The Effects of 2,4-D in Formation of Callus on Immature Zygotic Embryo of Papaya (Carica papaya L.)

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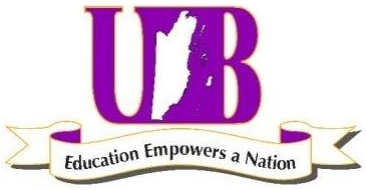
Under the Supervision of Dr. Dion Daniels



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Abstract

Papaya (*Carica papaya* L.) is a popular fruit that has been commercially produced in subtropical and tropical countries which includes Belize. This tropical fruit has been produced through micropropagation and tissue culture techniques. This study highlights the induction of callus using immature zygotic embryos of *C. papaya* through indirect somatic embryogenesis. Callus was induced on immature zygotic embryos after 4-5 weeks of culture on half strength Murashige and Skoog (1972) inorganic salts, which served as the basal medium supplemented with 5mg/L, 10mg/L and 15mg/L concentrations of 2,4-D in treatment 1,2 and 3 respectively. The highest percentage of callus formation was in treatment 2 supplemented with 10mg/L of 2,4-D, in which 60% of papaya zygotic embryos appeared to have callus formation. In treatment 1, a concentration of 5mg/L of 2,4-D was 50% effective in inducing callus formation on zygotic embryos. The effects of 2,4-D at a concentration of 15mg/L induced 33.33% callus formation but growth of callus was small. The study is viable in the production of *C. papaya* in Belize and would enable the laboratory propagation of papaya plant which can be use for further somatic embryogenesis studies to generate selected types of papaya plants *in vitro.*

Acknowledgement

My greatest gratitude goes to Dr. Dion Daniels for proof reading this research paper and for assisting me from the start of this research work. His guidance and note for improvement in this research paper is product of this remarkable and intriguing research of which I am very proud of having completed. A special recognition to Dr. Thippi Thiagarajan for his assistance in providing his published journal related to this project. Last, but not least, a special thanks to Dr. Caroline Herron for reviewing the abstract of this research paper and providing her professional comments. A special acknowledgment to my common-law husband, Glenford Guy, for his support and patience in assisting me throughout this project.

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Introduction

Biotechnology is the application of scientific techniques to modify and improve plants, animals, and microorganisms to enhance their value (Wieczorek, 2003). Plant Biotechnology in particular has paved the way to many commercial applications of plants such as production of pathogen free plants, mass production of plant specie, conservation of germplasm, genetic engineering, and many other applications. Papaya is a model plant in plant technology using tissue culture to improve the production of the fruit via somatic embryogenesis. Somatic embryogenesis is an ideal system for the investigation of the whole process of differentiation in plants, as well as the mechanism of expression of totipotency in plant cells. It has several specific advantages over the conventional micropropagation. Somatic embryos can differentiate either directly from the explant without an intervening callus phase or indirectly after a callus phase (Kamle et.al., 2011).

Papaya regeneration using zygotic embryos is viable via indirect somatic embryogenesis, a process in which somatic embryos are generated from *in vitro* cultured cells. Callus is defined as an unorganize tissue mass growing on solid subtrate (Kamle et.al., 2011). Callus forms naturally on plants in response to wounding, infestation, or grafting. The formation of callus is produced from a single differentiated cell, and many callus cells are totipotent, being able to regenerate the whole plant body. During callus formation, there is some degree of dedifferentiation; the changes that occur during development and specialization are reversed, both in morphology and metabolism. Callus formation can be induced by high hormone levels such as auxin and cytokinins in the semi-solid culture media (Ikeuchi, Sugimoto, & Iwase, 2013).

In a study conducted by Malabadi et al. (2011) showed that somatic embryos can be formed using immature papaya zygotic embryos after four to six weeks of culture on Murashige and

Skoog basal medium in the presence of thidiazuron (TDZ) in combination with dichlorophenoxy acetic acid (2, 4-D). The study also showed that somatic embryos never form on explants cultured on control medium lacking plant growth regulator (Malabadi et al., 2011). In a similar experiment conducted by Bhattacharya et.,al (2002) the MS medium was supplemented with 2, 4- D which supported formation of somatic embryos.

Micropropagation of papaya has become important for the multiplication of specific sex types of papaya, and in the multiplication of genetic transformation technologies (Jaime, et al., 2007). Generating Papaya plants that are resistant to diseases is the mantra of genetic transformation of somatic embryos produced through indirect somatic embryogenesis. Papaya is most commonly propagated by shoot tip or axillary bud. For this research, zygotic embryos excised from immature seeds were used to induce callus formation. Once callus with embryogenic potential is formed, it can be maintained in cell suspension but in this research the focus was solely on effects of potent auxin (2,4-D) in callus formation.

