Paclobutrazol (PBZ)-Cultar regulator in sugarcane: The effect of different PBZ-Cultar concentrations on Sugarcane *(Saccharum)* of variety *CPCL99-4455*

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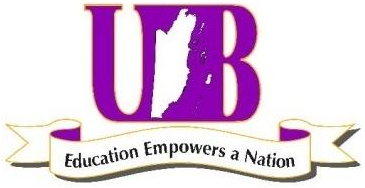

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Abstract

An experiment was conducted to investigate the effect of different Paclobutrazol (PBZ)-Cultar concentrations used in plant growth media (MS Media) on *in-vitro* multiplication of sugarcane variety CPCL99-4455. Paclobutrazol (PBZ)-Cultar is considered one of the most essential plant growth regulators that restricts vegetative growth and induces flowering in fruit species (Saxena et al., 2013). Four PBZ-Cultar treatments were used: T_1 was the negative control (0 g/ml per 100 ml H_2O stock solution), T_2 (0.25 g/ml per 100 ml H_2O stock solution), T_3 (0.4 g/ml per 100 ml H_2O stock solution), and T_4 (0.5 g/ml per 100 ml H_2O stock solution). The parameters used to determine PBZ-Cultar effects were average height per treatment, number of dead leaves and multiplication coefficient number. The results revealed that the culture medium supplemented with T_3 (0.4 g/ml per 100 ml H_2O stock solution) Cultar concentration, produced similar average heights in comparison to the T_4 (0.5 g/ml per 100 ml H_2O stock solution). T_3 showed to have the least number of dead leaves when sub-cultured after 24 days. Results also concluded that there was no significant difference between treatments T_3 and T_4 which obtained the highest multiplication coefficient number thus producing more plants per jar compared to treatments T_1 and T_2. Furthermore, it was also observed that the T_4 concentration had growth that produced coiling of leaves which made it difficult for the plant clumps to be divided. Using MS media treated with T_3 (0.4 g/ml per 100 ml H_2O stock solution) PBZ-Cultar concentration for *in-vitro* multiplication therefore increases production, reduces number of dead leaves and requires less plant growth regulator quantities. It also facilitates sub-culturing as it may reduce the time consumed during the process.

Keywords: Paclobutrazol (PBZ), Cultar, sugarcane, micro-propagation, MS medium, Sub-culturing, Multiplication coefficient

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1. Introduction

Raw sugar is derived from both cane sugar and sugar beet. Brazil and India are the world's two largest sugar producers. Together, they have accounted for over half the world's sugar cane 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 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 (Sugar Industry of Belize, 2010). Day by day increasing use of sugar and its relevant products have created a challenging situation for sugarcane researchers and growers.

However, the yield potential of 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 (Ali et al. 2008). The spectacular findings in plant tissue culture have attracted interest all over the world of agriculture. Specific methods have been generated for the propagation of genotype and efficient regeneration of plants by means of micro-propagation (Kyte and Kleyn, 1996).

The common sugarcane plant belonging to the genus *Saccharum* (Poaceae) is an important commercial and industrial crop which accounts for nearly 70% of sugar produced worldwide (Taylor and Koo, 2012). When comparing to other major crop efforts for improvement, sugarcane crops are limited and relatively recent in terms of inter-specific hybrid introduction. The progress of sugarcane plant breeding is hindered by its thin gene pool, complex genome, poor fertility and the long breeding or selection cycle (Taylor and Koo, 2012). The mentioned constraints, however, make sugarcane a good entrant for micro-propagation.

In sugarcane, micro-propagation 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 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 (Latimer & Scoggins 2012). These PGRs control plant height by inhibiting the production of gibberellins which is the primary plant hormone responsible for cell elongation. Furthermore, these growth-retardant effects are primarily seen in stem, petiole, and flower stalk tissues (Latimer & Scoggins 2012). 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 plant hardening phase. However, high concentrations of PBZ will deliver a plant that is unmanageable *in vitro* (Latimer & Scoggins 2012). 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 effect of Paclobutrazol (PBZ) – Cultar concentration on *in vitro* multiplication of sugarcane variety CPCL99-4455 in terms of height, number of dead leaves, and multiplication coefficient.

2. **Literature Review**

In the late fifteenth century, sugar used to be a luxury product when Spanish and Portuguese expanded sugarcane cultivation in countries such as Puerto Rico, Cuba and Brazil and exported them back to Europe to be refined (Pham, 2014). It wasn't until the seventeenth century that the British Government developed sugarcane cultivation areas and sugar mass production on West Indian islands. According to Pham (2014), sugar became a popular product for all classes ever since. As documented by Pham (2014), up until now, the sugar industry is one of the oldest food processing industries in the world, with more than one hundred (100) countries and territories participating in the value chain. As reported by Pham (2014), the scale of global sugar production is about 174.8 million tonnes in crop year.

Modern sugarcane cultivars are the products of crosses between species of the genus *Saccharum* that were made by breeders in the late 19th century (Cheavegatti-Gianotto et al., 2011). The most important species contributing to modern sugarcane varieties were *S. officinarum,* which was widely cultivated for its ability to accumulate sucrose in its stalks, and *S. spontaneum,* which is a vigorous, widely adapted wild species which contributed genes for disease and stress resistance (Cheavegatti-Gianotto et al., 2011).

Saccharum spp. is a member of the grass family (Poaceae). This grass is widely cultivated, providing around 70% of the world's sugar (Clayton et al. 2006). As stated by Clayton et al. (2006), sugarcane produces the highest number of calories per unit area of cultivation of any plant. This crop most likely originated in New Guinea, and was taken to the Americas by the explorer Christopher Columbus on his second expedition there in 1493 (Clayton et al. 2006). Sugar cane is now grown in more than 70 countries, mainly in the tropics, but also in some sub-tropical areas. *Saccharum* and its hybrids are grown for the production of sugar, ethanol, and other industrial uses in tropical and subtropical regions around the world (Biradar et al. 2009). The stems and the byproducts of the sugar industry are sometimes used for feeding to livestock (Biradar et al. 2009).

