture media used in the rooting phase were comprised of 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 with 1N hydrochloric acid (HCl) and/or 1N sodium hydroxide (NaOH) prior to sterilization and it was gelled with 6 g/L of agar.

The vessels were immediately wrapped with plastic wrap around the plastic cap. These culture vessels containing the culture media were then autoclaved at 121°C for 20 minutes at a pressure of 1 bar.

Different PBZ-Cultar concentrations used in the multiplication phase

The plant growth regulator used in this experiment was PBZ-Cultar. This growth regulator is a plant retardant that is well known for its ability to improve the yield of certain plants. According to the current protocol used at the UB Central Farm laboratory, 0.05% PBZ-Cultar is being used in the multiplication phase of sugarcane. In this experiment, three concentrations were studied and they were compared to the control. The concentrations studied were: T1 (0.00%), T2 (0.05%), T3 (0.08%), and T4 (0.10%). At the start of the experiment, twenty culture vessels of each of the treatments were used to subculture the sugarcane variety CPCL99-4455 and ten vitroplants were placed in each culture vessel. These culture vessels were then separated into groups of four so as to obtain five replicates. A randomized complete block design was used in this experiment. The treatments were randomly placed in the five different replicates. Additionally, guard plants were placed on each of the sides of the experimental setup in order to prevent any external contamination and to prevent additional light to the plants on the outside row.

Subculture was done every 28 days. A centimeter scale was gently drawn on the laminar flow with a pencil in order to keep a consistent size of the plantlets being sub-cultured. During subculturing, the plants were all cut at 1.5 cm in length. The parameters evaluated included plant height, number of dead leaves and multiplication coefficient. After evaluations at the two subcultures, the plants were transferred to the rooting phase, where they stayed for 28 days. Following this phase, they were transferred to the acclimatization phase to determine if PBZ-Cultar used in the multiplication phase had any effect in the overall growth and development of the plants in ex vitro conditions.

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 five 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

The data collected were analyzed using statistical computer software known as Statistical Package for Social Science (SPSS) and MegaStats. Analysis of Variance (ANOVA) and Dunnett's test were used, which allowed inferences to be made about the statistical differences between treatments in each of the parameters evaluated.

Results and Discussions

An analysis was conducted to assess the variances between four different treatments of PBZ-Cultar concentrations in the multiplication phase. These analyses allowed for inferences to be made about the variation in the mean heights, the number of dead leaves and multiplication coefficient of sugarcane (Saccharum officinarum) variety CPCL99-4455. Four PBZ-Cultar treatments were used: T1 (0.00%), T2 (0.05%), T3 (0.08%), and T4 (0.10%).

Different PBZ-Cultar concentrations used in the multiplication phase

It is believed that height decreases with an increase in the PBZ-Cultar concentration used in the culture media. The overall results illustrated that the mean height decreased with an increased PBZ-Cultar concentration at both subcultures. Based on the results obtained as seen in Table 1, T1, being the control, had a significant difference in height with respect to the other treatments in the first subculture. T2 also had a significant difference compared with T3 and T4. However, there wasn't any significant differences between T3 and T4.

Treatments	Height at 1st Subculture	Height at 2nd Subculture
T1 (0.00%)	4.55a	3.62a
T2 (0.05%)	3.80b	3.40a
T3 (0.08%)	3.21c	2.99b
T4 (0.10%)	2.97c	2.80b

Table 1. Plant height at first and second subculture

Different letters between treatments differ statistically for $p < 0.05$ according to Dunnett's test

The results showed that higher concentrations of PBZ-Cultar retarded the growth of the plants. At the second subculture, T1 and T2 had greater plant heights, which were significantly higher than the other two treatments. The mean height for T3 and T4 had no significant difference between them at the second subculture. It can be inferred from the results that there would be no difference in heights, up to the second subculture, if either T3 or T4 concentrations were used.

The results clearly indicate that the mean height decreased with an increased in PBZ-Cultar concentration in the culture medium in the multiplication phase.

Figure 1 shows the growth of plantlets when exposed to the different treatments of PBZ-Cultar concentrations just before the second subculture. further illustrates the decrease height with an increase in PBZ-Cultar concentration in the culture media. In this stage of micropropagation, the objective is not for the vitroplants to grow tall, but

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rather for there to be as many lateral shoots as possible. By controlling the plant height, then more nutrients can go into producing more lateral shoots.
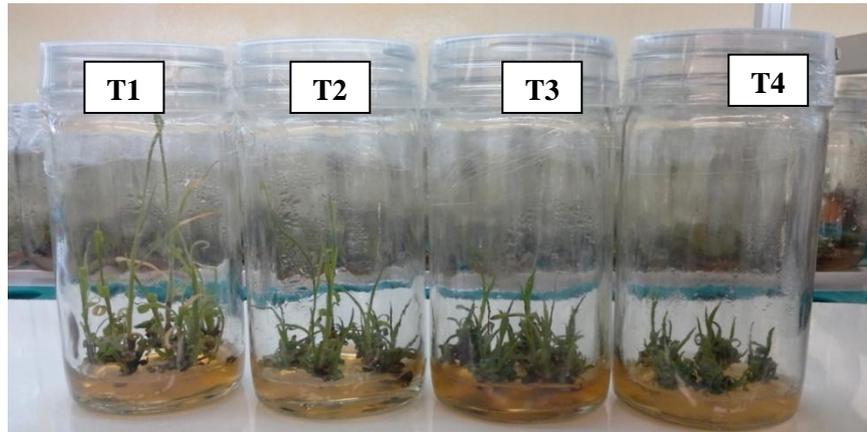
Figure 1. Plant growth in relation to different Cultar concentrations

Generally, the number of dead leaves would increase as plants grow higher because the nutrients are being exhausted and the carbon dioxide level inside the culture vessel is constantly being used up by the leaves. Most commonly, plants with larger heights will have larger leaves, which further cause the available carbon dioxide inside the vessel to be consumed at a faster rate. At the end of the second subculture, the total number of dead leaves per treatment was counted.