In brief, the study investigates if callus formed or not and once formed close observation was made to compare the characteristics of callus in each concentration of 2,4-D. Problems that are encountered when using indirect somatic embryogenesis are somaclonal variation and production of off-types papaya fruits. Hence the reason papaya fruit is not commonly micropropagated through somatic embrogenesis (Jaime, et al., 2007). However, callus formation through indirect somatic embrogenesis still remains important in genetic transformation. Genetic transformation is very important in producing superior cultivated varieties of *C. papaya* L. variety Maradol. Super varieties are more tolerant to viruses, herbicides, and increases the shelf life of the fruit.

This variety of papaya is produce largely by Brooks Tropicals Industry in the Corozal District of Belize.

Belize overtook Malaysia in 2007 to become the third largest papaya exporter. The main papaya growing region in Belize is the Orange Walk District located in the northwestern area of the country. Belize produces the small-sized Solo-type (Sunrise, Kapoho, and Tokita Sunrise) and large-sized type (Princess Scarlet, Maradol Rojo, and Tainung II) papaya cultivars. The decline in production between 2006 and 2010 was from 34,500 tons to 25,100 tons, or 37.45 percent is due in large part to the destruction caused by Hurricane Dean in 2007. Unlike most of the other leading exporters, only a small portion of the Belizean crop, less than 1 percent, is consumed locally. Papaya ranks third behind citrus and sugarcane in Belizean crop exports. In 2010, papaya exports were estimated to be about $12.6 million (Evans & Ballen, 2012).

Since Belize is becoming one of the leading countries in the export of Papaya, growers and industries on a whole should consider producing superior varieties of the fruit to solve issues of papaya disease. However, micropropagation of papaya can be done using shoot tip, axillary bud, and single node structures. Using these explants, multiple productions of the desired sex and variety of papaya can be obtained. Micropropagation will produce uniform plantlets which farmers can transplant to growing fields after acclimatization of the plantlet. This will allow them to re-cultivate the damage papaya fields caused by hurricanes in a relative short period of time. The aim of this research is to provide an overview of the effects of three different concentrations of 2, 4-D on callus formation on immature zygotic embryos of papaya explants; emphasis was on the papaya (*Carica papaya*) variety Maradol.

Literature Review

Papaya (*Carica papaya*) is an important tropical fruit that is easily grown by propagated seeds, however, through tissue culture propagation, papaya has been produced in an effort to combat many problems encountered in culturing this fruit *ex vitro*. Plant tissue culture refers to the *in vitro* cultivation of all part plants. In order to use Papaya in this model system, it is crucial to obtain a comprehensive review on the botanical, geographic, medicinal, economic and scientific use of this fruit.

Botanical Description

The botanical characteristics of the papaya tree and physiological description of the fruit are very important to properly identify the type of papaya. Papaya is a semi-woody tropical herb that has a single, straight and hollow stem which contains scars derived from the fallen fruit (Jaime, et al., 2007). The leaves of the papaya tree are large and palmately –lobed, normally exhibiting a spiral arrangement and clustered at the crown. The papaya fruit can exhibit varying shape of either round to an elongated shape. The semi-wild papaya trees bear very small fruit ranging from 2.5 to 15 cm in length (Jaime, et al., 2007). The papaya fruit is a very good indicator of the sex type of the tree. Fruits from female tree are round, male trees such as wild type are small or bear none, whereas fruits from hermaphrodite trees are elongated (Gunetilele & Jayatissa, 1990). The skin of papaya fruit is waxy and when the fruit is immature it is rich in white latex. When a papaya fruit ripens, the skin develops a yellow orange skin while the succulent flesh becomes aromatic, yellow orange which makes it obtain a sweet flavor. A mature papaya fruit contains numerous grey to black ovoid seeds that is attached to the succulent flesh by white fibrous tissue (Jaime, et al., 2007).

The classification of papaya has changed several times during the past years. Presently, papaya is grouped in the Caricaceae family which includes 35 latex-containing species in four genera, *Carica*, *Cylicomorpha*, *Jarilla*, and *Jacaratia* (Jaime, et al., 2007). The genus *Carica*, is represented by 22 species and is the only genus of the Caricaceae family that is cultivated as a fruit tree while the other three genera are grown as ornamentals. Another recent molecular evidence show that genetic distances occur between papaya and other related species. The taxonomic classification has been revised so that *C. papaya* remained as the only species within the genus Carica(Jaime, et al., 2007).

