EFFECT OF PRE-TREATMENT OF SUGAR CANE EXPLANTS (*SACCHARUM* SPP.) WITH CYTOKININ ENRICHED ESTABLISHMENT MEDIA

A Thesis Submitted to the University of Belize in Fulfillment of

BIOL 4992 - Independent Research

As Part of Bachelors of Science in Biology

Submitted by

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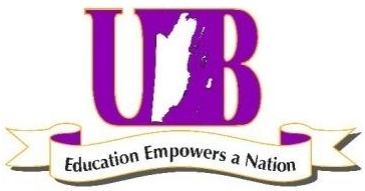

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Abstract

This study was conducted in order to find out whether the initial establishment of the Sugar Cane explants would be affected by the type of media in which it was initially placed. The importance of this research is that Sugar Cane is a very important economic crop not only in Belize but worldwide and finding a way to cheaply and successfully establish sugarcane explants would greatly benefit the industry. Two media treatments were used; establishment media and multiplication media, with the establishment media being supplemented with additional growth regulators such as IBA. The methods used to establishment of the Sugar Cane explants were the standard establishment techniques that have been described by various workers before. Preliminary results indicated all successfully established in both media used. The difference came in the establishment rate since the explants placed in the establishment media began shooting an average of two weeks earlier than those in the multiplication media alone.

Acknowledgements

This research was a long and trying process of learning and application. Without the people that were there to assist me along the way it would not have been possible to complete this paper. So, I would like to thank Dr. Stephen Williams for allowing me the use of the UB Central Farm micropropagation laboratory in order to conduct the experiments. Mr David Guerra, head of the micropropagation lab, was there to assist in every step of the way when it was time to do the actual process of micropropagation so special thanks goes out to him. Thanks are also in merit to Dr. Dion Daniels who was my supervisor in this endeavour. Finally, to all those people that were in some way involved in the process of helping me to complete this research paper a heartfelt thanks is in order as well.

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Introduction

Origins and Distribution:

Sugarcane (*Saccharum spp.*) is a tropical grass native to Asia where it has been grown in gardens for over 4,000 years. It is typically a plant that reaches a height of around three to four meters high and has a diameter of about 5 centimetres (Magness, 2014). The varieties that are used in modern plantations are hybrids that are formed from the complex and intensive selective breeding of species, especially those between the species *S. officinarum* and *S. spontaneum* (Regulator, 2012). The species *S. officinarum* is very efficient in storing sugars in its stem, but its low resistance to disease makes it a problem to grow. This species itself is thought to be a mix between three different species of cane (Regulator, 2012). It is thought that the place of origin of the sugar cane species *S. officinarum* was in Polynesia and that the species was then taken to the rest of Asia as humans travelled and spread from there (Daniels & Roach, 1987). The selection of sweet forms of *S. robustum* is thought to have been one of the ways by which the *S. officinarum* originated. Through the use and animals and their attraction to sweeter individual plants then it is possible that they would have been able to see and select which plants were the best as a source of food (Daniels & Roach, 1987). Methods for manufacturing sugar from sugarcane were developed in India about 400 BC. When Christopher Columbus came to the West Indies he brought the plant along with him and so today sugarcane is cultivated in tropical and sub-tropical regions throughout the world (Cope, 2010).

Botany of sugarcane:

The stem of the sugarcane plant is solid and unbranched and has a cross section that is roughly circular. The joints are clearly differentiated from one another and a clear demarcation of the node from the internode can be seen (Glyn, 2004). At the node a lateral bud is found on the axil of the leaf and it is a band that encircles the stem that contains a growth ring and root primordia. These buds, which can be situated on, or just above, the leaf scar, can have a small, round morphology with an adpressed position to the stalk, while others may be more prominent and pointed, depending on the variety (Glyn, 2004). Usually, only one bud is situated at each node and they are alternate in position from one node to the next when they are present in the stalk, but on occasion there may be more than one bud may be formed at a node (Glyn, 2004). The separation of the nodes on the stem is generally around 15 to 25 cm; but they tend to get closer to each other as the top of the stem is reached and where the elongation of the plant is taking place.

They also tend to be much closer to each other at the base of the plant where the new tillers are being produced (Glyn, 2004). A wax covered rind is also present on the stem. This rind encircles the soft parenchymal tissue that is found inside the plant and helps to prevent the loss of water from the stalk through the process of evaporation and also provides strength and rigidity (Glyn, 2004). The juice containing sugar is stored in the thin-walled, parenchymatous tissue (Glyn, 2004). At the base, multiple lateral shoots form and this makes it have multiple stems (Magness, 2014). It is these stems that then grow into the cane stalk and are then harvested for the different uses that sugar cane has (Muralles, Rice, & Baucum, 2013). The leaves of the sugarcane plant are attached to the stem at the bases of the nodes; they follow an alternate arrangement in two rows that are found opposite to each other on the sides of the stalk (Regulator, 2012). Each leaf has two parts to it: the sheath and the blade or lamina. The sheath has a tubular shape and has a broader base than that found at the top (Glyn, 2004). The sheath encircles the stalk tightly and a ligule separates it from the long, pointed leaf blade (Regulator, 2012). The free margins of the sheath are on the opposite side of the stalk from the bud, which it surrounds and protects (Glyn, 2004). The ligule is a membranous appendage that is formed through the elongation of parenchyma cells that do not and does not have vascular bundles. It has a translucent colouring and is hyaline when it is young, but as it ages it is dried up and changes colour and becomes torn (Regulator, 2012). The midrib, which is usually white and concave on the upper or adaxial surface, and pale green and convex on the abaxial side is strong and helps in the support of the leaf (Glyn, 2004). As it gets further away from the ligule the leaf starts to broaden and reaches as much as 10 cm in width at its broadest point before starting to narrow out as it reaches towards the tip (Glyn, 2004). Leaf length in sugarcane can be as much as 1 meter but variation exists and different varieties also have different leaf lengths (Glyn, 2004). Stomata are found on either side of the leaf, but the density of stomatal openings on the underside of the leaf can be twice as much as that of the upper surface. It has been estimated that a fully expanded leaf has about 30 million stomata (Glyn, 2004). The planting of a seed piece, or sett, of a sugarcane plant will then cause the development soon after of two types of roots: the sett roots and the shoot roots. The sett roots develop from the initials on the root band, meanwhile the shoot roots develop from the root primordia that are found on the new developing tillers (Glyn, 2004). Sett roots have a thin and branched morphology, while those of the tillers have a thicker and fleshier look with much less branching occurring in them (Daniels & Roach, 1987). The lifespan of the sett roots is limited

