A Study of Different Parameters during Establishment *In Vitro* of Pineapple (*Ananas comosus*)

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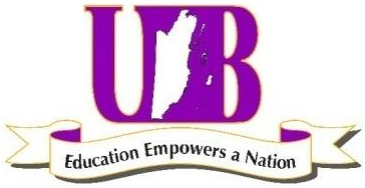

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ABSTRACT

Tissue culture refers to the *in vitro* aseptic culture of cells, tissues, organs or whole plant under controlled nutritional and environment conditions often to produce clones. Plant tissue culture technology is widely being used for large scale plant multiplication. This research aims to study two parameters, the effect of low temperature at different durations and the use of the different culture media according to physical state during the establishment of pineapple *in vitro*. Local and MD2 varieties of pineapple were cultured using the buds from the micro corms of the crowns as explants. For parameter one, explants of MD2 and local pineapple varieties were placed at a temperature of 4^{o}C for 24 and 48 hours. Explants from the MD2 variety was used in the control treatment (room temperature). With high percentage of contamination, no germination was observed in the local pineapple of both explants exposed to 24 and 48 hours of low temperature. Conversely, for MD2 variety, the explants subjected to room temperature had the greatest growth of explants followed by the 24 hours treatment. For parameter two, the local pineapple variety explants were cultured in liquid and semi-solid culture media. For an interval of four weeks both types of media was observed and over all showed no contamination, no growth or any difference at all. Through the understanding and use of tissue culture, thus mass production of this fruit can be acquired and hence improve the Belize's gross domestic product since it is one of the most economically important tropical fruits.

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INTRODUCTION

Plant research encompasses the growth of new plants in a controlled environment. These plants are those that have been genetically altered or that propagation of such plants are needed. These forms can be accomplished through tissue culture of tissues (explants) of a particular plant of interest (Traynor, et al., 2001). These explants is obtained from a single mother plant or they may be the result of genetic transformation of single plant cells which are then stimulated to grow and to ultimately develop into a whole plant. Thus, tissue culture is the process in which explants are cultured in a nutrient medium under sterile conditions inside the laboratory (*in vitro*). Tissue culture techniques are often used for commercial production of plants as well as for plant research. Using the appropriate growing conditions for each explant type, plants can be induced to rapidly produce new shoots, and with the addition of suitable hormones new roots (APS, 2015).

Plant Tissue Culture media preparation is based on the unique property of the cell-totipotency (TM Media, 2008). The cell-totipotency is the ability of the plant cell to regenerate into whole plant. In this process the excised bud is transferred into a vessel containing a sterile nutrient medium. The success of plant tissue culture media depends very much on the stage of explant selected, the sterilization period and the type of culture media used; different types of plants require different sets of culture media (TM Media, 2008). The rich plant tissue culture media provides a good nutrient source for bacteria and fungi, therefore precautions against microbial contamination must be taken in all in vitro procedures (TM Media, 2008).

Pineapple (*Ananas comosus* L.) is one of the most economically important tropical fruits (Duval et al., 2001). In terms of worldwide production, it is currently the third most important tropical fruit after bananas and mangoes (FAO, 2008). Pineapple, an herbaceous perennial plant

with short, stout stem and a rosette of waxy, strap like leaves, two and a half to five feet high with a spread of three to four feet. More than a hundred varieties exist, but only a few of them are cultivated commercially such as the successful MD2 variety. This MD2 is a hybrid pineapple with a golden skin color when mature (Purseglove, 2012). The use of byproducts of pineapple culture in feed production, canning and juice extraction has been encouraged. In animal food, leaves can be used in three forms: fresh, dried and in silage (Coppens and Leal 2003). Pineapple may offer additional advantages, such as its relevance as fiber source.

A major problem that both large scale commercial production of pineapple and the expansion of the existing small farms face is the difficulty in obtaining uniform planting material in large quantity due to the low rate of multiplication by conventional methods and the lack of high quality propagules. Using in vitro propagation of pineapples has a comparative advantage over the traditional methods as it leads to the production of large scale of disease free uniform planting materials in a short period of time. The objective of this research paper is to provide an outline on the effects of low temperature to break seed dormancy of the in vitro cultures of local and MD2 pineapple varieties and also to indicate the best culture medium (liquid or semi-solid media) for the growth of local pineapple.

