The Establishment of Surface Sterilization for the Microprogation of *Ficus carica* cv. BTM6 (Brown Turkey Modified 6)

By

Nur Athirah Binti Mohd Azam

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DECLARATION

I hereby declare that the work embodied in this report is the result of the original research and has not been submitted for a higher degree to any universities or institutions.

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Student

Name:

Date:

I certify that the report of this final year project entitled “The Establishment of Surface Sterilization for the Micropropagation of *Ficus carica* cv. BTM6 (Brown Turkey Modified 6)” by Nur Athirah Binti Mohd Azam, matric number F14a0222 has been examined and all the correction recommended by examiners have been done for the degree of Bachelor of Applied Science (Agriculture Technology) with Honours, Faculty of Agro-Based Industry, Universiti Malaysia Kelantan.

Approved by:

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Supervisor

Name:

Date:

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The Establishment of Surface Sterilization for the Micropropagation of *Ficus Carica* cv. BTM6 (Brown Turkey Modified 6)

ABSTRACT

Sterilization technique is the most crucial procedure in preparing explants for *in vitro* propagation. It is difficult to controlling the microbial contaminants especially in woody plant. Four methods of surface sterilization by using different concentration and time exposure of different sterilizing agents; Sodium hypochlorite (NaOCl) and Hydrogen peroxide (H_2O_2) with the presence and absence of a fungicide treatment (2000mg/L of THIRAM 80) were conducted in this study. The main purpose of this study is to establish the sterilization method for the *Ficus carica* cv.BTM6 explants. BTM6 was cultured from the shoot tips and nodal segment in MS media fortified with hormones of 2.0mg/L BAP and 0.2mg/L NAA. The results obtained indicate that these treatments of sterilization methods do not give positive effects in reducing the contamination on the BTM6 *in vitro* propagation. Thus, further study is needed to establish *in vitro* propagation of BTM6.

Keyword: *in vitro* propagation, sterilization method, contamination, BTM6, hormones

Pemantapan Pensterilan Permukaan Untuk Mikropropagasi *Ficus Carica*

cv.BTM6 (Brown Turkey Modified 6)

ABSTRAK

Teknik pensterilan ada salah satu teknik yang amat penting dalam penyediaan tisu tumbuhan dalam pembiakan tabung uji. Walau bagaimanapun, pengawalan kontaminasi mikrob adalah sukar terutamanya untuk tumbuhan berkayu. Empat kaedah pensterilan permukaan dengan menggunakan konsentrasi dan masa pendedahan oleh agen sterilan yang berbeza iaitu Natrium hipoklorik (NaOCl) dan Hidrogen peroksida (H_2O_2) dengan kehadiran dan tanpa rawatan racun anti kulat (2000mg/L THIRAM 80) telah dilakukan. Tujuan utama kajian ini adalah untuk memantapkan kaedah pensterilan yang terbaik untuk mikropropagasi BTM6. Pembiakan tabung uji BTM6 dikultur daripada hujung pucuk dan bahagian nod di dalam media MS yang telah ditambah dengan hormon 2.0mg/L BAP dan 0.2mg/L NAA. Keputusan yang diperolehi menunjukan bahawa kaedah pensterilan yang digunakan dalam kajian ini tidak memberikan kesan positif dalam mengawal kontaminasi dalam pembiakan tabung uji BTM6. Justeru, kajian lanjut perlu dilakukan untuk menghasilkan BTM6 secara tabung uji.

Kata kunci: pembiakan tabung uji, kaedah sterilan, kontaminasi, Brown Turkey Modified 6, hormon

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LIST OF SYMBOLS

G	Gram
L	Liter
Ml	Milliliter
M	Molarity
%	Percentage
Min	Minute
Mg	Milligram
^{0}C	Degree Celsius
Psi	Pound per square inch
SD	Standard deviation

LIST OF ABBREVIATIONS

NAA	α – Napthelene acetic acid
BAP	6 – benzyl aminopurine
MS	Murashige and Skoog
BTM6	Brown Turkey Modified 6
NaOH	Sodium hydroxide
NaOCl	Sodium hypochlorite
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
ANOVA	Analysis of variance

CHAPTER 1

INTRODUCTION

1.1 Research Background

Fig or its scientific name *Ficus carica* is a member of Moraceae family. It is a woody plant and contains high nutritive values which are good for health. It contains minerals such as calcium, copper, iron, magnesium, and potassium (Lewin, 2017). The fig fruits also can be eaten uncooked as a snack by peoples worldwide. The fig trees commonly are cultivated through vegetative propagation via cuttings, grafting and air layering (Fig Cultivation Information Guide, 2015). However, proper and special treatments are needed throughout this propagation in order to prevent fig trees from any infection of disease that will lead to death.

Furthermore, the multiplication rate of fig trees through vegetative propagation is quite low due to poor rooting and other factor like disease infestation (Danial, Ibrahim, Brkat, & Khalil, 2014). For this reason, some researchers have developed new solution to propagate the fig trees through tissue culture technology. Through this method, the growth rate of this plant could be increase. The common techniques of micropropagation used for fig trees are through apical bud and axillary buds which can induce multiple shoot and root. Another technique is through callus induction (Danial et al., 2014). Previous study reported that the fig shoot was successfully grown from the axillary bud of mature trees, from apical bud and from the calli of stem segment (Danial et al., 2014).

However, in order to conduct a proper micropropagation method for any plants or crops, there is one most crucial procedure that needs to be taken. This method could

determine the success or failure of the micropropagation technique. Surface sterilization is a mandatory process before any *in vitro* culture is conducted. This study is focusing on the establishment of surface sterilisation for *Ficus carica* L. (BTM6) by conducting several methods of surface sterilization.

1.2 Problem Statement

Generally, figs cultivation through vegetative propagation (cutting and grafting) mostly resulted in poor rooting of the trees which cause low of multiplication rates. Through tissue culture the root induction can be multiple (Mustapha & Taha, 2012). Furthermore, the cultivation of figs via vegetative propagations is not available for mass production (large production) which large amount of cuttings will be needed (Mustapha & Taha, 2012). The propagation of figs can be enhance via tissue culture which it will produce multiples of high quality breeds of trees (Danial et al., 2014).