and dies soon after the development and maturation of the tiller roots. They provide nutrient uptake to the plant at first but do not last longer than the development of the tiller roots (Development, 2013). The development of the roots in the subsequent phases of growth is then determined by the conditions that are present in the soil. If the conditions are favourable then the roots will develop and proliferate (Glyn, 2004). The shoot roots that develop along with the sett roots also have a limited lifespan, yet as each new shoot produces it new roots the roots system of the plant is renewed and keeps it from having no roots. This process is critical in the development of the plant since it not only keeps the plants from dying but it also allows it to adjust itself to various environmental conditions (Development, 2013).

Economic Importance:

When it is fully mature a stalk will contain a fiber content of around 11–16%, around 12–16% of soluble sugars, 2–3% non-sugars, and 63–73% water (Muralles, Rice, & Baucum, 2013). The average yield of cane stalk is 60–70 tonnes per hectare per year. However, this figure can vary between 30 and 180 tonnes per hectare depending on knowledge and crop management approach used in sugarcane cultivation. Sugarcane is a cash crop, but it is also used as livestock fodder (Magness, 2014). It is one of the most efficient converters of solar energy into organic substances, such as sugars, and many other forms of renewable energy. The first people to use the plant and domesticate it due to the presence of its sweet stem were the people in Polynesia (Jalaja, Neelamathi, & Sreenivasan, 2008). Nowadays, though, the sugarcane plant has emerged as a multipurpose crop that is used not only to produce sugar but is also used in a series of value added products such as paper, ethanol and other alcohol derived chemicals, animal feed, antibiotics, particle board, bio-fertilizer and raw material for generating electricity (Jalaja, Neelamathi, & Sreenivasan, 2008). Sugarcane (*Saccharum spp.* hybrids) is a crop with high economic significance as it accounts for more than 70% of the world's sugar production.

Seed and plant production:

Because of the extreme genetic variability in the seed population, sugarcane is traditionally propagated by vegetative cuttings (J., Arvinth, Sheeba, Kanchana, & Subramonian, 2006). The demands for true-to-type, disease free cane seedlings are being met currently by micropropagation of the sugarcane plants due to the high rates of demand. The seed-canes that are used in commercial scale farms or industries are currently produced either through direct organogenesis or through indirect somatic organogenesis (Burner & Grisham, 1995). In the

direct organogenesis method the apical meristem is the part of the plant that is used as the explant in the micropropagation. In the indirect somatic embryogenesis the immature leaf callus is the explant that is used (Brisibe, Miyake, Taniguchi, & Maeda, 1994). The limited area that is available for agricultural expansion coupled with the fact that agricultural land is now constantly being converted for non-agricultural purposes means that increased production in the sugarcane production sector will have to come from an increase in the yield per acre of sugarcane rather than an increase in the area of land that is being used to grow the crop (Hendre, Mascarenhas, Iyyar, Katwal, & Khuspe, 1983). Improved agronomic practices, use of required quantity of fertilizer at appropriate time, better irrigation facilities, comprehensive disease and pest management packages and regular development of improved varieties are the necessary inputs required for improving sugarcane production and productivity. Besides, availability of disease and pest-free, true to type planting material is an important prerequisite for achieving the desired yield improvement (Hendre, Mascarenhas, Iyyar, Katwal, & Khuspe, 1983).