LITERATURE REVIEW

Classification:

Kingdom: Plantae

Phylum: Anthophyta

Class: Liliopsida

Order: Poales

Family: Bromeliaceae

Genus: *Ananas*

Species: *Ananas comosus*

The family Bromeliaceae comprises of 56 genera with 2,921 species, classified into three subfamilies (Luther and Holst, 2004). This family, is set apart from other monocots by the unique scale like multicellular hairs and the unusual conduplicate and spiral stigmas. *Ananas* is the only genus whose flowers and bracts are completely merged into a single sorose-type parthenocarpic fruit (Bartholomew et al., 2003). Pineapple (*Ananas comosus*) has spiny or smooth leaves (Luther and Sieff, 1998).

Pineapple is a perennial monocarpic herb, are rosette forming, 2-4 feet tall and 3-4 feet wide. Stems are short and inconspicuous in the center of the rosette of long, linear leaves (Ray, 2002). The flowers, 100-200 in number are hermaphrodite and are self-incompatible. Anthesis usually takes place within a day after the flower is opened, and after this stylar canals are closed by mucilaginous plug (Bartholomew *et al.*, 2003). The ovaries, the bases of sepals and bracts and the cortex of the axis are the edible parts of the fruit (Ray, 2002).

The seeds are approximately 3-5 mm in length, and 1-2 mm in width, they are flat on one side and curved on the other with a pointed end. Seeds are desired only in breeding programs and are usually the result of hand pollination. The seeds are hard and slow to germinate. Treatment

with sulfuric acid achieves germination in 10 days, but higher rates of germination (75-90%) and more vigorous growth of seedlings result from planting untreated seeds under intermittent mist. The seedlings are then planted when they are 15-18 months old and will bear fruit 16-30 months later, however vegetatively propagated plants fruit in 15-22 months (Morton, 1987).

Origin

Pineapple is native to South America, the center of origin is thought to be in the northern Brazil, Colombia, Venezuela and northern Argentina. These regions contain the largest level of diversity within the species (Bartholomew *et al.*, 2003).

Chronicles of European explorers have described and mentioned pineapple domestication in parts of South America and in the Caribbean. Before the arrival of Columbus, pineapple were already part of the diet of the Native Americans (Collin, 1960). Two hypothesis on the possible origin of pineapple have been stated in Bartholomew *et al.* (2003); firstly it is suggested by Bertoni in 1919 that pineapples were domesticated by the Tupi-Guarani Indians from *A.comosus* var. *ananassoides.* Secondly, the genus could have been originated and located in an area within 10^{o}N-10^{o}S latitude and 55^{o}-75^{o}W longitude. Thus, also suggesting that south eastern Brazil could have been a secondary center of origin and distribution (Bartholomew *et al.* 2003).

Following the discovery of pineapple in South America, it was soon dispersed into other regions of the world by travelers and seafarers. Pineapple was introduced into the Philippones, Hawaii and Guam during the early 16^{th} century by the Spaniards and reached India and the east and west coasts of Africa by 1548. Pineapple were reported growing in China by the year 1594 and in South Africa in the year 1655. It was not until 1719 that pineapple plants were

successfully established in England in greenhouses (Purseglove 1972; Bartholomew *at al.*, 2003).

It was in the early 1493, when Christopher Columbus first brought the pineapple back from Guadeloupe to Spain's Queen Isabella, that no one in Europe had even seen anything like it. The Spanish saw the fruit's resemblance to a pine cone and first called it "Pine of the Indies." The English called it an apple because of its tasty fruits. Therefore, the name pineapple comes from the combination of the Spanish "pina" with the English "apple" (Ombrello).

Habitat

Pineapple inhabits a range from warm temperature moist to tropical very dry to wet forest life zones, it prefers light, permeable soil rich in organic matter with a pH between 5.5 and 6.0. This plant is reported to tolerate annual precipitation of 6.0 to 41.0 dm, and annual temperature of 16.2 to 27.4^{o}C (Duke, 1978). Pineapples thrive in climates that are uniformly warm. Leaf damage occurs at -2.2°C, and plants are killed at lower temperatures. Prolonged exposure at 5°C results in internal breakdown (Reed, 1976).

They are tolerant of a wide range of soils providing they possess good drainage, soil aeration, and a low percentage of lime. Sandy loam, mildly acid and of medium fertility, is best (Reed, 1976).