There wasn't a trend in terms of the number of dead leaves in the different concentrations used; however, in all the treatment that contained PBZ-Cultar, there were significantly less dead leaves when compared to the control (T1). There was no significant difference in the number of dead leaves between the treatments T2 and T4, while treatment T3 had the lowest number of dead leaves and the control had the highest number. These results clearly indicate that PBZ-Cultar had an effect in terms of lowering the number of dead leaves on the plantlets in vitro.

The number of dead leaves in the table below represent the total number from each treatment at the end of the second subculture.

Table 2. Number of total dead leaves after the second subculture.

Treatments	Number of Dead Leaves
T1 (0.00%)	64.8c
T2 (0.05%)	52.2b
T3 (0.08%)	43.9a
T4 (0.10%)	53.7b

Different letters between treatments differ statistically for $p<0.05$ according to Dunnett's test

Multiplication coefficient refers to the number of shoots or plantlets obtained per plant after subculturing. PBZ-Cultar has been popular for increasing yields during subculture therefore producing a higher multiplication coefficient. Generally, a higher concentration of the growth regulator will result in a higher multiplication coefficient and increase the number of shoots per plant. It can be noted that the highest multiplication coefficient was achieved by both T3 and T4 with an average mean of 2.8 for both treatments in the first subculture (Table 3). Based on the results, there is a significant difference in the multiplication coefficient between the treatments T3 and T4 when compared to T1 and T2 at both subculture intervals. However, treatments T1 and T2 had no significant difference between them. Similarly, no significant difference was observed between treatments T3 and T4.

Table 3. Multiplication coefficient at both subcultures

Treatments	Multiplication Coefficient (First subculture)	Multiplication Coefficient (Second subculture)
T1 (0.00%)	2.2b	2.2b
T2 (0.05%)	2.1b	2.3b
T3 (0.08%)	2.8a	2.8a
T4 (0.10%)	2.8a	2.9a

Different letters between treatments differ statistically for p<0.05 according to Dunnett's test

Valdes et al., (2017) carried out experiments using different concentrations of paclobutrazol; however, this was done in the acclimatization phase. Results showed that the best concentration for all the parameters evaluated was 0.015%. Berova and Zlatev (2000) stated that in Lycopersicon esculentum Mill, the reduced height and the increased thickness of the young plant stem, as well as the accelerated root formation are a significant advantage of PBZ treatment, contributing to the improvement of seedling quality at planting.

Rahman et al., (2016) applied PBZ that significantly reduced the growth of clonal palms with the optimal concentration of PBZ being 50 mg/L when applied as a foliar spray. PBZ-treated palms exhibited more compact structure as compared to untreated controls. Longitudinal sections of PBZ-treated oil palm clones revealed that both leaves and stems comprised of fewer cells each with a smaller volume. PBZ-treated plants exhibited a higher rate of photosynthesis compared to controls and this was correlated with an accumulation of starch in cells of the stem of the plants.

The results obtained in this study proved that PBZ is a growth retardant by minimizing cellular expansion.

Acclimatization phase

Being a growth retardant that would stimulate more lateral shoots rather than apical growth, it was important to study the residual effects of using PBZ-Cultar in the multiplication phase. The vitroplants from the experiment in vitro were planted in the greenhouse as described previously. The first parameter evaluated in the acclimatization phase was survival rates. All treatments had a survival rate of 98%. This high percentage is attributed to the cultural practices carried out in the greenhouse. The plants were transplanted in the late evening when the environmental temperature was not so elevated. The irrigation regimen also guarantees adequate relative humidity and moisture in the substrate, which are extremely important for survival at this stage (Rangel-Estrada et al., 2016).

The other parameters evaluated at the end of the acclimatization phase were plant height and number of leaves (Table 4). With respect to the plant height, T3 had the highest value (14.3 cm) with significant difference when compared to the other treatments. The lowest value was T4 and this could be attributed to the residual effects of the PBZ-Cultar used in the multiplication phase. Because of this effect in these ex vitro conditions, then 0.08% PBZ-Cultar is a more appropriate concentration to use in the multiplication phase.

Table 4. Plant height and number of leaves at the end of the acclimatization phase

Treatments	Plant height (cm)	No. of leaves
T1 (0.00%)	13.1 b	4.2 b
T2 (0.05%)	13.2 b	4.3 b
T3 (0.08%)	14.3 a	5.6 a
T4 (0.10%)	13.0 b	5.5 a

Different letters between treatments differ statistically for $p < 0.05$ according to Dunnett's test

The number of leaves in ex vitro conditions play a huge role in the overall growth and development of the plants because it determines the plant's photosynthetic capacity. With respect to the number of leaves, both treatments T3 and T4 had superior values, but without any significant differences between them; however, they were both significantly superior to T1 and T2. 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.

Conclusion

The results from this experiment indicate that PBZ-Cultar significantly reduced the plant height as well as number of dead leaves. It also had significant increase in the multiplication coefficient of the sugarcane variety CPCL99-4455 in the multiplication phase in vitro. Based on the results obtained from the acclimatization phase, the best concentration of PBZ-Cultar was 0.08%.

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