Fig trees are vulnerable toward disease such as fig rust, fig mosaic virus, stem fungal infection and nematode infection on root (Waterworth, 2015). Through *in vitro* propagation, disease-free plants that have high resistance toward any kind of pathogen could be produced (Pasqual & Ferreira, 2007).

Moreover, there is no feasible sterilization method specifically design for *Ficus carica* cv. BTM6. Thus, further study of sterilization method for BTM6 could be established through this research. Hence, a desired plant materials of BTM6 that are high resistant towards disease and have high qualities can be produced.

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1.3 Objectives

1. To establish surface sterilization method of *Ficus carica* cv. BTM6 by using different sterilizing agents.
2. To observe the effectiveness of fungicide used in the explants sterilization technique.

1.4 Scope of study

The scope of this study was focusing on the establishment of the surface sterilization for *Ficus carica* cv. BTM6. From the effects shown on the cultured explants, we could identify whether the sterilization methods treated for the explants is effective or ineffective. Thus, we could determine the most suitable surface sterilization technique for *Ficus carica* cv. BTM6. Different sterilizing agents (NaOCl and H_2O_2) and one fungicide treatment (THIRAM 80) have been used on explants during the sterilization procedures. The cultured explants were observed every weeks by observing the explants condition whether it are healthy, contaminate or dead.

1.5 Significance of study

The significance of this study is to provide further study on the establishment of surface sterilization for the micropropagation of *Ficus carica* cv. BTM6. Besides, specific sterilization technique for *Ficus carica* cv. BTM6 also could be identified. Hence, the best concentration and time exposure of surface sterilization for this study could be obtained.

CHAPTER 2

LITERATURE REVIEW

2.1 Biology and Morphology of *Ficus carica* L.

Ficus carica L. (2n = 6) or commonly known as fig comes from a family of Moraceae. The fig has over 1400 species with 40 genera. The figs are originated from Middle East and were cultivated alongside the valleys of the Arabic peninsula. It were distributed and grown wildly in the Mediterranean Basin to America. Figs can be characterized by its adaptation to various soil condition and climate. The fig germplasm are characterized by its various ecotypes that were identified through its fruit maturity, fruit shape and its skin colours (Saddoud, et al., 2008).

There are three types of fig that were commercially grown. The first one is the common type which develops fruit parthenocarpically. Seconds is Smyrna type which types that need pollination with pollen from caprifigs through caprification. The last type is the San Pedro type which parthenocarpically produces first crop and second crop after pollination (Gaaliche, Saddoud, & Mars, 2012). There are many varieties of figs that has been cultivated such as Celeste, Brown Turkey, Brunswick, Marseilles, Adriatic, Genoa, Purple Genoa, Black Ischia, Poona and others (Morton, 1987).

The fig tree can reach up to 3 m to 9 m in height. It is a perennial tree which has multiple branches. The leaves are broad and borne on long petiole. Fig trees are categorized as woody plant and it is also a monoecious plant which means the female and male flower are on the same plant. The fruit of this tree is borne on the axils of its leaves. The fig tree can live up until 50 to 150 years. Fig trees are grouped as crop plants and medicinal plant due to its fruits which are edible and have high nutritional value (Alaoui, 2000).

2.2 Climate Condition of Fig Tree

The growth and production of fig are highly dependent on climatic conditions. The figs can grow well at dry and warm temperate (mild – temperate) area such as in the Mediterranean area. Nevertheless, the figs also are found cultivated in tropical and sub – tropical area (Morton, 1987). The rainfall and cold temperature are factors that affect the growth development and yield loss of fig trees. Fig trees could not withstand the certain condition such as rainy and cold environment which it could cause them dying. Rains also will cause the fruits to split during the fruit development and ripening (Morton, 1987). Hence, the fig trees usually were cultivated in the netted house to prevent from any environmental conditions that are not suitable for its growth such as rain, hail and wind (Flaishman, Rodov, & Stover, 2008). The preferable climates of growing fig are semi-arid tropical and subtropical regions with good irrigation systems. However, too hot or warm area will cause the fruits to fall even though it is well irrigated (Morton, 1987).

2.3 Cultivation of Figs in Malaysia

Figs are propagated vegetatively via seeds, stem cuttings, air layering and grafting (Flaishman et al., 2008). Nowadays, there are several Malaysian farmers have been started to cultivate the figs widely and generate high income from it. The fig plantlets price could achieve up to RM4000 per plantlet according to its cultivar. There many cultivars of figs that have been cultivated in Malaysia. There are a few fig cultivars which obtain high demand from consumer, Masui and Jumbo that could reach up to 80 to 100 gram per fruit. This cultivar could be eaten freshly and can be used to make any downstream product such as jam, dried fruit and etc. (Hamzah, 2016)

There are many cultivars of fig trees that could be cultivated and suitable in Malaysia environmental condition such as Brown Turkey, Rimada, Tuka and Ponte TeresaTaiwan golden fig (TGF), BTM6, Purple Patlican, US – Conadria, Violet de Bordeaux, Dauphine fig Japan, Kapota and other (Hussin, 2016). In Malaysia, the cultivation of fig trees is also done through various methods vegetative such as, stem cuttings, air layering and grafting. For the early stage of planting, usually the air layering method is used. The cutting stems were wrapped into soil mixture of 70% top soil, 15% of peat moss and 15% of paddy hay to encourage the root formation. The soil condition is extremely important and must be treated well. It must not be too wet and the water must not be flooded to prevent root from become rot and die (Norizi, 2015).

The sprouted roots from the air layering plantlets will be transferred into medium such as coco peat in poly beg. The media for transplanting air layering plantlet were prepared in poly beg early. Then the air layering explant will be removed from the wrapped soil media and cleaned thoroughly without breaking any roots. Then, it was planted on the media with depth of 5 cm in deep (Yaacob, 2014).