Table 1. The Taxonomic classification of *C. papaya*.

Taxonomic Rank	**Classification**
Order	Brassicales
Family	Caricaceae
Genus	*Carica*
Species	*C. papaya*
Variety	Maradol

Geographic Distribution

Papaya is considered as a tropical and subtropical fruit because of the countries in which it is mostly grown. Papaya is an important fruit crop in Bangladesh and is one of the most versatile fruit which is used as a vegetable (Roy, et al., 2012). Japan has also been another country where papaya has been cultivated because of the prevailing warm temperatures. Papaya is traditionally considered as vegetable in Southeast Asia and immature green papaya fruits are sold in local markets for cooking (Tetsushi, et al., 2008). Papaya is also an important fruit in Kenya whereit has been micropropagated to combat constraints to the production of the fruit *ex vitro* (Wanzala,

et al., 2007). In Belize, papaya is consumed as a fruit and used in many dessert recipes. Papaya is also fermented and consumed as wine or as jam.

Medicinal Use

Several fruits around the world are consumed because of their natural medicinal benefits of which papaya fruit is of no exception. The major constituents in the papaya fruit are water and carbohydrates that are low in calories and rich in natural vitamins and minerals, particularly in vitamin A and C, ascorbic acid and potassium (Jaime, et al., 2007). Papayas are also rich in folate, magnesium, copper, pantothenic acid and fiber. They also have B vitamins, alpha and beta carotene, lutein and zeaxanthan, vitamin E, calcium, potassium, vitamin K and lycopene, the powerful antioxidant most commonly associated with tomatoes (Medical News , 2014).One of the medicinal uses of the papaya is the milk latex which is used to treat eczema, a fungal skin disease. Another benefit is from the papaya leaf extracts which is used to treat intestinal worms and boils. The beta-carotene found in papaya help lower asthma in patients who consume high quantities of papaya. Cancer is another disease that is prevented by eating papaya. The powerful antioxidant beta-carotene has been shown to play a role in reducing prostate cancer in younger men(Medical News , 2014). Papaya has also been consumed to help reduce many other sicknesses such as diabetes, heart diseases, and inflammation.

Economic Value in Belize

The papaya fruit is an export trade product in Belize besides the other three major products which are citrus, sugar and banana. In the release report from the Statistical Institute of Belize, in February 2015 alone, the export earnings of papaya were valued at an annual rate of 3% (2015). Papaya cultivation had its origin in South Mexico and Costa Rica. Total annual world production of papaya is estimated over 6.8 million tonnes of fruits. India leads the world in papaya

production with an annual output of about 4.5 million tons in 2010-2011 (Malabadi et al., 2011). Although papaya is mainly grown and consumed in developing countries it is also becoming an important fruit internationally as a fresh fruit and processed product. Papaya plants are also produced for papain and chymopapain, two industrially important proteolytic enzymes found in the milky white latex exuded by fruits (Malabadi et al., 2011). In general, female fruits tend to exude more papain than hermaphrodite fruits.

Papaya Use in Scientific Research

In scientific research, papaya is propagated through tissue culture models. The use of micropropagation techniques is an essential requirement in papaya cultivation that produce a large number of true to type high quality planting material. Furthermore, tissue culture techniques can be used as an important tool in crop improvement programs since they help to overcome the problems experienced in conventional breeding methods and in rapid cloned production of crops. Although the success of tissue cultured papaya clones has been reported, rooting of micropropagated plantlets is a major problem that is encountered. Therefore, somatic embryogenesis offers an attractive alternative, as the embryos are bipolar structures bearing both root and shoot apices. In addition, embryogenic cultures can optimally produce relatively large numbers of embryos per culture flask. In tissue culture, somatic embryogenesis is the most widely used regeneration system for many plant species. In plant species such as *C. papaya*, somatic embryogenesis is used to induce somatic embryos and callus formation. Apart from root explants, the ovules have been considered as the most suitable explants because of high regeneration potential (Bhattacharya et.,al 2002).

Materials and Methods

This Research was conducted at the Micropropagation laboratory at the University of Belize Belmopan Central Campus. This research was conducted during the period of March 27th, to May 5th, 2015.

PLANT MATERIAL

Papaya *Carica Papaya* L.variety Maradol was the mother plant used in this research. Immature seeds were dissected to obtain the immature zygotic embryo. The green fruits were first surface washed with liquid detergent and tap water for 5 minutes to get rid of dust particles. The fruits were surfaced sterilized with 70 % ethanol. Two green fruits were cut longitudinally with the sterilized knife for extraction of immature zygotic seeds. Immature zygotic embryos were dissected out of seed coat using forceps and scalpel under sterile condition in the Laminar Flow Chamber.