Literature Review

Over the recent years development of a number of techniques for the micropropagation of sugar seed production have been reported. [2]. One of the techniques that were developed in this time was that of apical meristem culture and its application was in the production of sugar cane mosaic virus free plants (Hendre, Mascarenhas, Nadgir, Pathak, & Jagannathan, 1983). Axillary bud culture is another method that was successfully used in the production of true to type clones in many sugar cane varieties (Sauvaire & Galzy, 1978). The standardized method for the production of sugarcane mosaic free virus plants was developed in 1983 and it used the apical meristem culture technique to do so (Hendre, Mascarenhas, Iyyar, Katwal, & Khuspe, 1983). A growing sugarcane plant will have a region found at its tip that consists of a region of cells that divide rapidly, known as the meristem. This area is usually surrounded be leaves that are developing and leaf sheaths as well (Hendre, Mascarenhas, Iyyar, Katwal, & Khuspe, 1983). Meristematic tissues are also found in the tips of axillary buds. The dormancy of these tissues is attributed to the activity of the apical meristem, which has dominant activity and causes the meristematic tissues in the axillary buds to be suppressed (Brisibe, Miyake, Taniguchi, & Maeda, 1994). Both apical and axillary bud meristems can be used in micropropagation so that meristem tip cultures can be initiated. A meristem in a sugarcane plant will usually have a length of 0.25 – 0.30 mm in length and will have a diameter of around 0.1 mm (Jalaja, Neelamathi, & Sreenivasan, 2008). By removing the sheaths of leaves that surround the tip of the sugarcane plant the area known as the meristem is exposed. The cells in this area of the plant are always in an embryonic state and even during vegetative growth they are in an active state (Jalaja, Neelamathi, & Sreenivasan, 2008). One of the reasons why this region of cells is used in the process of micropropagation is due to the high genetic stability of the cells. This will then give a higher chance that the plants that are produced from them will have the same genetic makeup as the parent plant that they were produced from (Jalaja, Neelamathi, & Sreenivasan, 2008). The quick regeneration and subsequent differentiation and growth of the sugar cane explants are beneficial since it allows for a larger amount of plants to be produced in a shorter amount of time. The effect that an auxin pre-treatment has on the regeneration of sugar cane explants was studied on midrib segment explants of sugarcane. In the experiment the starting materials were cultured on MS medium containing 3.0 mg/l 2, 4-D under continuous dark conditions (G., Arvinth, Sheeba, Kanchana, & Subramonian, 2006). To allow for the optimized and the most

efficient plant regeneration the midrib segments were transferred on the 8th day to an MS medium that was supplemented with different concentrations of Benzyl adenine (BA) (0.1–5.0 mg/l) either alone or along with 0.1 mg/l naphtaleneacetic acid (NAA) (G., Arvinth, Sheeba, Kanchana, & Subramonian, 2006). Within a week's time of culturing both starting materials had increased considerably in size. The midrib segments that were then obtained from these pre-treated materials had turned green within a week with shoot production starting when the explants were transferred over to regeneration medium and incubated in light-dark conditions (G., Arvinth, Sheeba, Kanchana, & Subramonian, 2006). As the pre-treatment period of the explants increased up until the 8th day the percentage of the plants that showed higher regeneration increased. Thereafter the percentage started to decrease (G., Arvinth, Sheeba, Kanchana, & Subramonian, 2006). With respect to the control plants that did not have a pre-treatment with the 2, 4-D, they showed very low regeneration (G., Arvinth, Sheeba, Kanchana, & Subramonian, 2006). The effect of BA on the regeneration of plants that had been pre-treated with the auxin was found to be critical in a dose-dependent manner. The establishment of a positive relationship between plant regeneration and BA concentration up to 2 mg/l was confirmed. The addition of NAA to the BA infused medium showed that even higher regeneration was possible. Meanwhile, medium that did not have any plant growth regulators or that had only NAA infused in it did not show any regeneration (G., Arvinth, Sheeba, Kanchana, & Subramonian, 2006). In order to demonstrate that the results that were gotten were not restricted to a single genotype, that the experiment was genotypically independent, it was repeated with several other genotypes of sugarcane and all were shown show regeneration. These observations revealed that the pathway of plant regeneration and regeneration from midrib segments is genotype-independent (G., Arvinth, Sheeba, Kanchana, & Subramonian, 2006). From the results it can be deduced that a period of pre-treatment with 2, 4-D of explants plays an important role on the regeneration of explants. The results also showed that with an increasing pre-treatment time there is also an increase in the regeneration and due to this it shows that there is an increase in the number of cells that are totipotent and available to form shoots (G., Arvinth, Sheeba, Kanchana, & Subramonian, 2006). Also the plants that were established on just the medium that contained BA showed very low regeneration and as such it could be deduced that BA by itself did not have a role in the primary phases of regeneration (G., Arvinth, Sheeba, Kanchana, & Subramonian, 2006). But, after pre-treatment with the auxin for 10 days, the

explants that where transferred to the medium with BA infused in it showed high regeneration which indicated that the BA has a larger role in the regeneration process afterwards (G., Arvinth, Sheeba, Kanchana, & Subramonian, 2006). The high regeneration and subsequent differentiation of explants pre-treated with an auxin and later transferred to a medium infused with BA showed that plant regeneration can be effectively regulated through an auxin pre-treatment and later cytokinin culturing (G., Arvinth, Sheeba, Kanchana, & Subramonian, 2006). In another experiment where a protocol for the establishment of sugarcane explants was being formulated the explants were first inoculated in media with different concentrations of BAP along with Kinetin; the variety used was CP77400 (Ali, Naz, Siddiqui, & Iqbal, 2008).

The results showed that the explants inoculated in MS medium containing 1.5 mg/l of BAP showed the best results for shoot formation with shoot proliferation response within 10 days of inoculation and a number of 1.8 shoots per explant. An increase in the concentration of BAP showed that there was a decrease in the shoot proliferation response and the time taken for shoot formation to occur was also delayed (Ali, Naz, Siddiqui, & Iqbal, 2008). Different sizes of shoot meristems were also used and ranged from 0.5 – 5 mm. The results that were gotten showed that survival and regeneration increased with a decrease in the size of the meristem until it reached a size of 3 mm, at which maximal survivability and regeneration was shown (Ali, Naz, Siddiqui, & Iqbal, 2008). At this size there was a survival rate of 100% survival and a regeneration potential of 90% within 12 days of inoculation in the media. Both solid and liquid media were used as well and best results were obtained on media that was solidified with Phytagel at 3.0 mg/l (Ali, Naz, Siddiqui, & Iqbal, 2008). So the optimal size for sugarcane explant inoculation was found to be 3 mm. The effect of cytokinins was also shown with BAP alone providing good results in shoot formation. But, media supplemented with 0.5 mg/l of BAP and 0.25 mg/l of Kinetin also showing positive results for shoot formation. The use of kinetin along with BAP for the establishment and shoot formation of sugarcane explants has widely been reported (Ali, Naz, Siddiqui, & Iqbal, 2008). In a second experiment where a protocol for sugarcane explant micropropagation was being sought it was found out that the cytokinin BAP was more effective in the establishment and shoot formation of the explants than Kinetin or indole-3-butyric acid (IBA) (Baksha, et al., 2002). Maximum shoot regeneration and shoot formation were gotten on media that was supplemented with 2.0 mg/l of BAP and 0.5 mg/l of IBA. This produced shoots within 2-3 weeks after inoculation (Baksha, et al., 2002). In a third experiment the effect of plant