The genus *Saccharum* was first described by Linnaeus (1753) in his book *Species Plantarum* (Cheavegatti-Gianotto et al., 2011). The generic name is derived from the Greek word *sakcharon,* which means *sugar* and was properly Latinized by the author (Cheavegatti-Gianotto et al., 2011).

Sugarcane Taxonomy:

Domain: Eukaryota
Kingdom: Plantae
Phylum: Anthophyta
Class: Equisetopsida
Order: Poales
Family: Poaceae
Genus: *Saccharum*

The application of plant growth regulators and the use of different culture media are common practices in the micro-propagation of crops. Much research has been conducted on the response of plants to plant growth regulators. Plant growth retardant induced manipulation in physiological activity, has been considered an important determinant of productivity enhancement in fruit crops (Saxena et al 2013). Among them, Paclobutrazol (PBZ) is considered as one of the important plant growth regulators which restricts vegetative growth and induces flowering in many fruit species including mango (Saxena et al 2013). Beneficial effects of paclobutrazol in induction of flowering in different mango cultivars have been reported (Yadava and Singh 1998; Singh and Singh 2003).

On a study conducted by Zaffar et al. (2014), green elongated shoots with similar sizes (4.0-6.0 cm) were cut out and incubated on MS basal medium supplemented with different combinations of paclobutrazol (0,5 and 10 mg/l) and sucrose (60,90 and 120 $g.1^{-1}$). After 8-10 weeks of observation, a small globular micro-corm was observed on the basal portion of the shoots (Zaffar et al., 2014). According to the research, the careful selection of healthy shoots is one of the rudiments to obtain micro corms *in vitro*, and support the present investigation where shoot length and quality were found to be important for better *in vitro* corm formation. The supplementation of paclobutrazol and sucrose in the medium was found to be beneficial for micro corm formation (Zaffar et al., 2014).

Klinac et al. (1991) investigated the effect of Paclobutrazol (Cultar) by applying it to two-year-old nashi trees either as a soil drench or as a series off our post-flowering foliar sprays. All cultivars treated with cultar showed a significant reduction in vegetative growth within the first season and for up to 4 years after initial application (Klinac et al. 1991). Most reduction in growth was obtained from soil applications. Least reduction in growth was from a foliar application at a lower rate. Over the period of the trial, total fruit numbers and yield, per tree,

were reduced by paclobutrazol application, though average fruit size was generally increased from Year 2 onwards. Treatment with paclobutrazol generally reduced the percentage of growth (Klinac et al. 1991). Soil treatment the cultivars, also produced a more even distribution of flowers and fruit throughout the canopy (Klinac et al. 1991).

Davis (1991) studied the regulation of tree growth and development with Triazole compound and further compared the plant cold hardiness with Paclobutrazol (PBZ)-treated plants. In his study with cherry trees, the altered dormancy characteristics in the paclobutrazol-treated trees were accompanied by reduced mid-winter cold hardiness of the flower buds (Davis, 1991). The treated trees had mid-winter T_{50} (temperature required to kill 50% of the buds) values that was about 2°C higher than non-treated controls. A similar observation has been made with several other *Prunus* species that were treated with paclobutrazol (Davis, 1991). These findings, however, are in contrast to research with a variety of herbaceous species where paclobutrazol increased cold hardiness. Further research is yet being done but this, in particular, concluded that too much or too little concentration introduced in plant media renders different results in terms of cold hardiness of specific plant species.

According to Quiroz (2014), all Sugarcane varieties are received at the Belize Sugar Industry (BSI) from a breeding program located in Barbados. A small number of seeds are brought and grown in a nursery until they can be planted in a testing plot. These seeds are then closely monitored for approximately ten months as they grow (Quiroz, 2014). The plants being grown are assessed in terms of specific standards according to BSI such as stunted growth, pale leaves, thin stalks etc. After being assessed, those who are able to meet the BSI's standards are then cut and their sets are replanted. These new cuts are further monitored for another 10 months and during those months, the plants that are not up to the required standards are then weeded out. Each plant is numbered at the time the replanting is done. This replanting procedure and weeding out of sub-standard plants goes on for years until the plant may show good growth. However, when plants are able to show good growth, those are then assessed in terms of sugar content.

Their content is compared to that of B79-474 which is the most popular variety used in Belize (Quiroz, 2014). This variety in particular is therefore used as a standard. In the event that it does meet all requirements in terms of growth and sugar content, the variety is then recorded. The letter being the location the variety was bred, the first two numbers indicate the year it was selected from the field, and then the number of the plant that was selected (from the field). The

sugarcane variety CPCL99-4455 was developed by Canal Point (Louisiana) and Florida Sugar Cane League in the year 1999 (Quiroz, 2014). The particular plant was plant number 4,455. This variety has been released in the US as it has already been proven that it shows good growth in that region (Acton, 2012).

3. Materials and Methods

The micro-propagation of sugarcane experiment was done between the periods of February 20 to April 18, 2015. This experiment was conducted at the tissue culture laboratory in the University of Belize Central Farm (UBCF) Campus. Already established sugarcane plants from the multiplication phase were used for this experiment.

3.1 General procedures and laboratory techniques

3.1.1 Instruments for sub-culture

The basic instruments such as the forceps, scalpel and glass tile were wrapped in foil, placed in a plastic bag and sealed in order for it to be autoclaved. They were autoclaved with exposure to high pressure saturated steam at 121^{o}C (249^{o}F) with a pressure of one (1.0) bar for 20 minutes. This ensured that the instruments to be used were properly sterilized. An autoclave tape was used which helped to indicate when the autoclaving process was complete by changing its color to black. Furthermore, prior to using the laminar flow chamber, the ultraviolet (UV) light was turned on for approximately twenty (20) minutes. The UV light was avoided as much as possible due to the fact that excessive exposure to this radiation may be harmful to human health. After leaving the UV light on for twenty (20) minutes, the laminar flow chamber was thoroughly sprayed with 70% alcohol and properly sanitized with sterilized tissue paper. Immediately after removing the plastic bag and foil wrap from the forceps and scalpel, it was inserted into the Germinator or also known as the Hot Bead Sterilizer. The glass tile was also unwrapped, sprayed with alcohol and flamed in order to maintain it properly sanitized. 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. The transparent glass plate was placed on top of the scale. The plants were all cut at 1.5 cm in length.