INSTRUMENTS

In the extraction of immature zygotic embryos, forceps, knife, scalpel and tile were used. Bunsen burner was used to flame the dissecting instruments. Jars of 250ml volumetric capacity were also used to hold the culture medium. Conical flask of 750ml volumetric capacity was used to prepare culture medium. Autoclave and Laminar Flow Chamber were used to sterilization.

EXPLANT CULTURE MEDIA

Semi-solid Medium

Immature zygotic embryos of Papaya were cultured on half strength Murashige and Skoog (1962) stock solution (MS 1,2, 3, 4, and 5) and supplemented with MS vitamins, 100mg/L of myo-inisitol, 400mg/L of L-glutamine, and 60g/L of sucrose. 300ml distilled water was then

added to the conical flask in order for the volume to be adjusted to the final volume mark, 750ml. The contents in the flask were stirred with stirring spoon to dissolve the contents. Each medium was supplemented with three different concentrations (5mg/L, 10mg/L, and 15mg/L) of dichlorophenoxy acetic acid (2, 4-D) respectively. The media in this study was adjusted to pH 5.8 using1 N sodium hydroxide (NaOH)or HCl with the use of a pH meter. The culture medium was gelled with 8g/L of agar. Glass jars of capacity 250ml volumetric capacity were used and 30 ml of culture medium was added to each jar and autoclaved at 121^0C at a pressure of 1.2kg/cm^3 for a period of 15 minutes (Figure 10 in Appendix).

TREATMENTS

The culture medium containing different concentration (treatments) of 2, 4 D was placed in jars and labeled treatment 1, 2,and 3 respectively. In treatment 1 (5mg/L 2,4-D) 9 jars were obtained, treatment 2 (10mg/L 2,4-D) 10 jars, and treatment 3 (15mg/L 2,4-D) 9 jars. In each jar 3 immature zygotic embryos were cultured on the basal medium. The jars were kept in a growth room enclosed in a box in total darkness at room temperature 27 ± 2^0 C for 4-5 weeks.

INOCULATION

Inoculation of zygotic embryos was conducted in the laminar flow chamber to reduce the risk of contamination (see figure 11 in Appendix). The laminar flow was cleaned with 70% ethanol before commencing work. The blades and forceps were immersed in 70% ethanol and were flamed on the Bunsen burner before cutting the papaya seeds. The zygotic embryos were removed from the immature seeds using the forceps. These were then swiped unto the culture media making sure the forceps did not pierce the media. During the inoculation period, hands and the laminar flow were often sprayed with 70% ethanol to reduce contamination to its minimal. A total o f three zygotic embryos were placed in each jar containing the culture media

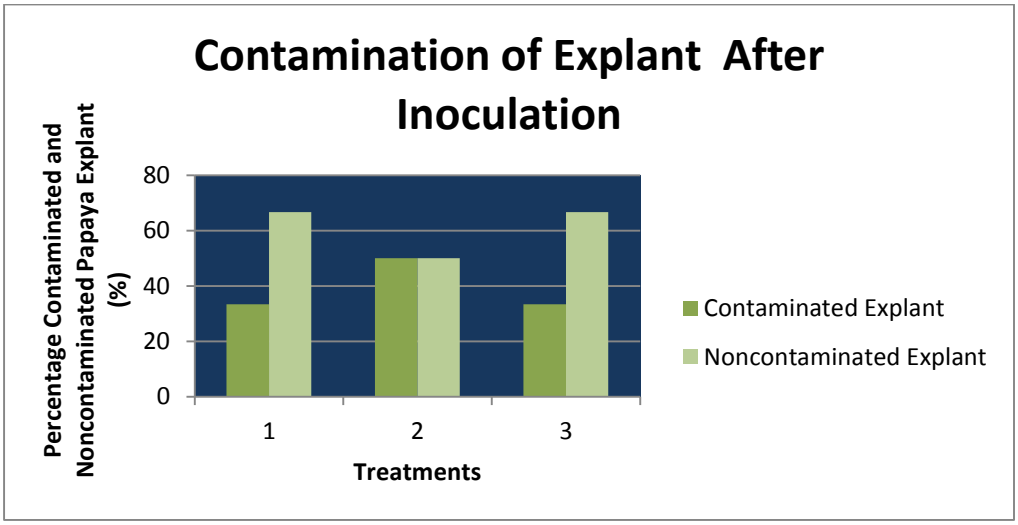
and respective 2,4-D concentration: 5mg/L, 10mg/L, and 15mg/L. A total of 28 jars containing 3 zygotic embryos were obtained in this research. The jars were then transported to the growth room where they were monitored once a week for callus formation (see Figure 6, 7, and 8).