Uses

Pineapple, is one of the most economically important tropical fruits, in terms of worldwide production, it is currently the third most important tropical fruit after bananas and mangoes (Farahani, 2013). Most pineapples are canned, juiced, jammed or eaten raw fruits are important source of vitamin A and B_1 and contain a protein digesting enzyme bromelain. Due to

the presence of this proteolytic enzyme, *A. Comosus* has various medicinal uses. It is currently marketed under the name Ananase to treat inflammation and related pain.

Bromelain also decreases osteoarthritic pain, for reducing swelling (inflammation), especially of the nose and sinuses also interfere with the growth of tumor cells and slow blood clotting. Due to High concentration of vitamin C it boosts immune system and acts as an antioxidant. The enzyme bromelain, also aids digestion by breaking down proteins. This is why pineapple juice is often used in marinades, as the bromelain helps to tenderize the meat (Farahani, 2013).

In respect to the pineapple yield worldwide, it is recorded that in 2013 Indonesia ranked first, Mexico ranking twelfth and Guatemala ranking twentieth third as illustrated in Figure 1. Belize ranked thirty ninth with 187.971 hectogram per hectare, Figure 2 illustrates the pineapple yields over the past years, and shows that the year 2009 has the most yield. Concerning the pineapples production quantity (tons) Belize ranked 60, with a value as number of 1.297 as illustrated in table 1 (FAOSTAT, 2013).

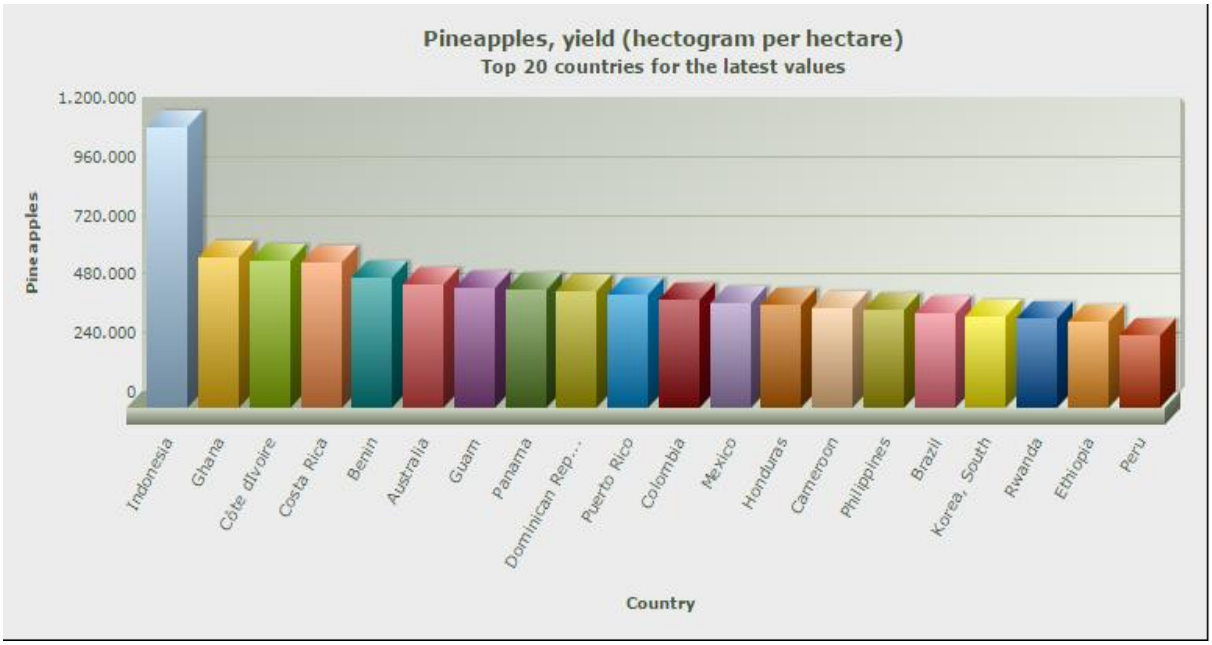

Figure 1: Showing the Top 20 Countries for the Pineapple Yields (Hectogram per Hectare).