3.1.2 Culture media preparation and jar sterilization

Culture vessels of 0.67 liter capacity were used for the sub-culturing process. Before placing the culture medium in it, these culture vessels were properly sterilized with 5% Clorox. The 5% Clorox solution was obtained by mixing 750ml of Clorox with 14,250ml of distilled water. The culture vessels were further immersed in the prepared Clorox solution for at least two (2) minutes, were then taken out and flipped over for them to dry off. While the jars were left to

dry off, the culture media was prepared in a total volume of 4.0 liters. A 4.0L culture media was prepared in order to suffice for approximately 64 jars with each jar containing 25ml of the multiplication media. The culture media was prepared using the standard MS Salts (Murashige and Skoog) solution (Table 2.0 of appendix 1) and the different concentrations of the growth regulator. Furthermore, a specific concentration of growth regulator was introduced in each liter of solution for experimental purposes.

While the solution was being boiled, the pH was being adjusted with Hydrochloric acid or Sodium Hydroxide in order to maintain it at a pH of 5.8. The media was boiled with all the ingredients until it became clear in appearance. This clarity indicated that the media was ready. Immediately after the media was ready, a 50ml measuring cylinder was obtained to measure 25ml of the culture media which was then estimated thereon in each jar using a liquid dispenser in order to speed up the process. Instantaneously after the media was dispensed into the jars, the culture vessels were properly capped and further sealed with plastic wrap around the cap ends.

3.2 Experimental detail and setup

The plant growth regulator used in this experiment was Cultar. This growth regular is a plant retardant that is well known for its ability to improve the yield of certain plants. According to the current protocol obtained from the UB Central Farm laboratory, a 25 g/ml per 100 ml H_2O stock solution of Cultar is being used in the multiplication media. For the purpose of this experiment, four PBZ-Cultar concentrations were tested: T_1 (0 g/ml per 100 ml H_2O stock solution), T_2 (0.25 g/ml per 100 ml H_2O stock solution), T_3 (0.4 g/ml per 100 ml H_2O stock solution), and T_4 (0.5 g/ml per 100 ml H_2O stock solution). At the start of the experiment, sixteen (16) jars of each of the treatments were used to plant the sugarcane variety CPCL99-4455. These jars were then separated into groups of four (4) so as to obtain four replicates with the four sets of treatments in each. The specific setup used in this experiment is known as the Randomized Complete Block Design (see Figure 1 in appendix 1). The treatments were randomly placed in the four different replicates. Additionally, a jar-wide set of Guard Plants were placed on each of the sides of the experimental setup (Figure 1, Appendix 1) in order to prevent any external contamination and to prevent additional light to the plants on the outside layers.

Figure 3.2.1. Diagram illustrates the experimental setup *in vitro* during multiplication phase

3.3 Statistical Analysis and results

The data was collected in terms of height, multiplication coefficient and number of dead leaves. These data sets were then analyzed using statistical computer software known as MegaStats, with the aim of determining the statistical differences, if any, among the results. More specifically, an Analysis of Variance (ANOVA) was conducted which allowed inferences to be made about the statistical differences in each of the experiments under study. A specific ANOVA test was conducted in each of the variables or data sets being height, number of dead leaves and finally the multiplication coefficient.

4. Analysis and Discussion

An analysis was conducted to assess the variances between four different treatments of PBZ-Cultar concentration. 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*). Four PBZ-Cultar treatments were used: T_1 (0 g/ml per 100 ml H_2O stock solution), T_2 (0.25 g/ml per 100 ml H_2O stock solution), T_3 (0.4 g/ml per 100 ml H_2O stock solution), and T_4 (0.5 g/ml per 100 ml H_2O stock solution).

4.1 Analysis for height of the four Treatments after the first subculture

Height is believed to decrease when there is an increase in the PBZ-Cultar concentration used in the growth media for the plants. Table 8.2.2 in appendix 2, presents a summary of the mean number and standard deviation for the four treatments (T1, T2, T3 and T4) after the first sub-culture was performed (24 days later). The overall results illustrate that the mean height decreased with an increased PBZ-Cultar concentration treatment at the end of the first subculture. Based on the results obtained, T1 had a significant difference in height to T2, T3 and T4. T2 had a difference with T4 and T3. However, it was noted that T4 and T3 treatments did not have a significant difference in the mean heights.

Table 4.1.1 Table showing mean values of the plant heights and its significant relationship

Treatments	Height after 1st Subculture
T1	4.552a
T2	3.800b
T3	3.214ce
T4	2.970de
Difference in letters indicate p<0.05	

As can be observed from the box-plot (Figure 8.3.1.1 in appendix 3), the height means for T1 and T2 lie above the average mean (higher heights), while the mean heights for T3 and T4 lie below the average mean (lower heights). The height means of T3 and T4 had no significant difference. It can be inferred from the results that there would be no difference in heights, up to the first subculture, if either T3 or T4 concentrations were used. T4 would however, expend an

additional 0.1 g/ml per 100 ml H_2O stock solution and would serve the same purpose as T3 therefore making it less effective to use.

4.2 Analysis for height of the four Treatments after the second subculture, at the end of the experiment

At the end of the second subculture, the mean heights were recorded. Table 8.2.4 in appendix 2 provides a summary of the mean and standard deviation for the four treatments under study. The results illustrate once more that the mean height decreased with an increased PBZ-Cultar concentration treatment medium. Furthermore, it can be deduced that at the end of the experimental period, the mean heights of T1 and T2 had no significant difference (.0846) and the mean heights of T3 with T4 did not have a significant difference either (See table 4.2.1 below).