2.4 Surface Sterilization Method

Surface sterilization is one of the most crucial procedures needed during the *in vitro* culture process. This technique is done in order to remove or eliminate all the microbial contaminant and excessive dust on surface and the interior of plant materials used in *in vitro* culture. This will reduce the chances of plant materials to get contaminated during the *in vitro* (Teixeira Da Silva, Winarto, Dobranszki, & Zeng, 1989). Hence, the surface sterilization is the most important procedures in aseptic establishment in *in vitro* culture.

There were many methods have been developed to reduce and eliminate the contamination during micropropagation. Contamination by fungi and bacteria on the *in vitro* culture is a serious problem can cause rooting problem and may lead the *in vitro* plantlets to disease vulnerable and worst condition which is death. This contamination also could be worst for plant materials that are from woody plant, because the plant materials are naturally exposed and infected by the microorganism from the outside environment. Thus, preparation of chemical solutions for surface sterilizing those explants must be prepared carefully (Mihaljevic et al., 2013).

Generally, the sterilizing agents or disinfectants used in sterilization process are calcium chloride ($CaCl_2$), ethanol, hydrogen peroxide (H_2O_2), mercuric chloride ($HgCl_2$), Silver nitrate ($AgNO_3$) and Sodium hypochlorite (NaOCl) (Mihaljevic et al., 2013). The disinfectant agents are toxic to the plant materials. Hence, the sterilization must be conducted carefully to prevent the cell tissue of the plant materials from die as well as removing the microbe contaminants. Higher presence of microbes could increase the mortality of the *in vitro* culture (Mihaljevic et al., 2013). Furthermore, anti – fungal agents (fungicide) also could be used to reduce the microbial contaminant on the exogenous and endogenous of the plant materials. Broad spectrum fungicides such as Benzimidazole (Benomyl) and Carbendazim (Bavistin) and antibiotics such as Ampicillin, Streptomycin and Tetracycline might be useful to reduce microbial activity on the explants during sterilization process and the explants were cultured (Eed et al., 2010). Basically, the surface sterilization process started with removing the excess contaminant on plant materials surface by rinse it under running tap water for several minutes. Then, the explants will be immersed into isopropyl alcohol (ethanol) for only seconds or a minute. The plants materials cannot be soaked to long in the ethanol because it is extremely phytotoxic and it could damage the cell tissue of explants. The tender tissue tends to get more damaged if it were exposed too long in alcohol. Thus, the normal concentration of alcohol used for surface sterilization is

70% (Michigan State University, 2015). Next, the explants will be immersed into sterilizing agents of disinfectants with addition of Tween – 20 (1 – 2 drops), detergent for several minutes. For sodium hypochlorite, usually it takes 10 minutes up to 20 minutes to immerse the explants, longer than that could give phytotoxicity to the explants same as other sterilizing agents. Last step of explants sterilization is rinsing, the explants will be rinsing with sterilized distilled water three to five times (Smith, 2013).

Besides, instead of focusing on sterilization process other aspects also must be considered. The media used also need to be in good condition, fresh media are most preferable. The tools used for the dissection process also needed to be sterilized well. The sterilization, dissection and in vitro culture procedures also must be conducted under aseptic (controlled) condition inside the laminar hood. If all of these factors were considered, all the superficial microbes and contaminant could be killed (George, 1993).

Sterilization process is different depend on the species of the plant materials and explant part used for the sterilization. Different part of explants have different surface of contaminant exposed. Thus, not every methods of surface sterilization can be applied to all different species of plant materials.

2.5 Micropropagation of *Ficus carica* L.

The production yield of fig trees all around the world are quite which it reaches 1,070,676 million with plantations that cover 426, 244 hectare. The countries that contributed large production of fig are Algeria, Iran, Egypt, Greece and Turkey (Pasqual & Ferreira, 2007). The fig trees were cultivated through tissue culture method which is in controlled to prevent it from any infection of diseases and to

obtain plantlets which are possibly free from any pathogen. Moreover, this technique is useful for the large production of fig tree. Before doing the *in vitro* culture the fig explants, the material collected (fig explants) must undergo sterilization process first. It will be washed under running tap water to remove out all the residue and unnecessary substances that could cause oxidation toward the explant for several hours. Then, the explant must be sterilized by using any substances with germicidal action such as ethanol and chlorine based compound (calcium hypochlorite or sodium hypochlorite). Surfactants (example: Tween 20) are used to increase the penetration on explant tissue to improve the contact latter with the tissues. The ethanol of 70% to 80% was used for several second to prevent the explant tissue from dehydrating faster. The explants are washed thoroughly around three to five times using sterile distilled water or deionized autoclaved water. Then, the explants are ready to be *in vitro* culture on culture medium. The most preferable media used for micropropagation of figs are MS (Murashige & Skoog) and WPM (Woody Plant Medium). The micropropagation of figs could be done using shoot tips, apical buds, leaf segment and other part (Pasqual & Ferreira, 2007). Hormones such as auxins and cytokinins also can be added to the media to increase the induction of shoot and root. According the precious report, culture media that have been fortified with 2.0mg/L 6 – benzyl aminopurine (BAP) and 0.2mg/L α – Napthelene acetic acid (NAA) showed a good development of *Ficus carica L*. shoot induction (Kumar, Radha, & Chitta, 1998).

CHAPTER 3

METHODOLOGY

3.1 Materials

3.1.1 Plant material

The explants of Brown Turkey Modified 6 were purchased from a fig grower in Kota Bharu, Kelantan. The explants were stored in Tissue Culture nursery at UMK Jeli Kampus.

3.1.2 Equipment and Apparatus

Equipments and apparatus that were used in this study were autoclave, analytic balance, drying oven, fridge, hot plate stirrer, laminar flow, pH meter**,** aluminium foil, beaker, blade, forceps, and scalpel, chopping board, petri dish, plastic wrapper, masking tape, measuring cylinder, test tube, spirit lamp, and schott bottle.