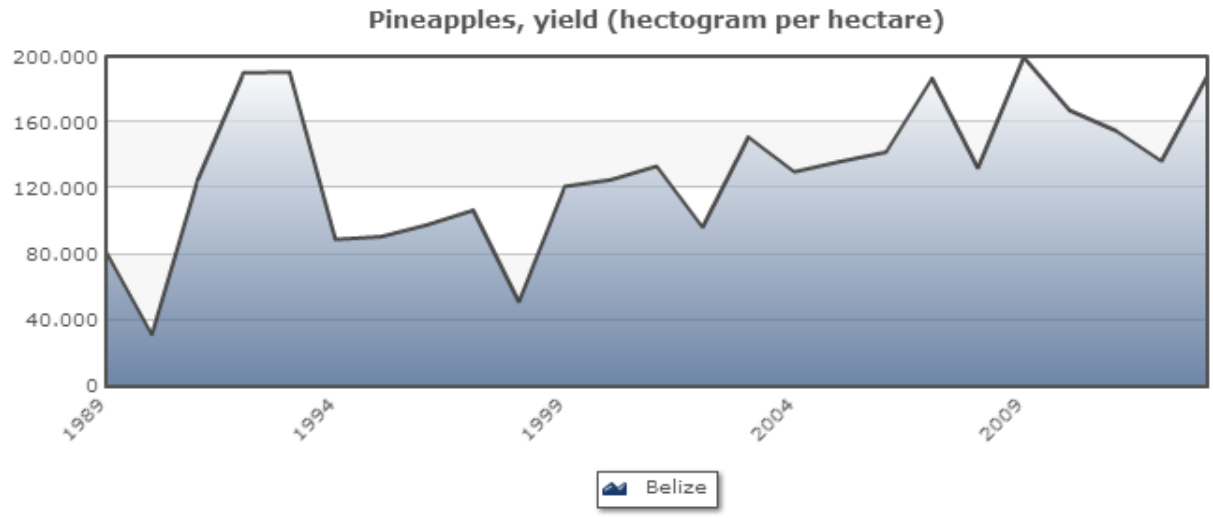

Figure 2: Showing the Pineapple Yields over the Past Years (1989 – 2013) in Belize

Data Set	Rank	Value	Date
Pineapples, production quantity (tons)	66	1.297	2013
Pineapples, area harvested (hectare)	70	69	2013
Pineapples, yield (hectogram per hectare)	39	187.971	2013

Table 1: Showing the pineapple production quantity, area harvested and yield in Belize

MATERIALS AND METHODS

The research was performed at the Micro-propagation Lab at the University Belize Belmopan Campus from March to May 2015.

In order to study two main parameters in the establishment of pineapple in vitro, two separate experiments were carried out. Experiment one, the effect of low temperature at different durations, and experiment two, the effect of types of media according to physical state.

Planting materials:

MD2 variety pineapple fruits (*Ananas comosus*) were obtained from the University of Belize, Central Farm Campus. While the local variety pineapple was purchased at the market in Belmopan City.

Experiment 1: The effect of low temperature at different time durations on the establishment of MD2 and local varieties of pineapple

Culture media: Semi solid culture media (1Liter)

Materials needed to prepare the culture media were obtained. Approximately 100mL of distilled water was poured in a beaker of one liter capacity. A volume of 20mL of each Murashige and Skoog stock solutions (MS 1, 2, 3, 4, 5) were added to the flask. A filter boat was obtained and placed over a weighing balance and tare, Myoinositol was then placed in the boat to obtain a weight of 0.1 grams. The obtained mass of Myoinositol was then added to the flask. Using the same weighing materials, 20grams of sucrose was weighed and also added to the flask. A micropipette was used to measure 1000µL of Thiamine (100mg/100mL) and poured in the flask containing the rest of the components. The tip of the micropipette was changed and a new

one was inserted so to measure 2000µL of 6BAP (50mg/100mL) and added to the same flask. This was also done for the auxin NAA (50mg/100mL). More distilled water was added to the flask just below the final volume mark. The solution was stirred to dissolve all the components. Using a pH meter and probe the pH of the media was adjusted to 5.7. The flask containing the culture media was then placed on a hot plate magnetic stirrer device for it to heat to approximately 50^{o}C. Meanwhile, using the weighing balance, the filter boat was placed over the balance and tare and then 7 grams of agar was weighed and added to the flask slowly when the media had acquired the desired temperature. The agar was cooked until it dissolved properly and with a magnetic stir it was stirred at the same time. When the media became clear, the heat was removed. Twenty five clean jar vessels were obtained, and 40mL of the media was poured in each vessel. The 25 jars along with the media were then sterilized and then stored for 4 days in the inoculation room.