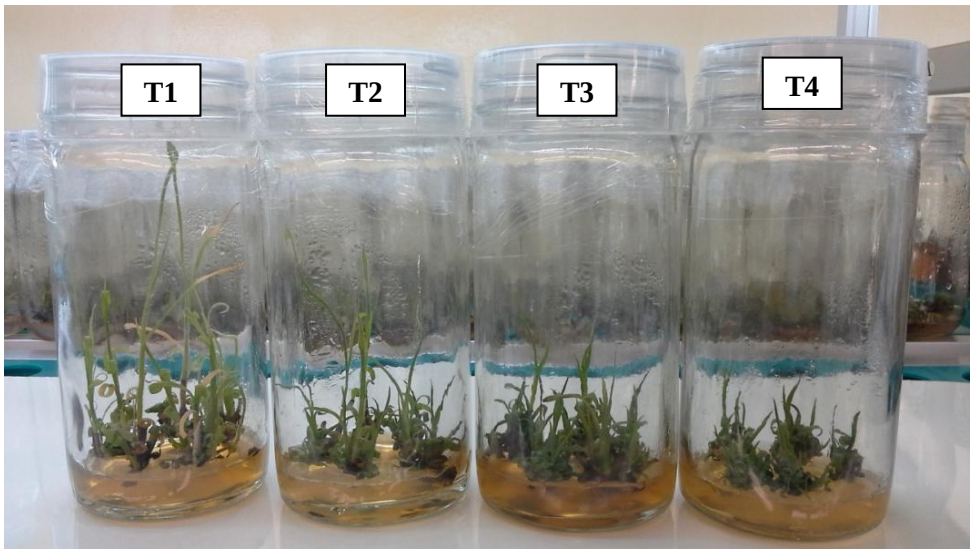
Table 4.2.1 Table showing mean values of the plant heights and its significant relationship

Treatments	Height after 1st Subculture
T1	3.62a
T2	3.40a
T3	2.99bd
T4	2.80cd
Difference in letters indicate p<0.05	

The height means for T1 and T2 (as seen in box-plot, figure 8.3.2.1 in appendix 3) lie above the average mean, while the mean heights for T3 and T4 lie below the average mean. It can be inferred from the results that there would be no difference in heights if T1 or T2 concentrations were used. Similarly, it is observed that T3 and T4 concentrations would also have no difference in plant growth/height when used in the culture media.

The following figure (Figure 4.2.1) depicts the growth of plantlets when exposed to the different treatments of PBZ Cultar concentrations. It further illustrates the decrease in height with an increase in Cultar concentration in the culture media.

Figure 4.2.1 Diagram illustrates growth of plantlets treated with different Cultar concentrations



4.3 Analysis for the number of dead leaves of the four treatments after the first subculture

Generally, the number of dead leaves in a subculture jar would increase as plants grow higher because the oxygen level inside the jar is constantly being used up by the leaves. Most commonly, plants with larger heights will have larger leaves which further cause the available oxygen inside the jar to be consumed at a faster rate. At the end of the first subculture, the number of dead leaves per jar in each of the treatments was counted. Table 8.2.6 in appendix 2 gives a summary of the mean and standard deviation of dead leaves count for the four different treatments.

Treatment T2 had a borderline significant difference in the number of dead leaves from T3 ($p=0.0495$). Similarly, the number of dead leaves between T3 and T4 also had a borderline difference. There was no significant difference in the number of dead leaves between the treatments T2 and T4 ($p=0.7168$). Overall, treatment T3 had the lowest number of dead leaves while treatment T1 had the highest number of dead leaves. The T2 and T4 treatments had no significant difference in terms of number of dead leaves (refer to table 4.3.1).

Table 4.3.1 Table showing mean values of the number of dead leaves and its significant relationship among the four treatments

Treatments	Height after 1st Subculture
T1	64.8a
T2	52.2be
T3	43.9c
T4	53.7de
Difference in letters indicate p<0.05	

Figure 4.3.1 Diagram illustrating dead leaves separated from initial clumps during subculture

4.4 Analysis for the multiplication coefficient number of the four treatments after the first subculture

Multiplication coefficient refers to the number of new shoots or plantlets obtained per plant. PBZ-Cultar has been popular for increasing yield during subculture therefore producing a higher multiplication coefficient value. Generally, a higher concentration of the growth regulator will result in a higher multiplication coefficient number per plant. Five clumps, each having three single plants were placed in each jar. To obtain the multiplication coefficient number, the number of total clumps resulting from each jar was recorded. Based on the data from table 8.2.8 in appendix 2 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. The analysis of variance shows that there was a significant difference between the mean multiplication coefficient numbers of the treatments. Based on the results presented in table 4.4.1, it can be concluded that there is a significant difference in multiplication coefficient number between the treatments T4 when compared to T1 and T2; as well as treatment T3 when compared to T1 and T2. However, treatments T1 and T2

had no significant difference in terms of multiplication coefficient. Similarly, no significant different was observed between treatments T3 and T4 (refer to table 4.4.1).

Table 4.4.1 Table showing mean values of the multiplication coefficient and its significant relationship among the other four treatments

Treatments	Height after 1st Subculture
T1	2.2a
T2	2.1a
T3	2.8b
T4	2.8b
Difference in letters indicate p<0.05	

Treatment T3 and T4 have a value that is above the mean average (refer to figure 8.3.4.1 appendix 3). Treatment T1 and T2 both have a mean that is below the mean multiplication coefficient average. The observed results conclude that treatment T3 and T4 had no difference between both; however, they had a higher multiplication coefficient than treatment T1 and T2.