growth regulators was also measured on the establishment and shoot generation of the explants. The results showed that the apical meristems that had been inoculated in the media started elongation only a few days after they had been inoculated. Shoot initiation had started after 15 days of explantation (Khan, Dahot, Yasmin, Khatri, Seema, & Naqvi, 2006). Excessive phenolization was observed to occur in the explants that were not kept in the dark, meanwhile a 20 day incubation period in the dark showed favourable results (Khan, Dahot, Yasmin, Khatri, Seema, & Naqvi, 2006). With respect to the growth regulators the results were as follows: a higher explant establishment, shoot formation and multiplication were found to occur in media that contained Kinetin, IBA and indole-3-acetic acid (IAA). A concentration of 4.5 mg/l of BAP was found to be ideal for explants but variations in genotype were present (Khan, Dahot, Yasmin, Khatri, Seema, & Naqvi, 2006). The concentration of sucrose that was present in the media was also found to have an impact on the regeneration and the production of shoots. Depending on the genotype of the explant, a 4% or 6% sucrose concentration in the media was found to have effects on the explants (Khan, Dahot, Yasmin, Khatri, Seema, & Naqvi, 2006). Based on the results it is deduced that the production of shoots in sugarcane explants is not only on the growth regulators but also on the genotype of the explant. There is a complex interaction between genotype/media composition, including sucrose concentration, which affects the establishment and the production of shoots by explants (Khan, Dahot, Yasmin, Khatri, Seema, & Naqvi, 2006). The results also showed that the addition of IAA, IBA and Kinetin enhanced the production of sugar cane plantlets (Khan, Dahot, Yasmin, Khatri, Seema, & Naqvi, 2006). The use of 4% sucrose concentration was found to be the best when the plant was being regenerated and when shoot production was the goal. The same concentration could be used for the multiplication phase of the micropropagation process (Khan, Dahot, Yasmin, Khatri, Seema, & Naqvi, 2006).

Methodology

Equipment/materials needed:

The equipment and materials that were used in the process of this research were the following items: Jars of sterile water (5 shoots per jar), empty sterilized jars (enough for 5 shoots per jar), and jars with antioxidant solution inside (50 mg/l of ascorbic acid and 50 mg/l citric acid) (enough to rinse shoots with the antioxidant 2 times). Sterilized absorbent paper (at least one piece per jar), sterilized cloths for cleaning laminar flow cabinet, sterilized beaker for disposal of water, sterilized bags for disposal of plant debris and disinfectant used for cleaning vegetables (Microdyn or plant preservative)

Methods:

Preparation of the Media

Establishment Media: the media was prepared first by placing 1000 ml of water in a 6 l beaker. The beaker was placed on a hot plate and a stirring magnet was placed inside. The liquid components of the media were then added along with the sucrose. A total of 5 g of sucrose was added to the solution. The liquid components of the media were gotten from prepared stock solutions so a total of 20 ml of each of the components were added to the water, these included: micronutrient solution, calcium chloride, Potassium Iodide, Fe-EDTA solution, Vitamin Heinz solution, Vitamin B and Plant Growth Regulators. The solution was then adjusted to a pH of 5.7 using NaOH and HCl. The solution was then left to heat. After a high enough temperature was reached 2.5 g of Phytagel was added to the solution and allowed to mix properly. The solution was then poured in glass jars and test tubes at 20 cm^3 per vessel. The media was then autoclaved to sterilize it.

Multiplication Media: the process for the making of the multiplication media was the same as that of the establishment media. The difference came when it was time to add the liquid components of the media. In the establishment media additional plant growth regulators had been added. In this media only the basic nutrients were placed.

*The exact concentrations of each of the components can be found in the Appendix.

Harvesting and Preparing Shoots for Cold Storage

While in the field, the shoots were cut approximately 2ft long, with the leaves trimmed all the way to where to where they converged at the stalk. On the shoots, the terminal node of the cane stalk was included. These were then transported to the laboratory cleaning station. Once the

stalks were taken to the laboratory's cleaning station, one leaf layer was removed and the top part of shoots was cut off. The shoots were then washed with a mixture of soap, bleach and water to clean and remove soil material. Next the shoots (10 each) were wrapped in parcels with wrapping paper and stored in the refrigerator at 4°C for 5 days.

Preparing Shoots in Lab for first Thermotherapy treatment

After a 5 day period the shoots were removed from the refrigerator. They were unwrapped and another layer of leaves was peeled off. The stalks were cut so that the shoots were approximately 6-8 inches long. The shoots were then placed in a water bath at 50°C for 20 mins. The shoots were kept in constant motion so that the tissues would not suffer from heat stress. The jars with sterilized water were placed in the water bath so that the temperature of the water inside jars would equalize with the water in the bath. The jars would be used in a later step. Then the shoots were placed in large containers and taken to the sterile room.