Sterilization:

The 25 culture vessels were placed in the autoclave at 121^{o}C; 1.2 Kg/cm^{2} for a period of 15 minutes for the purpose of sterilization. The instruments such as the forceps, sterile blade handles, cutting tiles were each wrapped separately with aluminum foil and placed in the autoclave along with the culture media vessels.

Treatments:

Three treatments were carried out; control, 24 hours and 48 hours. The crowns from the MD2 and local varieties were removed from their fruit. The crowns leaves were carefully excised in order to obtain the micro-corm from each without damaging the buds. The micro-corms were then placed on jars containing sterilized distilled water.

Control treatment: The MD2 pineapple variety was used. The micro-corms were used at room temperature and immediately cut into longitudinal quarters and inoculated.

24 hours treatment: Local and MD2 pineapple varieties micro-corms were subjected to 24 hours at a temperature of 4ºC. After this time period the second inoculation took place.

48 hours treatment: Local and MD2 pineapple varieties micro-corms were subjected to 48 hours at a temperature of 4ºC. After this time period the last inoculation was carried out.

Inoculation:

The inoculation phase was done in the inoculation room, under a laminar flow. This was done to reduce contamination. Using 70% ethanol the laminar flow and hands were sterilized before starting to work. The instruments sterilized in the autoclave were brought to the laminar flow. The blade along with blade handles and forceps were immersed in 70% ethanol. The micro-corms subjected to low temperature at different durations were inoculated separately. Inoculation occurred every 24 hours. The jars containing the micro-corms were brought to the laminar flow, were removed from the sterilized distilled water and cut longitudinal. These cut micro-corms were placed in a clean sterilized jar containing 4% sodium hypochlorite and shaken for 30 minutes. It was then rinsed twice.

The explants used were the buds, which were excised from the micro-corms with a sterile blade on top of the sterilized tile. This was done closely to the rear of the laminar flow filter wall to minimize contamination. The blade and forceps were heated using the Bunsen burner flame before excising the buds to reduce any form of contamination. The jars' mouth were heated on

the flame before and after placing the explants in them. Five explants were placed in each jar as shown in figure 3. After inoculation the jars were placed in the growth room at a temperature of 27+ 24^{o}C at low light conditions.

This experiment resulted with 25 jars containing 5 explants each. There were 5 jars for each treatment.

Local 24 hours: 5 jars

Local 48 hours: 5 jars

MD2 24 hours: 5 jars

MD2 48 hours: 5 jars

Control room temperature: 5jars

Figure 3: Showing the buds of the micro-corms (explants) in the semi-solid culture media used in Experiment 1

Experiment 2: The physical state of culture media - the effect of using semi-solid and liquid media during the establishment phase of local pineapple variety.

Culture media: Semi solid and liquid culture media (200 mL each)

The preparation of the semi-solid and liquid culture media is similar as to that of experiment 1. The only difference is the quantity of components added, the other procedures remained the same. Approximately 40mL of distilled water was poured in a flask of 500 mL capacity. A volume of 8mL of each Murashige and Skoog stock solutions (MS 1, 2, 3, 4, 5) were added to the flask. For the Myoinositol 0.04 grams was weighed, which was added to the flask. Eight grams of sucrose was weighed and added to the flask. A micropipette was used to measure 400µL of Thiamine (100mg/100mL) and poured in the flask containing the rest of the components. The tip of the micropipette was changed and a new one was inserted and 800µL of 6BAP and NAA (50mg/100mL) was measured and added to the same flask. More distilled water was added to the flask just below the final volume mark of 400mL. The solution was stirred to dissolve all the components. Using a pH meter and probe the pH of the media was adjusted to 5.7.

The solution was divided into two, 200mL was used for the semi-solid media and the remaining 200mL was used for the liquid media and poured in two different flasks. For the semi-solid media, one of the flasks containing the media was placed on a hot plate magnetic stirrer device for it to heat to approximately 50^{o}C. Meanwhile, using the weighing balance, the filter boat was placed over the balance and tare. After this, 1.4grams of agar was weighed and added to the flask slowly when it had acquired the desired temperature. The agar was cooked until it dissolved properly and with a magnetic stir the media was stirred at the same time. When

the media became clear, the heat was removed. Ten clean test tubes were acquired, and 20mL of the media was poured in each vessel.