Figure 4.4.1 Figure illustrates the number of clumps obtained from a single jar; each clump having three single plants

5. **Conclusion**

The results from this experiment indicate that there is a significant difference in height among the four Paclobutrazol (Cultar) concentrations used as treatments. However, no significant difference was seen in heights between treatment T3 and treatment T4 during the first subculture. Similarly, treatments T3 and T4 had no significant difference at the end of the second subculture. It was also concluded that there was no significant difference in height between treatments T1 and T2 at the end of the experiment. In terms of the number of dead leaves, treatment T3 had the lowest count of dead leaves and had marginal significant difference with the rest of the treatments. Treatment T1, having no Cultar concentration, had the highest number of dead leaves. Moreover, treatments T3 and T4 did not have a significant difference in terms of multiplication coefficient. Additionally, these two treatments had the highest multiplication coefficient among the four treatments. Based on the results, it can be concluded that using treatment T3 in replacement of T1, T2 and T4 would encourage yield and efficiency in the *in vitro* multiplication phase. Using treatment T3 will therefore require the use of less growth regulator; will increase the production of plantlets (multiplication coefficient); and will reduce the number of dead leaves.

6. **Recommendations**

The current use of 0.025 Cultar concentrations at the UBCF tissue culture lab has the same results as if no Cultar was added to the culture media. It is therefore recommended to increase the Cultar concentration to 0.04 which will also increase production. Plants to be treated with Plant Growth Regulators (PGRs) should be healthy, turgid, and unstressed–never wilted. In order to obtain more accurate results, a larger sample of sugarcane plantlets should be used for the experiment. It is recommended that all necessary procedures for sterilization prior and during the subculture process be taken in order to avoid contamination. The sub-cultured plants will have to be further monitored *ex vitro* in order to assess its growth and survival potential.

7. **Bibliography**

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8. **Appendices**

Appendix 8.1 Experimental Design and Media Preparation

Table 8.1.1 Table illustrates the MS Salts (Murashige and Skoog) quantity along with plant growth regulator concentration used according to the protocol.

Sugarcane Multiplication Media				
Components	**Multiplication** (Phase II)			
	1 L	**2 L**	**4 L**	**5 L**
Macronutrients	20mL	40mL	80mL	100mL
Micronutrients	20mL	40mL	80mL	100mL
KI	20mL	40mL	80mL	100mL
$CaCl_2$	20mL	40mL	80mL	100mL
Fe-EDTA	20mL	40mL	80mL	100mL
Inositol	0.1g	0.2g	0.4g	0.5g
Sucrose	30g	60g	120g	150g
Vit. B^+	20mL	40mL	80mL	100mL
Kinetin	5mL	10mL	20mL	25mL
6-BAP	0.6mL	1.2mL	2.4mL	3mL
IAA	5mL	10mL	20mL	25mL
Cultar	***0.25mL**	0.50mL	1.0mL	1.25mL

Note: pH was adjusted to 5.8 after both liquid and solid components are added and after media has been made up accordingly.

Figure 8.1.2 Table presents the experimental layout (Randomized complete block design)

		Guard plants (GPs) – 1 jar wide		
Rep. 1	GPs	Q1 (4 jars)	Q2 (4 jars)	GPs
		Q3 (4 jars)	Q4 (4 jars)	
Rep. 2	GPs	Q1 (4 jars)	Q2 (4 jars)	GPs
		Q3 (4 jars)	Q4 (4 jars)	
Rep. 3	GPs	Q1 (4 jars)	Q2 (4 jars)	GPs
		Q3 (4 jars)	Q4 (4 jars)	
Rep. 4	GPs	Q1 (4 jars)	Q2 (4 jars)	GPs
		Q3 (4 jars)	Q4 (4 jars)	
		GPs	GPs	

Note: All jars in each replicate were maintained under the same environmental conditions as practicable.

Appendix 8.2 Descriptive Statistics

Table 8.2.1 Table provides actual height readings of the different treatments per replicate at the end of the first subculture (4 jars per quadrant in each replicate)

Replicate 1				Replicate 2				Replicate 3				Replicate 4			
T1	**T2**	**T3**	**T4**	**T1**	**T2**	**T3**	**T4**	**T1**	**T2**	**T3**	**T4**	**T1**	**T2**	**T3**	**T4**
6.0	6.0	4.0	3.8	3.8	2.5	4.0	3.8	6.5	3.0	2.0	3.5	5.0	4.0	3.0	2.5
5.5	5.0	3.0	3.0	2.0	4.5	3.8	4.5	5.0	2.5	3.0	3.0	5.5	3.5	2.5	3.0
6.0	3.0	3.5	3.0	3.0	3.0	3.8	3.0	4.8	3.5	2.5	2.0	5.5	5.0	4.0	2.0
5.5	6.0	4.5	3.0	4.5	3.0	3.0	4.0	4.5	4.0	3.5	2.5	5.5	3.5	3.0	4.0
6.5	5.5	3.5	3.5	4.0	3.5	4.5	3.5	4.5	3.5	4.0	2.5	3.5	4.5	4.0	4.5
4.0	4.0	3.0	3.5	5.0	5.0	2.5	2.0	4.0	3.5	2.5	3.0	4.5	4.0	4.0	3.0
3.0	4.0	4.0	2.5	6.0	3.5	3.0	3.0	4.0	2.5	4.0	3.0	4.5	3.5	3.5	3.0
4.0	4.0	2.0	3.0	5.5	6.0	3.5	2.5	6.0	3.0	4.0	3.5	5.0	3.0	4.0	4.5
4.0	4.5	4.0	3.0	5.0	4.0	3.0	3.0	3.0	4.0	3.5	2.0	6.0	2.5	2.5	3.8
4.0	5.5	2.5	3.0	4.0	4.5	2.5	3.0	4.5	4.0	2.0	2.0	5.0	5.0	4.5	3.8
5.5	5.5	3.5	3.0	4.0	3.0	3.0	3.0	5.0	3.0	2.5	2.0	4.0	3.5	5.0	3.0
4.0	3.0	2.5	3.5	4.0	2.5	2.0	2.0	4.0	4.5	3.5	2.5	4.5	3.0	3.0	2.5
4.5	5.5	3.0	3.0	3.5	3.0	2.5	2.5	4.0	4.0	2.5	3.8	4.0	3.5	2.5	2.5
6.5	4.0	4.0	2.5	5.5	4.0	2.0	3.0	3.0	4.5	4.5	4.0	5.5	3.5	3.0	2.5
5.5	3.0	3.0	2.5	4.0	4.0	3.5	3.0	3.0	4.0	4.0	3.5	3.5	3.0	3.0	2.0
4.0	3.0	2.5	3.0	4.5	3.0	4.0	4.0	4.0	4.5	3.5	2.0	6.0	2.0	4.0	2.0
3.0	2.0	3.0	2.0	4.0	2.0	2.5	3.5	4.5	5.0	2.5	2.5	4.5	2.5	4.0	2.0
3.5	4.5	3.0	3.5	4.0	3.0	3.0	3.5	5.0	5.0	4.0	2.0	4.5	3.5	4.0	1.8
5.0	3.5	2.0	2.0	4.5	3.0	4.0	2.0	6.5	4.5	3.0	3.5	5.5	3.5	2.0	3.0
3.0	6.0	2.0	3.5	4.0	3.5	3.0	3.8	4.5	4.0	3.0	4.0	4.0	3.0	2.0	3.0