Result and Discussion

CONTAMINATION

Contamination of papaya explants was evaluated for each treatment after 3 weeks of inoculation. This was evident in all three treatments but the percentage at which contamination occurred varied. Treatment 2 had the highest percentage of contamination where 50% of the papaya explants were contaminated and the other 50% were non-contaminated. The percentage contamination in treatment 1 and 3 was lower than treatment 2. In treatment 1, 33.33% of the inoculated papaya explants were contaminated and 66.67% were non-contaminated. In treatment 3, 33.33% of the papaya explants were contaminated and the remaining 66.67% were non-contaminated (See Figure 2 below). Contamination of papaya explants were observed as a black pigmentation growing on the zygotic embryos as well as an opaque substance encircling it. The contaminated papaya explants were removed from the carton box which also contained non-contaminated papaya explants. The removal of contaminated culture vessels was important as a precautionary method to prevent further contamination of papaya explants. The remaining non-contaminated papaya explants were further observed for 1-2 weeks for callus formation.

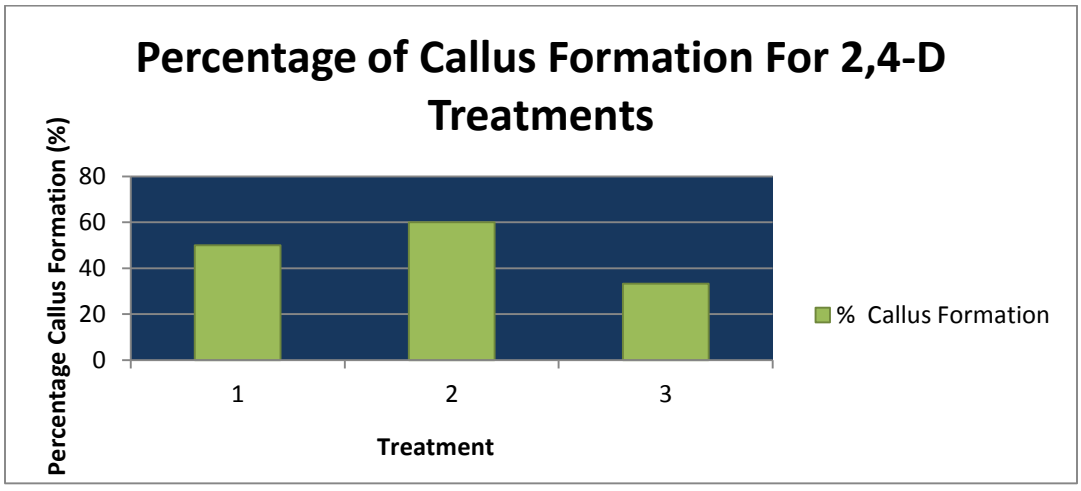
Figure 1. Percentage of Contamination After Inoculation of Immature Zygotic Embryos.



CALLUS FORMATION

The aim of this experiment was to determine the effects of three different concentrations of 2, 4-D on callus formation. Through the process of Indirect Somatic Embryogenesis callus can be formed as an intervening phase before somatic embryos formation initiates. 2,4-D, as the sole growth regulator used in this experiment is a very potent auxin that can induce callus growth on explants and which also can have adverse effects if the concentration use is inappropriate for the explants being tested. The results obtained show that 2,4-D induced callus after 4-5 weeks inoculation of immature zygotic embryos from *C. papaya* variety Maradol. Although callus was induced in the three different concentrations of 2,4-D, the percentage at which it formed for each jar varied. The highest callus formation was produced in treatment 2 supplemented with 10mg/L of 2,4-D and treatment 1 supplemented with 5mg/L of 2,4-D. The percentage average callus formation in treatment 2 was 60 % and in treatment 2 it was 50% (See Figure 2 below). Callus formation was observed in treatment 3 which contained 15mg/L 2,4-D. In treatment 3, the percentage of callus formation was 33.33% which indicates that 15mg/L of 2,4-D is least effective for induction of callus when compared to the other treatments.

Figure 2. Shows the Percentage of Callus Formation for the Three Different Concentrations of 2,4-D in Vessel.



Callus formation was clearly visible on immature zygotic embryos in all three treatments. The small structures emerged as distinct translucent pale yellow aggregates. Callus formation was observed after 4-5 weeks of inoculation of papaya explants. The characteristics of callus observed in each treatment was noted and comparison was made to determine the effects of 2,4-D (see table 5 in Appendix). The callus formed in treatment 1 had loosely compacted aggregates of tissue cells and was observed to have the second largest callus when compared to callus formed in treatment 2.