For the liquid media, the other flask containing the remaining 200mL of media was stirred in order to dissolve all the contents within it. Ten other clean test tubes were obtained, and M shaped double strips of filter paper were placed in them. After this, approximately 20mL of the media was added to each vessel.

The 20 test tubes were then placed in a test tube rack and placed in the autoclave.

Sterilization:

The process of sterilization of vessels was done the same way as that of experiment 1. The 20 culture vessels along with all instruments were placed in the autoclave at 121^{o}C; 1.2 Kg/cm^2 for a period of 15 minutes for the purpose of sterilization.

Inoculation:

The inoculation phase was done in the inoculation room, under a laminar flow as in experiment 1. Sterilization of the laminar flow and hands with 70% ethanol was performed. The explants used were the buds as well, which were removed from the micro-corms with a sterile blade on top of the sterilized tile. This was done closely to the rear of the laminar flow filter wall to minimize contamination. The blade and forceps are heated using the Bunsen burner flame before excising the buds to reduce any form of contamination. The test tubes' mouth were heated on the flame before and after placing the explants in them. In each test tube one explant was inserted. For the liquid media, the buds were placed into the culture media on top of the filter paper as shown in figure 4. It was ensured that the buds were not placed too deep. After inoculation the twenty test tubes were placed in the growth room at a temperature of 27$\pm$ 24^{o}C at

low light conditions on one test tube rack. Observations were recorded every week, to see if any the difference between the two.

Figure 4: Showing the buds of the micro-corms in semi-solid media (above) and liquid media (below) used in experiment 2

RESULTS AND DISCUSSION

The purpose of this research was to study two parameters on the establishment phase of the in vitro of pineapple. Thus, two experiments were carried out separately. In experiment one, the effect of low temperature at different durations on the growth of local and MD2 pineapple varieties was studied. In experiment two, the local pineapple variety was studied according to the physical state of the culture media, thus using semi-solid and liquid media.

Experiment 1: The effect of low temperature at different time durations on the establishment of MD2 and local varieties of pineapple

In terms of contamination, the results show that the local variety of pineapple at both 24 and 48 hours duration of low temperature was 100% contaminated with fungus. For the MD2 variety it was determined that the explants subjected to 48 hours of low temperature had 100% contamination when compared to the 24 hours period, that showed 60% of fungal contamination and the control treatment with only 20% contamination.

Since fungal contamination was observed on all the explants of the local variety (as shown in figure 5) of both treatments, no growth occurred. Due to this reason, observations for the 24 hour treatment was terminated four weeks after inoculation.

Figure 5: Showing fungal contamination on all explants subjected to 24 hours treatment of low temperature

Since the remainder explants were only of MD2 variety, for experiment one, the research focused only on this variety. From the results it was observed that the 48 hours treatment had the most contamination as previously mentioned, followed by the 24 hours treatment and the control explants with the least form of contamination. Black puffy and white liquidly fungus was mostly observed on top of the culture media and explants. This may be due to improper sterilization of the laminar flow, hands, explants, or utensils used.

Thus, the best treatment to break the dormancy of MD2 pineapple variety was the control treatment followed by the 24 hours treatment. The 48 hours treatment showed to have the least growth as shown in figure 6. There were five culture vessels for each treatment and each vessel contained five explants. So, to determine which treatment was the best, the explants within each jar that showed growth was determined.

Figure 6: Showing the growth of the explants subjected to different treatments in vitro

The null hypothesis (H_0) for experiment one states that there is no significant difference in mean value between the three treatments. Table 2 in the appendix, presents the F statistics being 2, with a p-value of 0.1780. The p-value showed to be more than the accepted 95% confidence interval (0.05). Therefore statistically significant, the null hypothesis is rejected. There is at least one different mean growth number of explants among the three treatments.

In determining in which treatment resulted as the best, table 2 shows that the control had the highest standard deviation of 1.34, followed by the 24 hours treatment with a standard

deviation of 0.84 and lastly the 48 hours treatment with a value of 0.45. Hence buds from the micro-corm MD2 pineapple were proved to grow best when treated at room temperature (control). This is supported by the results shown in figure 9, which illustrates that the mean of the control is above the rest of the other means. The boxplots in figure 10, 11 and 12 (appendix), also supports this claim.

Experiment 2: The physical state of culture media - the effect of using semi-solid and liquid media during the establishment phase of local pineapple variety.