Table 8.2.2 Table summarizes descriptive statistics of the height for the four treatments.

Mean	*n*	*Std. Dev*	
4.552	80	0.9716	T1
3.800	80	0.9892	T2
3.214	80	0.7537	T3
2.970	80	0.6954	T4
3.634	320	1.0533	Total

Table 8.2.3 Table provides actual height readings of the different treatments per replicate at the end of the second subculture (6 jars per quadrant in each replicate)

Replicate 1				Replicate 2				Replicate 3				Replicate 4			
T1	**T2**	**T3**	**T4**	**T1**	**T2**	**T3**	**T4**	**T1**	**T2**	**T3**	**T4**	**T1**	**T2**	**T3**	**T4**
4.0	3.5	3.0	2.0	3.5	4.0	5.0	3.5	5.5	3.5	2.5	2.5	4.5	3.0	2.5	3.0
5.0	3.5	3.0	2.5	3.0	4.5	5.0	3.0	2.5	3.0	3.0	3.5	5.0	2.0	2.5	1.5
4.0	4.0	2.0	2.5	2.5	3.5	4.0	2.5	4.5	4.0	2.5	2.0	3.0	2.5	2.0	2.5
4.0	5.5	4.0	3.5	2.0	3.5	3.5	2.0	3.5	5.5	2.0	2.5	3.0	2.0	2.0	2.0
3.5	4.5	2.5	4.0	4.0	4.5	4.0	4.0	3.5	4.0	3.0	5.0	4.0	2.0	2.5	3.0
4.0	4.0	2.0	3.0	2.5	3.0	2.5	2.5	2.0	5.0	2.5	2.5	2.5	2.0	2.5	1.5
3.0	6.0	2.0	3.5	2.5	5.0	5.0	2.5	3.5	4.0	3.5	2.5	5.0	4.0	2.5	2.0
6.0	3.0	2.0	2.5	2.0	4.5	4.0	2.0	4.0	4.0	2.0	2.0	3.0	3.0	2.0	2.5
5.0	2.5	3.5	4.0	2.0	5.0	2.5	2.0	4.0	4.0	3.5	2.5	4.5	2.0	3.0	3.0
4.5	3.0	3.5	4.5	3.5	3.0	2.5	3.5	5.5	3.0	2.5	2.0	3.5	2.0	2.5	2.0
5.0	3.0	3.5	2.5	2.0	5.0	3.0	2.0	5.0	4.0	2.5	2.0	4.0	2.0	2.0	2.0
4.5	5.0	3.0	2.5	3.5	3.0	4.5	3.5	3.0	3.0	2.0	2.5	3.5	4.5	2.0	2.5
5.5	6.0	2.5	3.5	3.0	2.0	3.0	3.0	6.0	2.5	3.0	2.0	4.0	2.0	2.0	2.0
4.0	3.5	5.0	3.0	2.0	2.5	2.5	2.0	5.0	4.0	2.0	2.5	4.0	2.5	3.0	2.5
3.5	5.0	3.5	2.5	2.5	3.0	5.0	2.5	3.0	3.5	3.0	3.0	4.0	3.0	2.0	2.5
3.5	2.5	4.0	3.5	3.5	2.5	2.0	3.5	3.0	3.0	2.5	3.0	3.5	6.0	3.5	2.0
5.0	3.5	5.0	2.0	2.0	2.5	5.0	2.0	4.0	4.0	3.0	3.5	5.0	4.0	3.5	2.0
5.0	3.5	3.0	2.0	3.0	4.0	4.0	3.0	2.5	4.0	4.5	3.5	4.5	2.0	2.0	2.5
5.0	2.5	2.5	3.0	2.5	5.0	5.0	2.5	2.0	2.0	3.5	2.5	3.0	2.5	4.5	2.0
4.5	4.0	4.0	2.0	2.0	4.5	4.0	2.0	2.0	3.0	3.0	2.5	2.0	2.0	2.0	2.0
2.5	3.5	2.5	4.0	4.0	2.0	3.5	4.0	5.5	2.5	3.0	4.0	3.0	2.5	2.5	3.5
3.5	4.0	4.5	5.0	2.0	3.0	2.5	2.0	3.0	2.5	3.0	3.0	3.5	4.0	3.0	2.0
3.0	4.5	2.5	5.0	2.5	2.5	4.5	2.5	7.0	3.5	2.0	3.5	3.0	4.0	2.5	2.5
4.5	3.0	4.0	3.5	2.5	3.0	3.0	2.5	3.0	4.0	2.0	4.0	2.0	3.0	2.0	2.5
3.5	2.5	3.5	6.0	3.0	4.0	2.0	3.0	6.0	3.0	1.5	3.0	3.0	2.0	2.0	3.5
2.0	2.0	3.5	3.0	2.5	3.0	4.5	2.5	6.0	3.5	2.0	2.5	3.5	4.0	2.5	2.5
4.5	3.5	2.0	3.5	3.0	3.5	4.5	3.0	5.0	3.0	2.0	2.5	3.5	2.5	3.0	1.5
3.5	4.0	3.5	3.5	2.5	2.0	3.0	2.5	5.0	4.5	2.0	3.0	3.0	2.0	3.0	1.5
5.0	3.0	2.0	3.0	2.5	3.0	3.0	2.5	5.0	3.0	2.5	3.5	3.0	3.0	2.5	2.0
3.5	4.5	2.0	5.0	3.0	2.5	2.5	3.0	3.0	4.5	3.0	3.0	4.0	4.0	4.0	2.0

Table 8.2.4 Table summarizes descriptive statistics of the height for the four treatments.