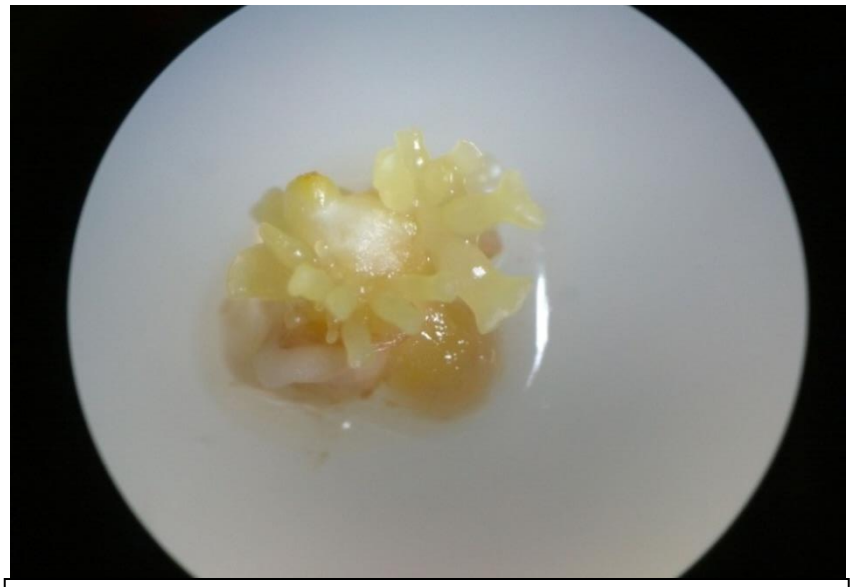


Figure 3. Callus Formation on Zygotic Embryos in Treatment 1 (Containing 5mg/L of 2,4–D) Viewed Under Stereo

The callus formed in treatment 2 was observed as having well bulbous aggregates of tissue cells on the immature zygotic embryos. The callus appeared as a pale white tissue and was observed to be the largest when compared to treatment 1 and 3. The callus observed under the stereo microscope had a more distinctive structure seeming as it was advancing to the somatic embryogenic phase.

Figure 4. Callus Formation on Zygotic Embryos in Treatment 2 (Containing 10 mg/L of 2,4–D) Viewed Under Stereo Microscope

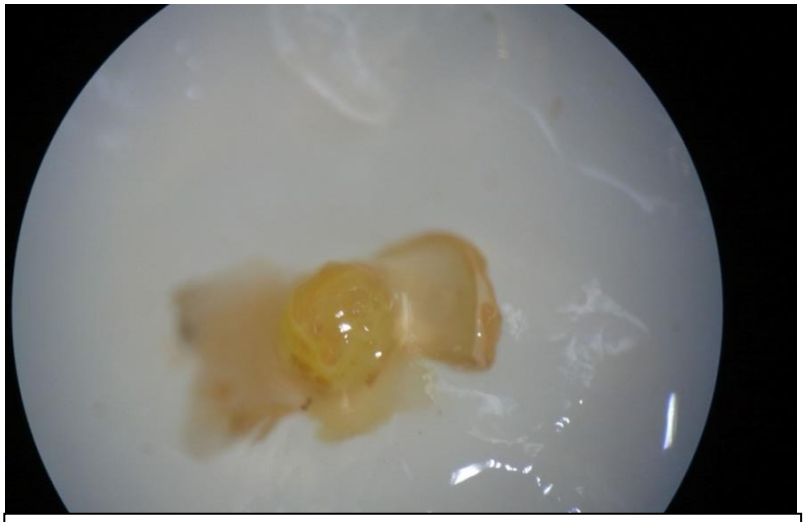


Figure 5. Callus Formation on Zygotic Embryos in Treatment 2 (Containing 15 mg/L of 2,4-D) Viewed Under Stereo Microscope

In treatment 3, the callus formed was not clearly visible and appeared as a mass of translucent cells tightly compacted together. Since 15mg/L of 2,4-D had lowest percentage of callus formation, this is evident in the picture shown on the left. Based on the evaluation conducted, 2,4-D is an important plant growth regulator for the induction of callus formation but there were major differences in the quality and size of the callus formed. It can be seen that according to figure 4, the callus formed in the culture medium supplemented with 10mg/L of 2,4-D produced the best callus when compared to callus formed in treatment 1 and 3.