For this experiment the physical state of the culture media on the local pineapple was studied. Observations were taken over a period of four weeks. The explants found on the semi-solid media and liquid media showed no growth or fungal contamination. Hence, there was no difference detected on the two types of culture media according to the physical state used for the tissue culturing of pineapple as shown in figure 7 and 8 below.

Figure 7: Showing the local pineapple explants on liquid media

Figure 8: Showing the local pineapple explants on semi-solid media

CONCLUSION

The effect of low temperature at different durations was the first parameter studied during the establishment in vitro of two varieties of pineapple. The local pineapple explants subjected to both treatments resulted fully contaminated with fungus hence there was no growth observed. However, the MD2 pineapple resulted growing best at room temperature and thus having far less fungal contamination.

The second parameter studied was the type of culture media according to the physical state. Results showed that tissue culturing local pineapple buds using liquid or semi-solid media presents the same outcome. No growth or contamination was observed on both types of media.

RECOMMENDATIONS

For better results in studying the effect of low temperature, it would be recommendable to use more effective sterilization techniques in further research. Also, a longer period of observations should be performed for at least two trials. These recommendations also apply in studying the physical state of the culture media used in the tissue culture of local pineapple.

Further research it is recommended to determine why local pineapple showed higher fungal contamination level when compared to the MD2 variety. Through the understanding of this, hence large scale production of this fruit can be acquired. By doing so then it improves Belize's gross domestic product since it is one of the most economically important tropical fruits.

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APPENDIX

Table 2: Showing a one factor ANOVA to determine if there is a significant difference between three treatments on the number of explants growth of MD2 pineapple.

One factor ANOVA

Mean	*n*	*Std. Dev*	
0.2	5	0.45	48 hours
0.8	5	0.84	24 hours
1.4	5	1.34	Control
0.8	15	1.01	Total

ANOVA table

Source	*SS*	*df*	*MS*	*F*	*p-value*
Treatment	3.60	2	1.800	2.00	.1780
Error	10.80	12	0.900		
Total	14.40	14			

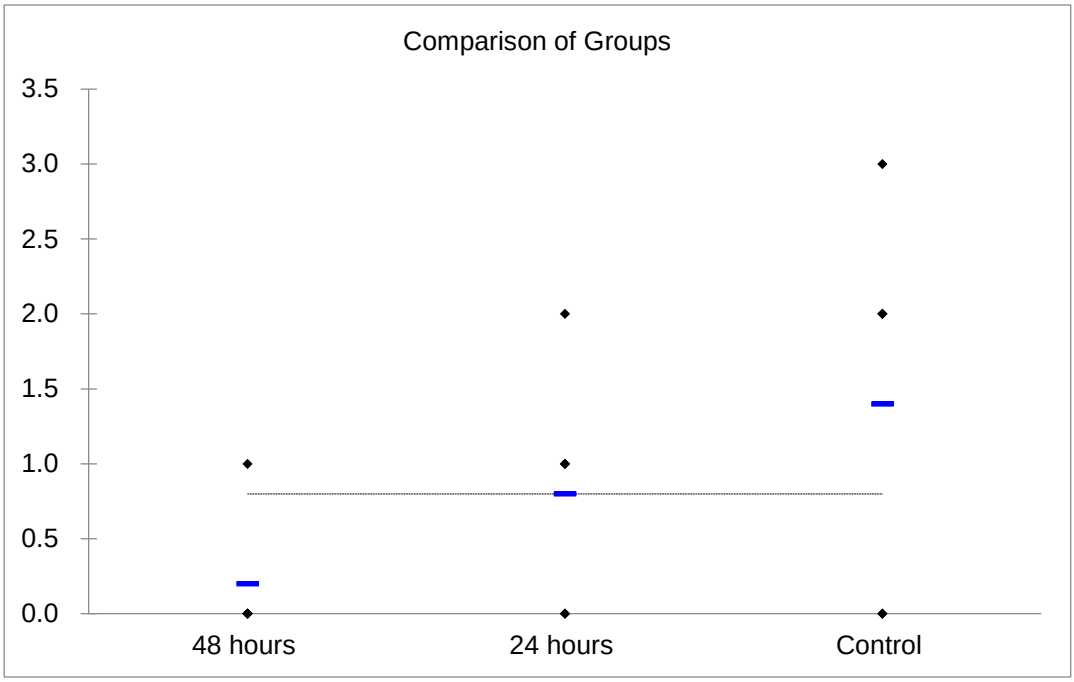

Figure 9: Shows a graph making comparisons between the three different treatments used on the establishment of MD2 pineapple