Mean	*n*	*Std. Dev*	
3.62	120	1.121	T1
3.40	120	1.001	T2
2.99	120	0.914	T3
2.80	120	0.824	T4
3.20	480	1.022	Total

Table 8.2.5 Table provides actual number of dead leaves count per jar number of the different treatments recorded at the end of the first subculture

Jar No.	Treatments			
	T1	**T2**	**T3**	**T4**
1	63	43	54	56
2	62	31	48	29
3	67	40	34	59
4	66	60	55	56
5	64	70	48	55
6	95	40	51	67
7	77	55	44	61
8	65	62	52	70
9	55	62	34	56
10	52	51	40	52
11	56	79	35	52
12	38	43	45	57
13	65	37	37	57
14	76	54	51	55
15	80	71	37	34
16	56	37	38	43

Table 8.2.6 Table summarizes descriptive statistics of the number of dead leaves for the four different treatments under study

Mean	*n*	*Std. Dev*	
64.8	16	13.09	T1
52.2	16	14.22	T2
43.9	16	7.51	T3
53.7	16	10.59	T4
53.7	64	13.61	Total

Table 8.2.7 Table provides multiplication coefficient values per jar number of the different treatments recorded at the end of the first subculture

Jar No.	Treatments			
	T1	T2	T3	T4
1	2.0	2.0	2.0	2.0
2	2.0	2.0	2.0	2.0
3	2.0	2.0	3.0	3.0
4	2.0	2.0	3.0	3.0
5	2.0	2.0	3.0	2.0
6	3.0	3.0	2.0	3.0
7	1.0	2.0	3.0	2.0
8	2.0	2.0	3.0	3.0
9	2.0	2.0	2.0	3.0
10	2.0	3.0	3.0	4.0
11	3.0	2.0	3.0	3.0
12	3.0	2.0	3.0	3.0
13	3.0	2.0	2.5	2.0
14	2.0	2.0	3.0	3.0
15	2.0	2.0	3.5	3.0
16	2.0	2.0	3.0	3.0

Table 8.2.8 Table summarizes descriptive statistics of the multiplication coefficient for the four different treatments under study

Mean	*n*	*Std. Dev*	
2.2	16	0.54	T1
2.1	16	0.34	T2
2.8	16	0.48	T3
2.8	16	0.58	T4
2.5	64	0.57	Total

Appendix 8.3 Statistical Analysis (Analysis of Variance – ANOVA)

8.3.1 One-factor Analysis of Variance for height of the four Treatments after the first subculture

The p-value is considered significant when it is less than *alpha* (0.05). According to the analysis, the F value (53.34) provides a p-value ($p=6.39E^{-28}$) that is less than 0.05.

Table 8.3.1.1 Table provides statistical results of the analysis of variance of the four treatments.

ANOVA table

Source	*SS*	*df*	*MS*	*F*	*p-value*
Treatment	118.9739	3	39.65798	53.34	$6.39E^{-28}$
Error	234.9538	316	0.74352		
Total	353.9277	319			

After performing a *post hoc* analysis, the variation among the four treatments was noted. Table 3.1.2.2 shows the different treatments that had a significant relationship in terms of height.

Table 8.3.1.2 Table illustrates relationship in mean heights between the four treatments

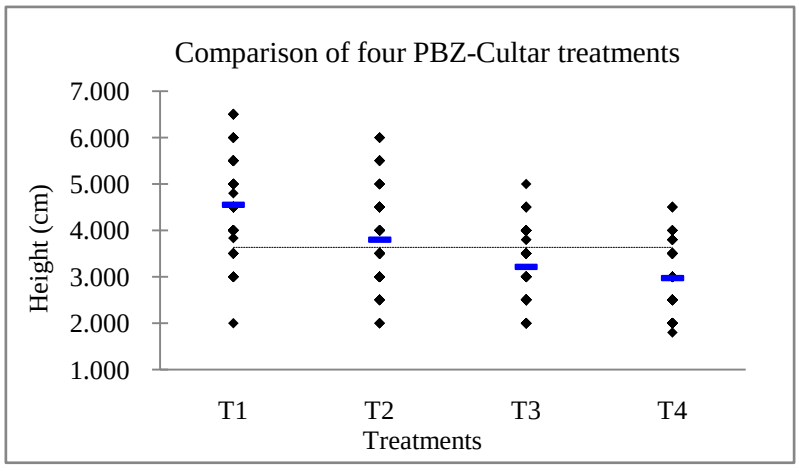
Post hoc analysis

p-values for pairwise t-tests

		T4	T3	T2	T1
		2.970	3.214	3.800	4.552
T4	2.970				
T3	3.214	.0748			
T2	3.800	3.32E-09	2.28E-05		
T1	4.552	3.67E-26	5.13E-20	7.33E-08	

The mean height is illustrated for the four different treatments (T1, T2, T3 and T4) and is represented by a horizontal line that is among the series of dots (See box-plot figure 1.1).

Figure 8.3.1.1 Dot-plot comparing the mean heights for the four different PBZ-Cultar treatments



3.2 One-factor Analysis of Variance for height of the four Treatments after the second subculture, at the end of the experiment

The p-value is considered significant when it is less than *alpha* (0.05). The analysis of variance (as seen in table 2.2) further proves that at least one or more mean variances are different between the four concentrations used in the experiment ($F=18.05$, $p=4.15E^{-11}$).

Table 8.3.2.1 Table provides statistical results of the analysis of variance for the mean height of the four treatments.