3.1.3 Chemicals

Chemicals used in the explant sterilization process were 70% and 95% alcohol (ethanol), distilled water, fungicide (THIRAM 80), commercial bleach (sodium hypochlorite), and sodium peroxide. The chemicals used to prepare MS media are as followed in Table 3.1.

Table 3.1: Chemicals composition in Murashige and Skoog media

Chemicals		**Amount used**
Macronutrient stock solution	**1X (g/L)**	**20X (g/500mL)**
Ammonium nitrate (NH_4NO_3)	1.65g	16.5g
Potassium nitrate (KNO_3)	1.90g	19.0g
Calcium Chloride ($CaCl_2 2H_2O$)	0.44g	4.4g
Magnesium sulphate ($MgSO_4.7H_2O$)	0.37g	3.7g
Potassium Dihydrogen Orthophosphate (KH_2PO_4)	0.17g	1.7g
Micronutrient stock solution	**1X (g/L)**	**200X (g/500mL)**
Manganese Sulphate ($MnSO_4.7H_2O$)	0.02230g	2.23g
Zinc sulphate ($ZnSO_4.H_2O$)	0.00860g	0.86g
Potassium Iodide (KI)	0.000830g	0.083g
Cupric Sulphate ($CuSO_4.5H_2O$)	0.0000250g	0.0025g
Sodium Molybdate ($Na_2.MoO_4.2H_2O$)	0.00025g	0.025g
Cobaltus chloride ($CoCl_2 6H_2O$)	0.0000250g	0.0025g
Boric Acid (H_3BO_3)	0.00620g	0.62g

Vitamin stock solution	**1X (g/L)**	**500X (g/125mL)**
Nicotinic acid (Vitamin B3)	0.0005g	0.0313g
Thiamine HCL (Vitamin B1)	0.001g	0.0625g
Pyridoxine (Vitamin B6)	0.0005g	0.0313g
Myo – inositol	0.1g	6.25g
Glycine ($C_2H_5NO_2$)	0.002g	0.125g
Ferum stock solution	**1X (g/L)**	**200X (g/500mL)**
Sodium EDTA (Na_2.EDTA.2H_2O)	0.0278g	2.78g
Ferrous Sulphate ($FeSO_4$.7H_2O)	0.0373g	3.73g
Hormones		**Concentration/L**
Auxin α – Napthelene acetic acid (NAA)		0.2mg/L
Cytokinin 6 – benzyl aminopurine (BAP)		2.0mg/L
Others		**Amount/ L**
Sucrose		30g
Agar / Gelrite		8g / 2.75g
pH (0.1M NaOH and 0.1M HCl)		5.7 – 5.8

3.2 Methodology

3.2.1 MS Media Preparation

The MS media was prepared according to the listed chemicals in the Table 3.1. About 200ml of distilled water was added into a 1 L r beaker. The beaker was put on the plate stirrer to make sure all the mixture inside the beaker was well stirred. 30g of sucrose was added into the mixture solution and dissolved well. Then, 50mL of macronutrient solution, 5mL of micronutrient, 5 mL of iron stock and 1mL of vitamin solution. Next, hormones solution of auxin (0.2mg/L of Napthelene acetic acid) and cytokinin (2.0mg/L of benzyl aminopurine) were added into the media. Then, the water volume was adjusted to 400mL. the pH value of the mixture solution were stabilized by using 1.0M NaOH and 1.0M HCl until it reached to 5.7 – 5.8 of pH value. After that, 8g of agar or 2.75g of Gelrite was added into the mixture solution and stirred well. The MS media solution was heated inside a microwave for several minutes until the agar inside the solution melted. Then, the amount of MS media solution was adjusted until reach 1000mL or 1L. The MS media solution was filled into the culture tube and autoclaved at 15psi and 120^0C for about 15 minutes. The diagrams were simplified as in Figure 3.1.

3.2.2 Explant Sterilization

The shoot tips (apical buds and leaves) and nodal segment of *Ficus carica* cv. BTM6 explants were taken and cleaned thoroughly under running tap water for 30 minutes. Next, the explants were cut separately to separate the leaves, apical buds and node segment. The explants were placed in laminar flow to carry out the sterilizing method under sterile condition. The cut explants were soaked into 70% of alcohol (ethanol) for 1 minute. After a minute, the explants were immersed into

different sterilizing agents with concentration of 4% and 10% and addition of 2 drops of Tween – 20 for both treatments with and without fungicide. The fungicide used in this sterilization method is THIRAM 80 with concentration of 2000mg/L (20g/L) with 10 minutes of time exposure. The sterilization methods for the explants were divide into four methods are as followed in Table 3.2, 3.3, 3.4 and 3.5.

Table 3.2 First sterilization method for BTM6 explants (NaOCl without fungicide)

Type sterilizing agents	Treatments	Concentration (%)	Time exposure (min)	Fungicide treatment
Sodium hypochlorite	T1	4	10	Absent
	T2	4	20	
	T3	4	30	
	T4	10	10	
	T5	10	20	
	T6	10	30	

Table 3.3 Second Sterilization method for BTM6 explants (NaOCl with fungicide)

Type sterilizing agents	Treatments	Concentration (%)	Time exposure (min)	Fungicide treatment
Sodium hypochlorite	T7	4	10	Present
	T8	4	20	
	T9	4	30	
	T10	10	10	
	T11	10	20	
	T12	10	30	

Table 3.4 Third Sterilization method for BTM6 explants (H_2O_2 without fungicide)

Type sterilizing agents	Treatments	Concentration (%)	Time exposure (min)	Fungicide treatment
Hydrogen peroxide	T13	4	10	Absent
	T14	4	20	
	T15	4	30	
	T16	10	10	
	T17	10	20	
	T18	10	30	

Table 3.5 Fourth sterilization method for BTM6 explants (H_2O_2 with fungicide)

Type sterilizing agents	Treatments	Concentration (%)	Time exposure (min)	Fungicide treatment
Hydrogen peroxide	T19	4	10	Present
	T20	4	20	
	T21	4	30	
	T22	10	10	
	T23	10	20	
	T24	10	30	

3.2.3 Explant *in Vitro* Culture

The sterilized explants (apical buds, leaves and nodal segments) were cut accordingly into small pieces. Then, the explants were culture vertically and horizontally on nutrient media; Murashige and Skoog that has been supplemented with 2.0mg/L 6-benzylaminopurine + 0.2mg/L 1-napthaleneacetic acid. The cultures were incubated at 25 ± 1°C under 16 hour photoperiod. The uncontaminated regenerated shoots were subculture on fresh medium every 2 week.