The results obtained in this experiment supports similar experiments done by Perez et.al (2009) which proved that callus can be induced on immature zygotic embryos of *C. papaya* L. Maradol at a concentration of 15mg/L of 2,4-D. However, literature sources were not found which tested for induction of callus at concentrations of 5mg/L and 10mg/L of 2,4-D.

Conclusion

In this research, 2, 4-D induced callus formation on immature zygotic embryos of *C. papaya* L. Maradol at concentrations of 5mg/L and 10mg/L and 15/mg/L. The best callus formed and with the highest percentage was in treatment 2 containing a concentration of 10mg/L of 2,4-D. The growing papaya industries in Belize can implement the *in vitro* production of *C. papaya* L. Maradol through the process of indirect somatic embryogenesis to combat papaya diseases and destruction by hurricanes that threatens the production of the fruit by propagating selected types.

Recommendations

- For best results, it is recommended that the immature zygotic embryos are obtained from good quality papaya grown in industry fields as these fruits undergo good management control and monitoring of papaya fruits.
- Production of sample having >30 culture vessels is recommended to better interpret data by using statistical analysis.
- It is recommended that the other varieties of papaya grown in Belize be tested in different concentrations of 2,4-D to enhance knowledge of indirect somatic embryogenesis in these varieties.
- Callus produce high variations in papaya plantlets, but through direct somatic embryogenesis variation is reduced. An attempt to produce plantlets through direct somatic embryogenesis is recommended for selected types.
- For statistical analysis, it is recommended that the callus is weighed in each treatment in an attempt to discover which concentrations produce the highest mass of callus.

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Appendix

Tables

Table 2 . Shows Measurement of Murashige and Skoog (1962) Inorganic Salts

MS	Inorganic Salt	Amount (g/L)	Sampling
1	NH_4NO_3	82.5	20ml
	KNO_3	95.0	
2	$MgSO_4.7H_2O$	18.5	20ml
	$ZnSO_4.7H_2O$	0.43	
	$MnSO_4.4H_2O$	1.115	
	$CuSO_4.5H_2O$	0.00125	
3	$CaCl_2.2H_2O$	22.0	20ml
	KI	0.042	
	$CoCl_2.6H_2O$	0.00125	
4	KH_2PO_4	8.5	20ml
	H_3BO_3	0.31	
	$NaMoO_4.H2O$	0.013	
5	Na_2-EDTA	1.865	20ml
	$FeSO_4.7H_2O$	1.39	

Table 3. Showing the Materials Used to Prepare Culture Media for Explants Inoculation

Mineral Nutrients and Organic Salts Used	Amount
Ms Half Strength	100%
MS Vitamins	10ml/L
MyoIsotol	100mg/L
L-glutamine	400mg/L
Sucrose	60g/L
2,4-D	5mg/L
	10mg/L
	15mg/L
Agar	8g/L

Table 4. Showing the Percentage of Callus Formation in MS Basal Medium for Three Different Concentrations of 2, 4 D.

Treatment	# of Jars Containing Callus	Total # of Non-contaminated explants	% Callus Formation
1	3	6	50.00
2	3	5	60.00
3	2	6	33.33

Table 5. Brief Comparative Description of Callus Formation in Each Treatment after 4-5 Weeks of Inoculation

Treatment	# of Jars with Callus after Removal of Contaminated Explants	Observation
1	3	After 4-5 weeks of inoculation, callus was observed on zygotic embryo explants. The mass of cells appeared off white and no jars were contaminated after the first evaluation for contamination.
2	3	Callus formation was observed and the mass appeared pale yellow. The callus formed was the largest when compared to the other treatments. No contamination was seen in the jars.
3	2	Least amount of callus formed in 2,4-D [15]. The mass of cells were not clearly visible even when observed under a stereo microscope. No Contamination was observed.

Table 6. Global Export of Papaya from 2002-2009 (in metric tons) (Adapted from Evans and Ballen, 2012).

Countries	2002	2003	2004	2005	2006	2007	2008	2009	% 2007–09
Mexico	68,558	74,814	96,525	83,159	94,891	101,306	90,316	134,960	40.9
Brazil	28,541	39,492	35,930	38,757	32,475	32,267	29,968	27,554	11.2
Belize	11,307	16,886	28,751	28,635	34,475	33,341	28,967	27,152	11.2
Malaysia	60,892	71,473	58,149	42,008	50,545	26,938	24,168	24,301	9.4
India	3,452	3,550	3,475	6,434	10,344	10,880	13,834	17,573	5.3
United States	7,106	7,046	9,789	10,704	3,586	9,604	9,031	8,090	3.3
Netherlands	3,362	10,548	9,554	9,402	9,392	8,625	7,596	8,023	3.0
Guatemala	2,475	1,750	1,069	3,915	3,638	6,680	9,794	7,375	3.0
Ecuador	1,976	4,477	7,196	5,373	5,549	5,486	4,372	5,370	1.9
Belgium	655	295	980	793	731	527	593	2,496	0.5
Others	24,352	23,327	25,449	24,884	25,373	40,103	25,193	16,790	10.3
TOTAL	**212,676**	**253,658**	**276,867**	**254,064**	**270,999**	**275,757**	**243,832**	**279,684**	**100.0**