ANOVA table

Source	*SS*	*df*	*MS*	*F*	*p-value*
Treatment	51.060	3	17.0200	18.05	$4.15E^{-11}$
Error	448.885	476	0.9430		
Total	499.945	479			

A *post hoc* analysis revealed the variation between the four treatments. As can be noted in table 2.3, T1 had a significant difference in height to T3 and T4, those being $7.37E^{-07}$ and $1.24E^{-10}$ respectively. T2 had a difference in height with T3 and T4 (0.0011 and$1.66E^{-06}$ respectively).

Table 8.3.2.2 Table illustrates relationship in mean heights between the four treatments

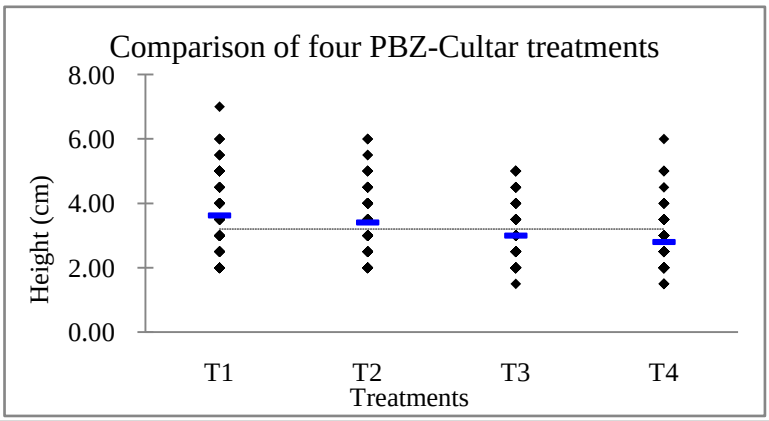
Post hoc analysis

p-values for pairwise t-tests

		T4	T3	T2	T1
		2.80	2.99	3.40	3.62
T4	2.80				
T3	2.99	.1189			
T2	3.40	1.66E-06	.0011		
T1	3.62	1.24E-10	7.37E-07	.0846	

The box plot (Figure 2.1) presents the mean heights for the four different treatments (T1, T2, T3 and T4) shown by a horizontal line that is among the series of dots.

Figure 8.3.2.1 Dot-plot comparing the mean heights for the four different PBZ-Cultar treatments



3.3 One-factor Analysis of Variance for the number of dead leaves of the four treatments after the first subculture

The results show that the F-value (8.70) provides a p-value of 0.0001 which is considered statistically significant (see table 3.3.1 below). The p-value is considered significant when it is less than *alpha* (0.05).

Table 8.3.3.1 Table provides statistical results of the analysis of variance for the four treatments in terms of dead leaves count

ANOVA table

Source	*SS*	*df*	*MS*	*F*	*p-value*
Treatment	3,537.19	3	1,179.063	8.70	.0001
Error	8,131.25	60	135.521		
Total	11,668.44	63			

A *post hoc* analysis revealed the variation between the four treatments. Based on the results from table 3.2, the number of dead leaves counted for concentration T1 had a significant difference when compared to T3, T2 and T4, those being 4.06E-06, 0.0032 and 0.0089 respectively.

Table 8.3.3.2 Table illustrates relationship in mean number of dead leaves between the four treatments

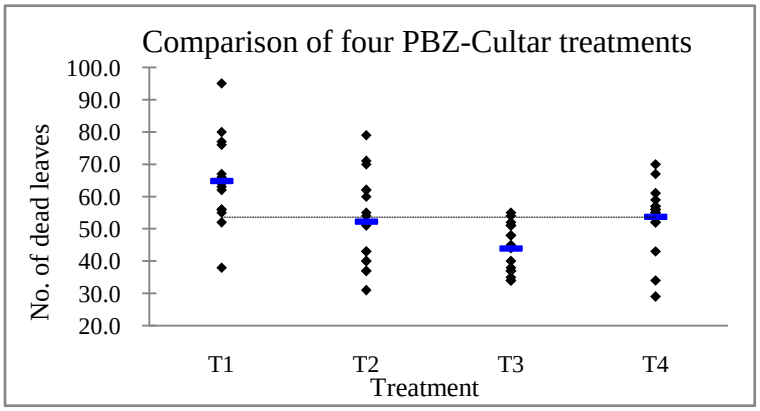
Post hoc analysis

p-values for pairwise t-tests

		T3	T2	T4	T1
		43.9	52.2	53.7	64.8
T3	43.9				
T2	52.2	.0495			
T4	53.7	.0211	.7168		
T1	64.8	4.06E-06	.0032	.0089	

The box plot (Figure 3.1) presents the mean in regards to number of dead leaves for the four different treatments (T1, T2, T3 and T4) shown by a horizontal line among the series of dots.

Figure 8.3.3.1 Dot-plot comparing the number of dead leaves counted during the first subculture for the four different PBZ-Cultar treatments



3.4 One-factor Analysis of Variance for the multiplication coefficient number of the four treatments after the first subculture

The F-value (7.72) in the analysis of variance test results in a p-value of 0.002. The p-value is considered significant when it is less than *alpha* (0.05).

Table 8.3.4.1 Table provides statistical results of the analysis of variance for the four treatments in terms of multiplication coefficient

ANOVA table

Source	*SS*	*df*	*MS*	*F*	*p-value*
Treatment	5.67	3	1.891	7.72	.0002
Error	14.69	60	0.245		
Total	20.36	63			

A *post hoc* analysis was conducted to determine the variation between the PBZ-Cultar concentration treatments.

Table 8.3.4.2 Table illustrates relationship in the mean multiplication coefficient between the four treatments

Post hoc analysis
p-values for pairwise t-tests

		T2 2.1	T1 2.2	T3 2.8	T4 2.8
T2	2.1				
T1	2.2	.7221			
T3	2.8	.0007	.0021		
T4	2.8	.0007	.0021	1.0000	

The box plot (Figure 4.1) presents the multiplication coefficient mean for the four different treatments (T1, T2, T3 and T4); shown by a horizontal line that is among the series of dots.

Figure 8.3.4.1 Dot-plot comparing the multiplication coefficient number during the first subculture for the four different PBZ-Cultar treatments