3.3 Statistical Analysis

The replication of this experiment was repeated or replicated three times. There were four methods of sterilization in this study which are treatment of Sodium hypochlorite without fungicide, Sodium hypochlorite with fungicide, Hydrogen peroxide without fungicide and Hydrogen peroxide with fungicide. The data of contamination, death and survived rate were collected every week and analyzed by using analysis of variance (ANOVA) through SPSS software application.

3.4 Flowcharts

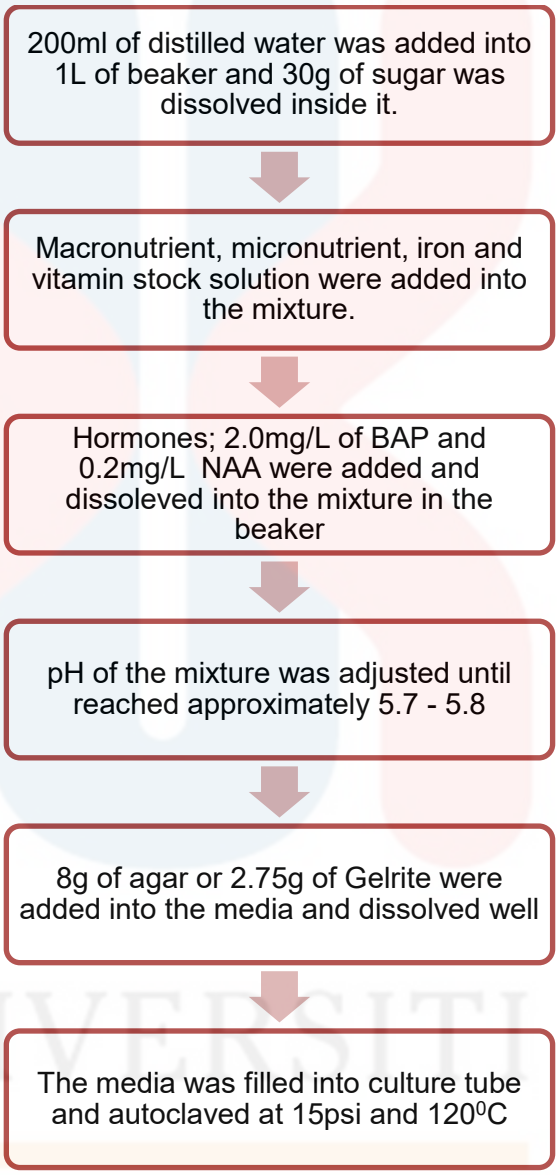

Figure 3.1 Flowchart of MS media preparation

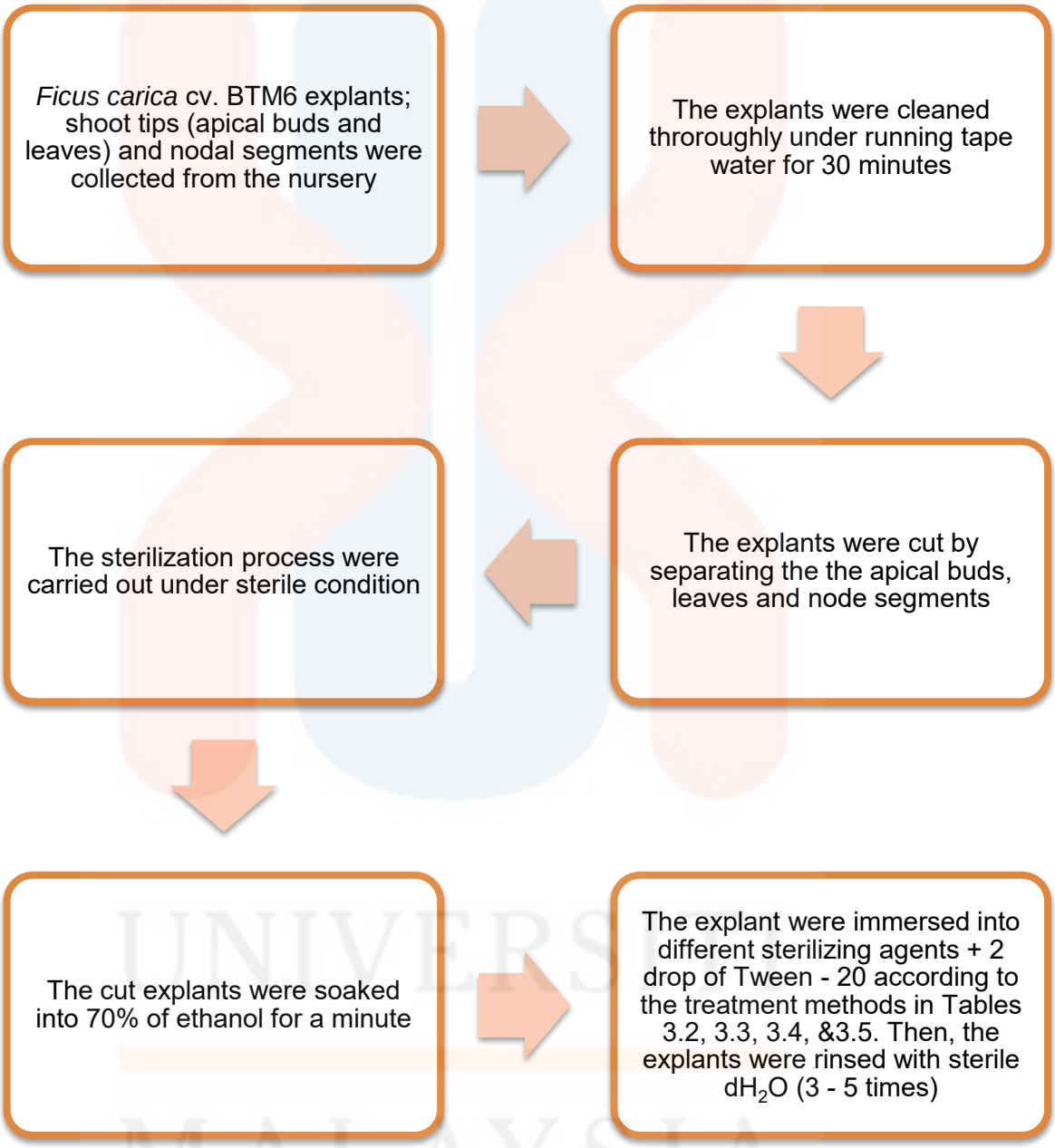

Figure 3.2 Flowchart of surface sterilization

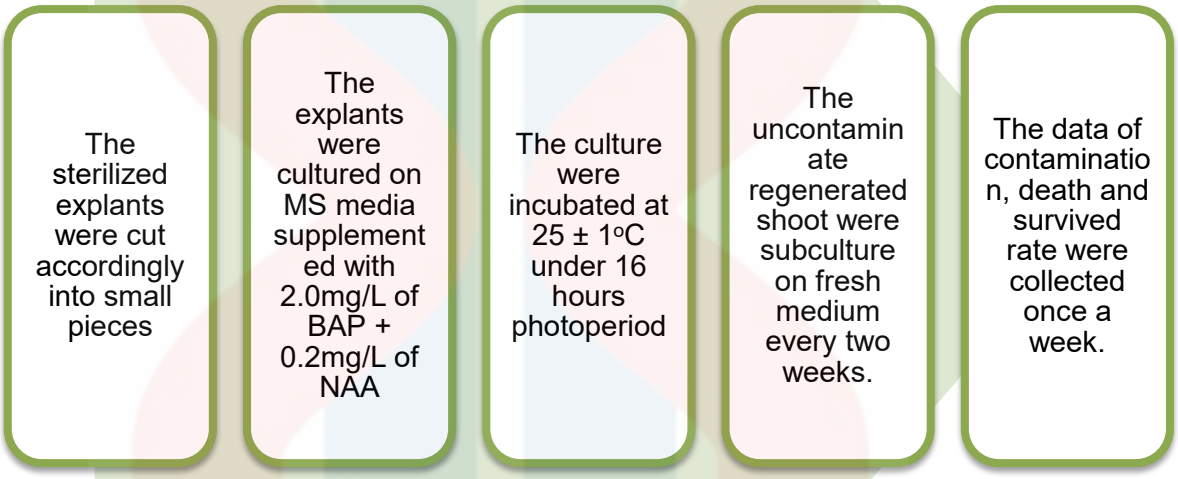

Figure 3.3 Flowchart of *Ficus carica* cv. BTM6 *In vitro* cultures

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Sterilization methods

There are two types of sterilizing agents; Sodium hypochlorite (NaOCl) and Hydrogen peroxide (H_2O_2) that were used during the sterilization procedure of BTM6 explant. A fungicide which is THIRAM 80 with concentration of 2000mg/L and 10 minutes of time exposure was used in this sterilization process as aid to reduce and remove any contaminant on the explants. This sterilization method was divided into four methods. The first method is the treatment of Sodium hypochlorite without fungicide treatment. Second is the treatment of Sodium hypochlorite with fungicide. Third, treatments of Hydrogen peroxide without fungicide and the last one was treatment of Hydrogen peroxide with fungicide. Different concentration of sterilizing agents and time exposure were used as treatments to examine the best condition for the explants response.

4.2 Contamination, Death and Survived Rate of the Explants

The contaminations of explants were identified based on the fungus and bacterial infection on the explant *in vitro* cultured as shown in Figure 4.1. As for the dead plants, it was identified based on the color of explant cultured as shown in Figure 4.2, the explant undergoes chlorosis which make the explant become pale or yellow. The explant was considered dead as the explant became pale (loss of chlorophyll).