Further reduction in Shoot Size and Shoot Sterilization in Sterilized Room

In the sterile room, another layer of leaves was removed using an alcohol sterilized knife. The shoots were also cut so that they were approximately 3-4 inches long. The shoots were then placed inside of a sterilized jar. Once all of the shoots were placed inside the jar, the inside was sprayed with 70% alcohol. A forceps was then used to set fire to the shoots inside of the jar. The forceps were moved around the rim of the jar. The cover was then replaced so that the flame would be put out.

Extracting Meristem in Laminar Flow Cabinet

The sterilized jars with shoots were then moved into the laminar flow cabinet. Water with disinfectant was then added to the jars. The shoots were then taken out of the jar one at a time. The leaves were then progressively peeled back and a scalpel was then used to cut the shoot top. The leaves were continually removed until the shoot was just over an inch long. The shoots were then placed into the jar with the disinfectant and the process was repeated until all shoots were in the jar with disinfectant. The shoots were rinsed with sterile water for 2-3 times. The sterile water was then disposed of and the jars were filled with antioxidant solution. The explants were left in the antioxidant solution for 1-2 mins. The antioxidant was then removed and fresh antioxidant solution was added. The explants were again left for 1-2 mins in the antioxidant solution. The jars of sterile water were collected from the water bath and taken to the laminar

flow cabinet. The shoots were then transferred into the warmed distilled water and the jars were sealed with plastic wrap. The jars, along with the shoots, were then returned to the water bath at 50°C. The jars were left in the water bath for 10 mins. The jars were kept in constant motion to prevent the plant tissues from being damaged by the heat. The jars with shoots were then returned to the laminar flow chamber. The water from the jars with shoots was then removed. The shoots were then removed from the jar and placed on absorbent paper to remove excess water. The shoots were then placed on the media upside down if in tubes or, if in jars, on side with shoot slightly buried in the media. The container was then covered and sealed with plastic wrap.

Experiment 1: Establishing the explants in establishment media

In the first experiment after the meristems had been successfully extracted they were then established in establishment media. This media the explants were then left in this media for a period of a week after which they were then transferred to multiplication media. These explants were then treated the same as the plants that were established in the multiplication media alone.

Experiment 2: Establishing the explants in multiplication media

In the second experiment the meristems were established in multiplication media directly after they had been successfully extracted. This media was the regular multiplication media in which explants are placed when they are in the multiplication phase.

Results & Discussion

The results gotten for this research were in line with what was expected. The data shows that the explants that were inoculated in the establishment media had both a faster regeneration time and a faster shoot production when compared to the explants that were established in the multiplication media alone. In terms of the survivability of the explants, it can be seen in table 1 that both media types had the same survivability. Both media types ended up having 6 samples per media type. The reason for the low survivability of the explants was due to inexperience in the field of micropropagation on the part of the researcher. Establishment was carried out for three sessions, 20 explants were established the first time, 14 the second and 6 the third for each of the media types. Due to a lack of experience, all of the explants that were established during the first two sessions had died due to contamination of the explants. The third session saw success in the first week's establishment of the explants and for the duration of the observations the survivability of both media types was observed to be the same. In light of this it can be deduced that both media types help the plant in its survival. This was also the case in the pre-treatment of sugarcane midrib segments with an auxin. Both samples that had been pre-treated with the growth regulator and those that had been used as a control saw the same survivability (G., Arvinth, Sheeba, Kanchana, & Subramonian, 2006). In this sense, the use of the establishment media is not needed since the survivability of the explants in either of the media should be equal. Another similarity that was seen in both of the explants was in terms of their phenolization characteristics.

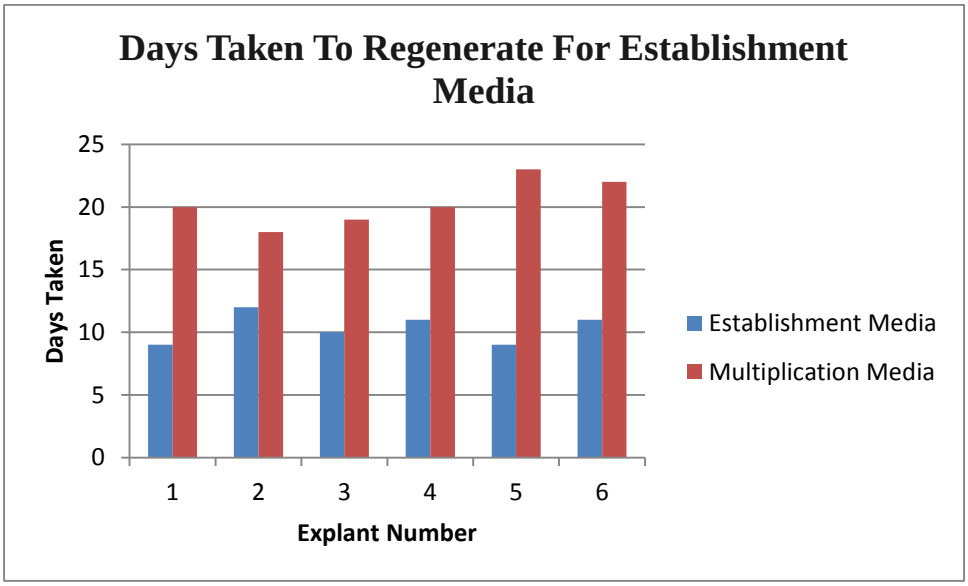
Table 1: Average statistics gathered for the two media types of over a period of 2 months.