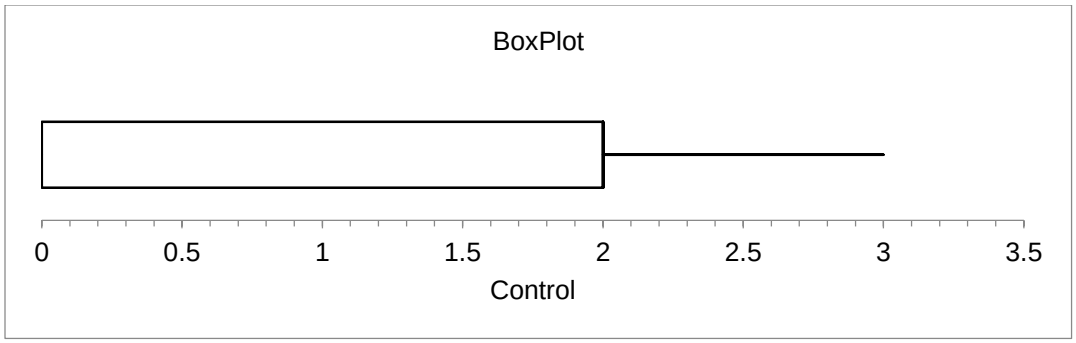

Figure 10: Showing a box plot for the results of the control treatment

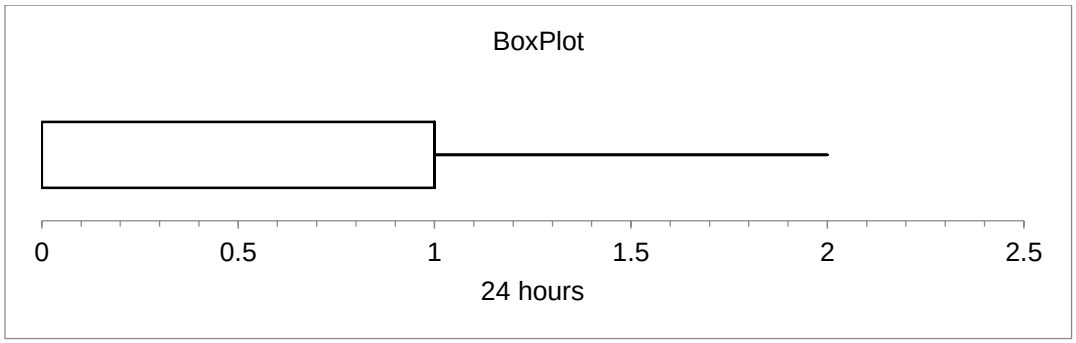

Figure 11: Showing a box plot for the results of the 24 hours treatment

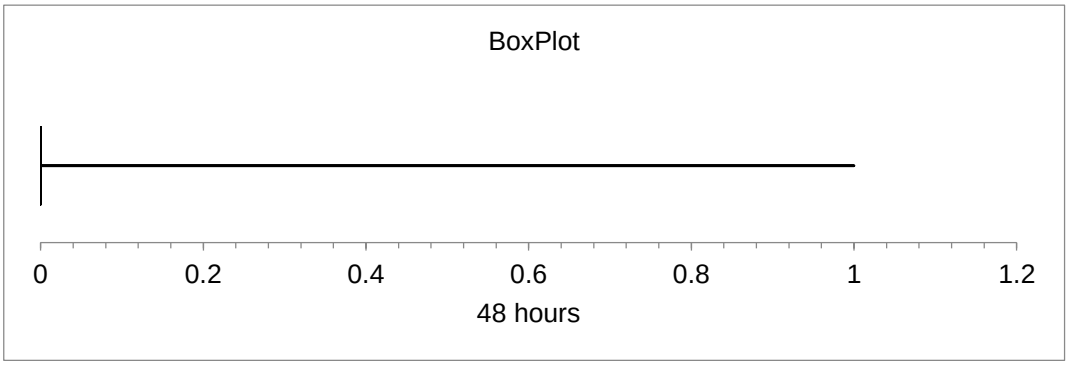

Figure 12: Showing a box plot for the results of the 48 hours treatment