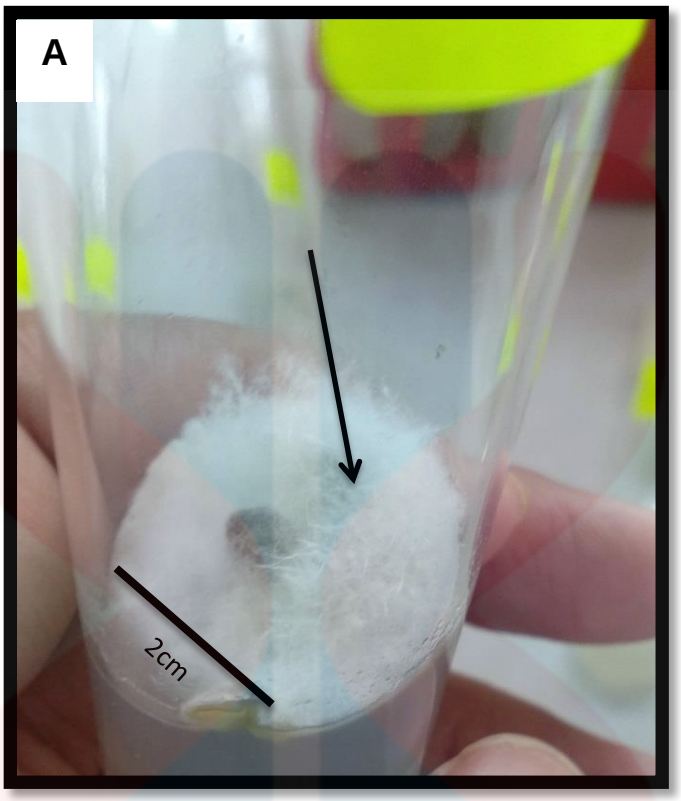

Figure 4.1: A) Explant cultured infected by fungus

Figure 4.2: B) Dead explant through chlorosis

The data of contamination, death and survived rate data were analyzed more detailed by using two – way analysis variance (ANOVA) through SPSS software application. This data were analyzed by using two – way ANOVA because it contained two independent variables which are concentration of the sterilizing agents and time of exposure. This analysis was also conducted to identify the significant of the sterilization methods used on the micropropagation of *Ficus carica* cv. BTM6. The differences between treatments used in the sterilization methods were considered not significant because the P-value ≥ 0.05 which mean that the treatments used do not give any effect on the micropropagation of BTM6 explants.

4.3 Duncan's Multiple Range Test (DMRT)

The mean ± SD values were compared by using Duncan's multiple range test (DMRT). The relation between concentrations of sterilizing agents and time exposure for each treatments showed P-value higher than 0.05 (P-value ≥ 0.05). These means, it were considered not significant since the p≥ 0.05. All treatments do not any effect on the *in vitro* establishment of BTM6 explants. Tables and figures below show the mean ± SD of all treatments obtained from the Duncan's multiple range test (DMRT) based on each sterilization methods used.

Table 4.1 The contamination, death and survived rate in the first sterilization method. (NaOCl without fungicide)

Treatments	Contamination rate (%)	Death rate (%)	Survived rate (%)
T1	$100^{b} \pm 0.00$	0	0
T2	$93.33^{ab} \pm 11.55$	$6.67^{ab} \pm 11.55$	0
T3	$93.33^{b} \pm 11.55$	$6.67^{b} \pm 11.55$	0
T4	$100^{b} \pm 0.00$	0	0
T5	$86.67^{ab} \pm 11.55$	$6.67^{ab} \pm 11.55$	$6.67^{ab} \pm 11.55$
T6	$73.33^{b} \pm 11.55$	$26.67^{b} \pm 11.55$	0

Data were subjected to mean ± SD

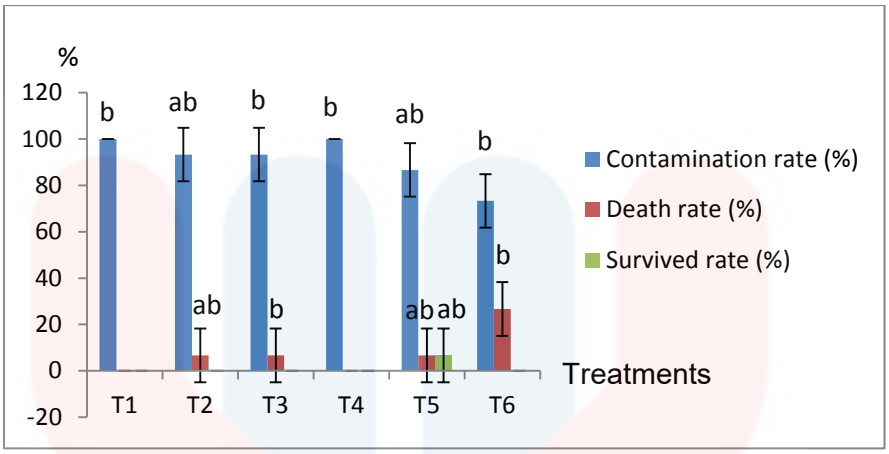

Figure 4.3 The contamination, death and survived rate in the first sterilization method

Based on Table 4.1 and Figure 4.3, T1 and T4 show the highest contamination rate which is $100^{b} \pm 0.00$. Both of the treatments consists one subset, which means that there is no significance different of the treatments; both treatments do not give effect on the *in vitro* establishment of BTM6 explants. T6 shows the lowest rate of contamination which is $73.33^{b} \pm 11.55$. There are few treatments contained two subsets (T2 and T5), which might give significant different between the treatments. However, since the significance value $P \geq 0.05$, it is indicate that the treatments do not give any effect on the explants. As for the death rate, T6 indicates the highest death rate with $26.67^{b} \pm 11.55$ and the lowest rate is 0 indicated by T1 and T4. Despite of the contamination and death of explants, there was one explant managed to survive which in T5 (Figure 4.4) with survived rate of $6.67^{ab} \pm 11.55$. Although, there was one explant survived at T5, the significance value of it still indicates higher than 0.05. Overall, all of the treatments do not give full effect on the *in vitro* establishment of BTM6 explants.

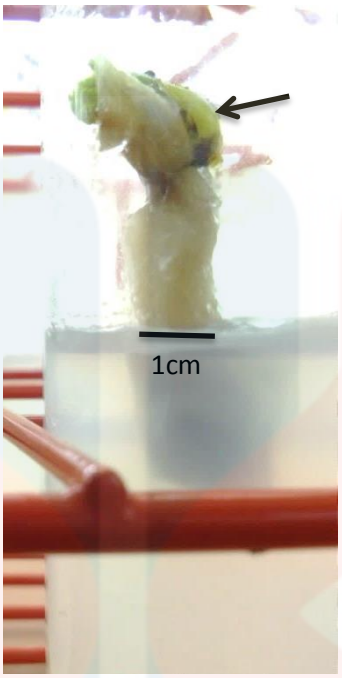

Figure 4.4: Survived explant that was treated with treatment at T5

Table 4.2 The contamination, death and survived rate in the second sterilization method (NaOCl with fungicide)

Treatments	Contamination rate (%)	Death rate (%)	Survived rate (%)
T7	73.33[a] ± 23.09	26.67[a] ± 23.09	0
T8	86.67[a] ± 11.55	13.33[a] ± 11.55	0
T9	73.33[a] ± 11.55	26.67[a] ± 11.55	0
T10	86.67[a] ± 11.55	13.33[a] ± 11.55	0
T11	66.67[a] ± 11.55	33.33[a] ± 11.55	0
T12	60[a] ± 20.00	40[a] ± 20.00	0