Media type	No. of Explant Inoculated	No. of Explant Regenerated	Days to Regenerate	Average No. of Initial Shoots	Shoot Initiation	Days to Shoot Visible
Establishment	40	6	10.3	0	33.5	43.3

Multiplication	40	6	20.3	0	N/A	N/A

The table above shows the general statistics that the explants inoculated in the two media got after observation for two months. Both media were seen to have had the same number of explants regenerate. Regeneration time for the explants in the multiplication media was double that of those in the establishment media. Shoot formation was not observed in the explants established in the multiplication media.

Figure 1: Chart Showing Difference in Regeneration Time of the Explants



The figure above is showing the difference in regeneration time of the explants in the different treatments. From the graph it can be seen that the explants in the establishment media had a shorter regeneration time than those in the establishment media.

Now in regards to the effect that the pre-treatment of the explants in an establishment media that has been infused with PGRs the difference comes in regards to the regeneration time and the generation of shoots. As can be seen in table 1, and when tables two and three are compared, there is a notable difference in the number of days that the explants take to regenerate. In the explants that were inoculated in the establishment media an average of 10.3 days was taken to for the explant to regenerate. Meanwhile, in the explants that had been established in the

multiplication media alone an average of 20.3 days was taken for the explants to regenerate. A statistical test that was run on the data showed that the difference between the two data sets was significant, as can be seen in table 5. The alpha for the analysis was 0.05 but when the p. value for the statistical analysis was returned there was a p. value of 4.4813E-5. This p. value was considerably lower than the alpha value, this meant that the difference between the two data sets was significant and that the establishment media did have a significant effect on the regeneration rate of the sugarcane explants. The reason for this can be explained by the presence of the cytokinins in the establishment media. The presence of gibberellins, IBA and other growth stimulating cytokinins means that during the 8 day period where the plants were in the establishment media their cells were stimulated by the cytokinins and cell division and growth were started. This was shown in the establishment of a micropropagation protocol where higher concentrations of cytokinins helped to stimulate the growth and the regeneration of the explants (Ali, Naz, Siddiqui, & Iqbal, 2008). In this case the establishment media had these cytokinins in higher concentrations than the multiplication media as well and sometimes it had cytokinins present in it there were not present at all in the multiplication media. In regards to the production of shoots there was a marked difference in the amount of time that both explants took to produce them. In the explants that had been inoculated previously in the establishment media the start of shooting and the visible production of shoots came at an average of 33.5 and 43.3 days, respectively. The explants that had been inoculated on the multiplication media showed no signs of shooting until the last day when observations were taken. An explanation for this may be found in the previously mentioned regeneration rate. The explants that had been inoculated in the establishment media had a much shorter regeneration time. This meant that they had undergone some form of growth and as such it would take a shorter time for them to start the development of shoots. The explants that had been inoculated in the multiplication media had taken a much longer time to regenerate and so their production of shoots would be delayed in reference to those from the other set.

Table 2: Results gotten for the explants inoculated in the Establishment media

Establishment Media					
Explant Number	Days to Regenerate	No. of Initial Shoots	Final Number of Shoots	Shoot Initiation	Days to Shoot Visible

1	9	0	1	32	42
2	12	0	1	35	44
3	10	0	1	36	45
4	11	0	1	33	46
5	9	0	1	30	40
6	11	0	1	35	43
Average	10.3	0	1	33.5	43.3

The table above shows the data gotten for each of the explants that were inoculated in the establishment media for a period of 8 days. The explants that were first inoculated in this medium were seen to begin shoot production after a period of around a month with shoots visible after an average of 43 days.

Table 3: Results gotten for the explants inoculated in the Multiplication media

Multiplication Media					
Explant Number	Days to Regenerate	No. of initial Shoots	Final number of shoots	Shoot Initiation	Days to Shoot
1	20	0	0	N/A	N/A
2	18	0	0	N/A	N/A
3	19	0	0	N/A	N/A
4	20	0	0	N/A	N/A
5	23	0	0	N/A	N/A
6	22	0	0	N/A	N/A
Average	20.3	0	0	#DIV/0!	#DIV/0!

The table above shows the data gotten for the explants that were inoculated in the multiplication media. These explants were inoculated directly into the multiplication media with subsequent weekly changes into media of the same type. These explants were not seen to start any shoot formation up to the time of the last recording of data.

Now when table 4 is seen and the characteristics for the explants in the two media are compared, it can be seen that both of the explants had phenolization characteristics that were the same. Both of the explants had high phenolization rates and their media had to be changed on a weekly basis. Both of the plants had been kept in light/dark conditions with 16 hours of light and 8 hours of

darkness. High phenolization rates and light availability has also been noted in other researches whereby plants that were incubated under conditions that had high light availability also show high rates of phenolization (Khan, Dahot, Yasmin, Khatri, Seema, & Naqvi, 2006).

Table 4: Phenolization characteristics and other qualitative data

Media Type	Phenolization Rate	Media Change Frequency	Conditions	Appearance	Media appearance
Establishment	High	Weekly	Light/Dark	Dark with phenols	Dark Around Explant, Light Brown Throughout
Multiplication	High	Weekly	Light/Dark	Dark with phenols	Dark Around Explant, Brown Color Throughout

The table above is showing some of the phenolization characteristics that were present in the explants when they were inoculated in both media. The explants in the establishment media could only be measured once when they were in the establishment media but their performance was still noted when they were transferred over to the multiplication media. The phenolization characteristics of both explants were the same. High phenolization rates were noted for both explants and weekly media transfers had to be done because of this.