Figures

Photograph of Plant Material and Treatments

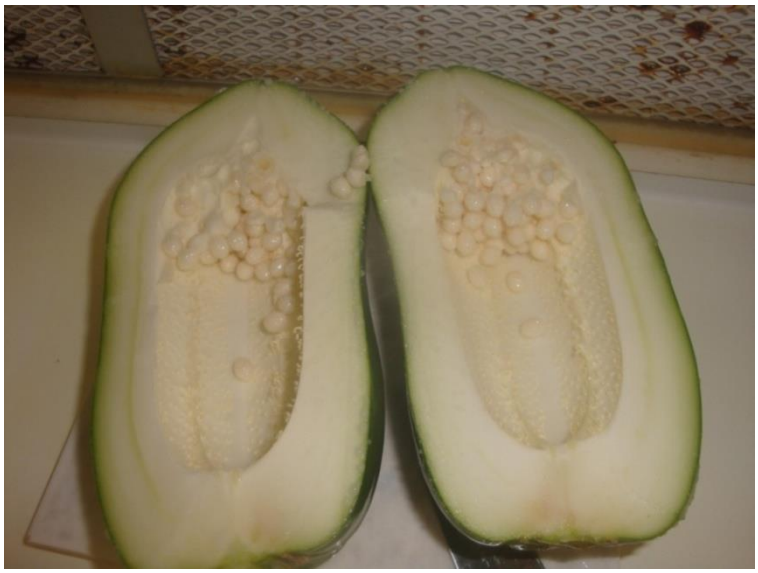

Figure 6. Mature Green Papaya Fruit Dissected. Inside are the Papaya Seeds Containing immature Zygotic Embryos

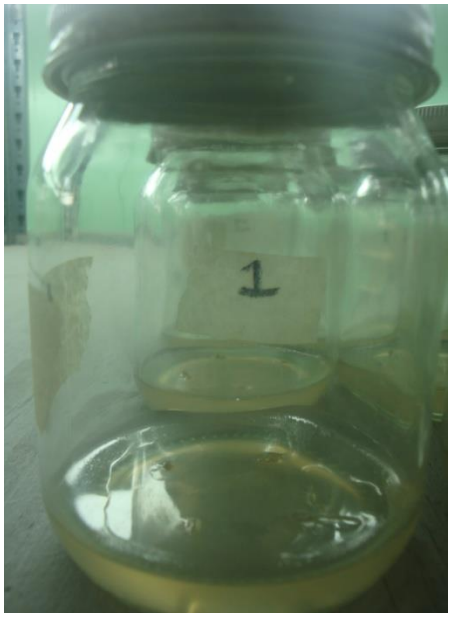

Figure 7 (Left) Treatment 1

Figure. 8, (Middle) Treatment 2

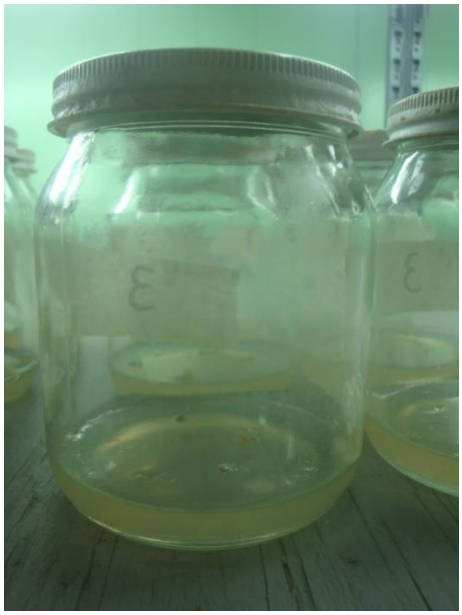

Figure 9. (Right) Treatment 3

Photographs of Medium Culture Preparation

Figure 10. MS Stock Solution 1, 2,3,4 and 5 used

Figure 11. Media placed in Autoclave

Photograph of Area Where Explants was Inoculated

Figure 12. Inoculation of Explant done in Laminar Flow Chamber