Table 5: Statistical analysis of regeneration time for two samples

t-Test: Two-Sample Assuming Unequal Variances		
	20	*9*
Mean	20.4	10.6
Variance	4.3	1.3
Observations	5	5
Hypothesized Mean Difference	0	
df	6	
t Stat	9.260129589	

P(T<=t) one-tail	4.4813E-05	
t Critical one-tail	1.943180281	
P(T<=t) two-tail	8.96261E-05	
t Critical two-tail	2.446911851	

The table above is showing the T-test that was performed on the data for regeneration time to see if there was any significant difference in the between the two. The alpha for the test was a 0.05 and with the p. value having a value of 4.4813E-05 then it is possible to deduce that the difference seen between the two data sets is significant and that a pre-treatment with establishment media does help in a faster regeneration, and establishment, of sugarcane explants.

Conclusions

After the performing the research and the analysis of the data that was gotten from the observations there are several deductions that can be made from the results. The first is that in regards to the survival of explants in media there is no difference between the two media that were tested. So in strict terms of whether establishment media is necessary for the establishment of sugarcane explants then it can be concluded from this research that the use of establishment media is not necessary. Now in regards as to the effectiveness of the use of establishment media in the establishment of the sugarcane explants another conclusion can be gotten. With the increased rate of regeneration through the use of establishment media then it can be concluded that the use of establishment media is beneficial and there is merit in its use. A final conclusion that can be drawn is that through the reading of literature, and through the results gotten, it can be safely deduced that a period of dark incubation would help to reduce the phenolization rate that the plants go through and help in the increase of media transfer intervals of the explants.

Recommendations

Recommendations in terms of how this experiment could have been improved are in terms of the repetition of the experiment with a larger amount of samples for each of the media types. Due to time and explant availability constraints there was little that could be done in this research but a larger and more detailed research on the effect that these different media types would have on the establishment of the explants would be ideal. Another recommendation for the improvement of this experiment would be in the measurement of the phenolization characteristics of the explants. By placing numerical values such as phenolization distance or percent phenolization of the explant then a better idea of what is going on in the explants can be better understood and compared between the two media types. Other ways in which this research could be improved are notable but these two were two of the most noteworthy improvements that could be done to this research. Now recommendations in terms of how this research can be used in practicality is that if micropropagation of a sugarcane explant is to be done then the results of this research show that there is a benefit to using an establishment media that is infused with cytokinins. This can then be used to produce explants faster and make the operation more effective.

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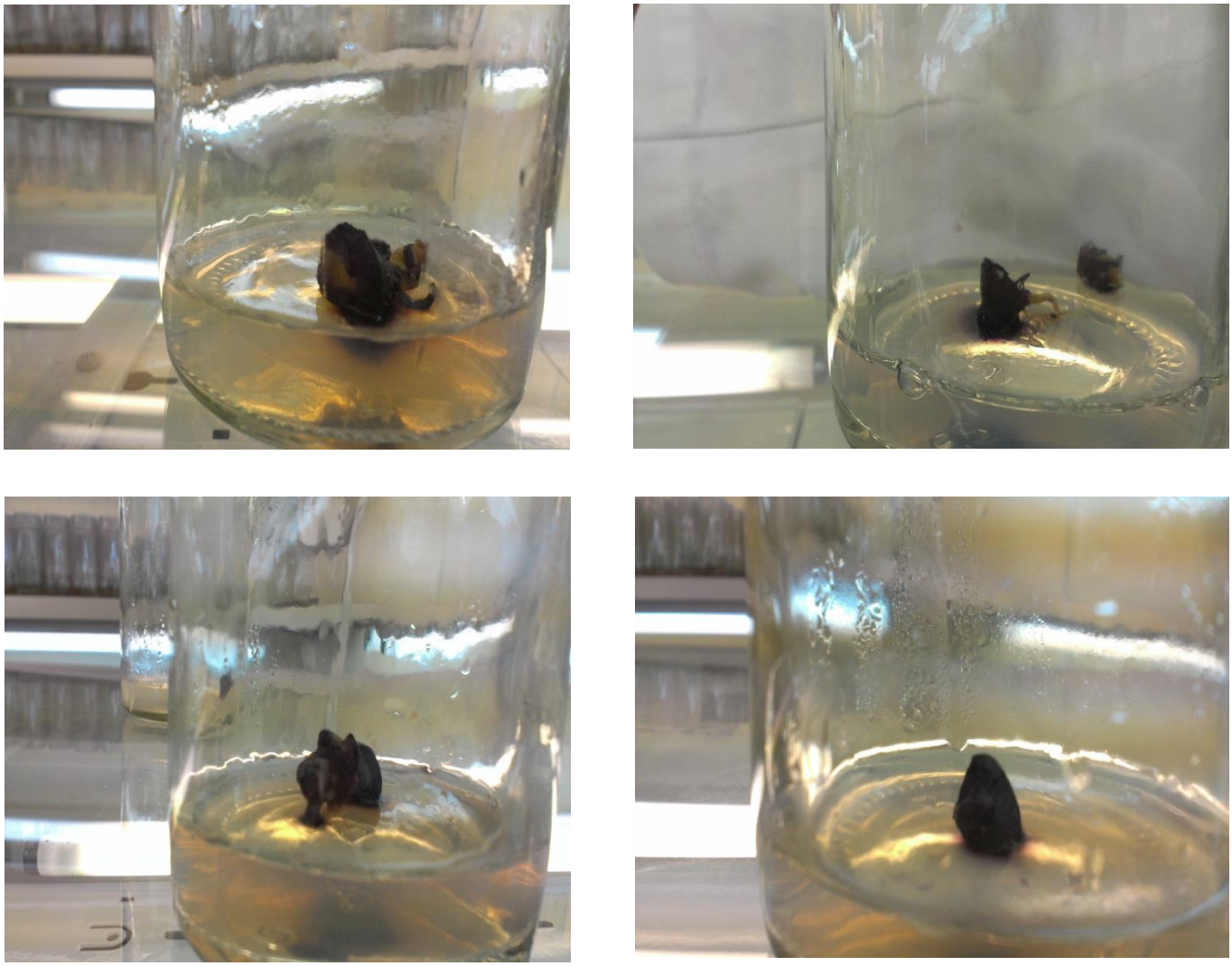
Appendix

Figure 2: Photographs of Explants in Establishment Media



The photographs above are showing the explants as they were in the establishment media. As it can be seen they were heavily phenolized due to the exposure to the light.

Figure 3: Concentration of Different Solutions in the Media

Sugar Cane Media Stock Solutions

Store all stock solutions in amber bottles / Add one chemical at a time (until dissolved) to avoid precipitation. Before use always check for precipitate or other "foreign bodies" in stock solutions. If contaminated make fresh solution.

Macronutrient Stock Solution - Solution A	**g/L**	✓
Ammonium Nitrate (NH_4NO_3)	82.5	
Potassium Nitrate (KNO_3)	95	
Magnesium sulphate 7-hydrate ($MgSO_4$ $7H_2O$)	18.5	
Potassium di-hidrogen phosphate monobasic. (KH_2PO_4)	8.5	

CAN BE STORED FOR SEVERAL WEEKS IN REFRIGERATOR AT 2-4 ºC. At San Rafael this stock is not stored in the refrigerator.

Micronutrient Stock Solution - Solution B	**g/L**	✓
Boric acid. H_3BO_3	0.310	
Manganese sulphate hydrate $MgSO_4$ H_2O	0.845	
Zinc sulphate 7-Hydrate. $ZnSO_4$ $x7H_2O$	0.430	
Sodium molybdate 2-hidrate. $NaMoO_4$ $x\ 2H_2O$	0.0125	
Copper II sulphate 5-hidrate. $CuSO_4$ $x\ 5H_2O$	0.00125	
Cobalt II cloride 6-hidrate. $CoCl_2$ $x\ 6H_2O$	0.00125	

CAN BE STORED FOR UPTO A YEAR IN REFRIGERATOR AT 2-4 ºC

Calcium Chloride ($CaCl_2$ $2H_20$) - Solution C	**g /L**	✓
Dissolve in distilled water store in refrigerator - Soln should be clear. If soln not clear add drops of dilute HCL until clear then adjust to pH 7.	22	

Potassium Iodide (KI) - Solution D	**g /L**	✓
Dissolve in distilled water store in refrigerator in amber glass	0.042	

Fe-EDTA Stock Solution - Solution E	g /L	✓
Na EDTA in 100 ml boiling distilled water	1.865	
$FeSO_4$ 7 H_20 in 100 ml boiling distilled water	1.390	

Add Fe over Na solution, when solution turns yellow bring to 500 ml in volumetric flask. Store in dark brown glass contrainer.

Vitamin Heinz Stock Solution	g /L	✓
Nicotinic acid	0.025	
Pyridoxine B6	0.025	
Thiamine (Vit B1)	0.050	
Glycine	0.100	

Dissolve the reagents separately and bring up to volume. Store in refridgerator in a brown bottle.

VITAMIN STOCK SOLUTIONS CAN BE STORED IN REFRIGERATOR IN AMBER GLASS FOR 2-3 MONTHS

Vitamin B^+	g /L	✓
Nicrotinic acid	0.250	
Pyridoxine	0.050	
Thiamine	0.025	
Gylcine	0.150	
Arginine	2.500	
Biotin	0.050	

Dissolve in distilled water and store in refridge

VITAMIN STOCK SOLUTIONS CAN BE STORED IN REFRIGERATOR FOR 2-3 MONTHS

HORMONES - aim to make fresh stock solutions each week - store all stock solns in amber containers	*Solvent*	*Dilute nt*	*Powder Storage*	*Liquid (stock) concn*	✓	*Liquid (stock) Storage*	*Final Working concn. (in literature)(mg / l)*
Indole-3-acetic acid (IAA) - Auxin (stock in refridge lasts only 2-4 days)	Ethanol or 1N NaOH	Water	- O ºC (freezer)	13 mg / 100 ml		- O ºC (freezer)	0.01 - 3.0
Indole-3-butyric acid (IBA) - Auxin	Ethanol or 1N NaOH	Water	2 - 8 ºC (refridge)	25 mg / 25 ml		- O ºC (freezer)	0.1 - 10

1-naphthaleneacetic acid - Auxin	1N NaoH	Water	2 - 8 °C (refridge)	13mg/100 mL		2 - 8 °C (refridge no more tn 2 wks)	
6-Benzylaminopurine (6-BAP) - Cytokinin	1N Na OH	Water	Room temp	25 mg / 25 ml		2 - 8 °C (refridge no more tn 2 wks)	0.5 - 5.0
Kinetin - Cytokinin	1N NaOH	Water	- O °C (freezer)	20 mg / 100 ml		- O °C (freezer)	0.1-5.0
Gibberellic Acid (GA_3) -	Ethanol	Water	Room temp	10 mg / 100 ml		2 - 8 °C (refridge)	0.01 - 5.0
CULTAR - (paclobutrazol - PBZ)	Water	Water	Refridge	1 ml / 9 ml		Refridge	