Data were subjected to mean ± SD

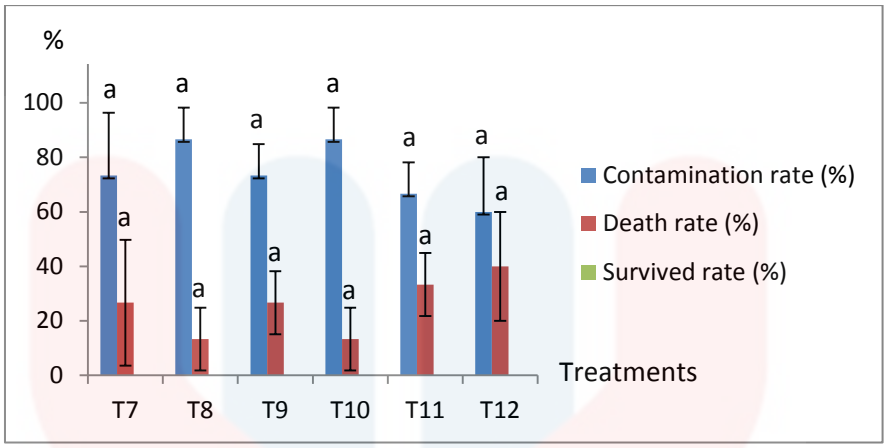

Figure 4.5 The contamination, death and survived rate in the second sterilization method (NaOCl with fungicide)

For the second method, based on the Duncan's MRT, all of the treatments consists only one subset which means there is no significance effect of the treatments on the BTM6 explants. The significance value of each treatment also indicates values higher than 0.05 (not significant).

According to Table 4.2 and Figure 4.5, T8 and T10 indicate higher contamination rates which are $86.67^{a} \pm 11.55$ for both and lowest rate at T12 with $60^{a} \pm 20.00$. As for the death rate, the highest death rate indicated by T12 ($40^{a} \pm 20.00$) and the lowest indicates by T8 and T10 ($13.33^{a} \pm 11.55$). Overall, all of the treatments indicate P-value ≥ 0.05, null hypothesis is accepted. This stated that the treatments do not give any effects on the *in vitro* establishment of BTM6 explants.

Table 4.3 The contamination, death and survived rate in the third sterilization method (H_2O_2 without fungicide).

Treatments	Contamination rate (%)	Death rate (%)	Survived rate (%)
T13	100 [a] ± 0.00	0	0
T14	100 [a] ± 0.00	0	0
T15	93.33 [a] ± 11.55	6.67 [a] ± 11.55	0
T16	66.67 [a] ± 57.74	33.33 [a] ± 57.74	0
T17	86.67 [a] ± 11.55	13.33 [a] ± 11.55	0
T18	66.67 [a] ± 57.74	33.33 [a] ± 57.74	0

Data were subjected to mean ± SD

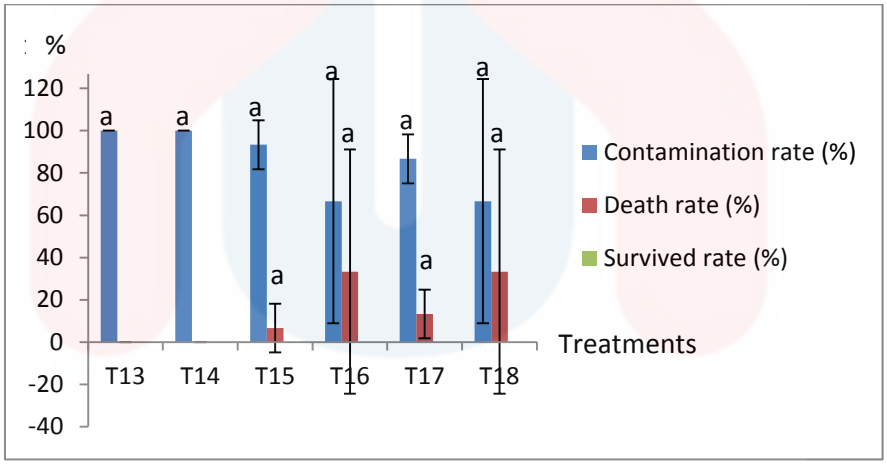

Figure 4.6 The contamination, death and survived rate in the third sterilization method (H_2O_2 without fungicide).

According to Table 4.3 and Figure 4.6, it shows the mean ± SD for the third sterilization method (H_2O_2 without fungicide). Based on the result, T13 and T14 indicated higher contamination rate which are 100 [a] ± 0.00 for both treatments and T18 indicated the lowest contamination rate which is 66.67 [a] ± 57.74. As for the death rate, the highest rate is 33.33 [a] ± 57.74 which indicated by T16 and T18 and the lowest rate is 0 which indicated by T13 and T14. There is none of the explants survived for this method.

Since, the significance values for all of these treatments indicate value ≥ 0.05, there is no significant different among the treatments used in this sterilization methods. This sterilization method does not give positive effect in controlling the contamination for the *in vitro* establishment of BTM6 explants.

Table 4.4 The contamination, death and survived rate in the fourth sterilization method (H_2O_2 with fungicide).

Treatments	Contamination rate (%)	Death rate (%)	Survived rate (%)
T19	$93.33^a \pm 11.55$	$6.67^a \pm 11.55$	0
T20	$66.67^a \pm 57.74$	$33.33^a \pm 57.74$	0
T21	$100^a \pm 0.00$	0	0
T22	$80^a \pm 34.64$	$20^a \pm 34.64$	0
T23	$86.67^a \pm 23.09$	$13.33^a \pm 23.09$	0
T24	$66.67^a \pm 23.09$	$33.33^a \pm 23.09$	0

Data were subjected to mean ± SD

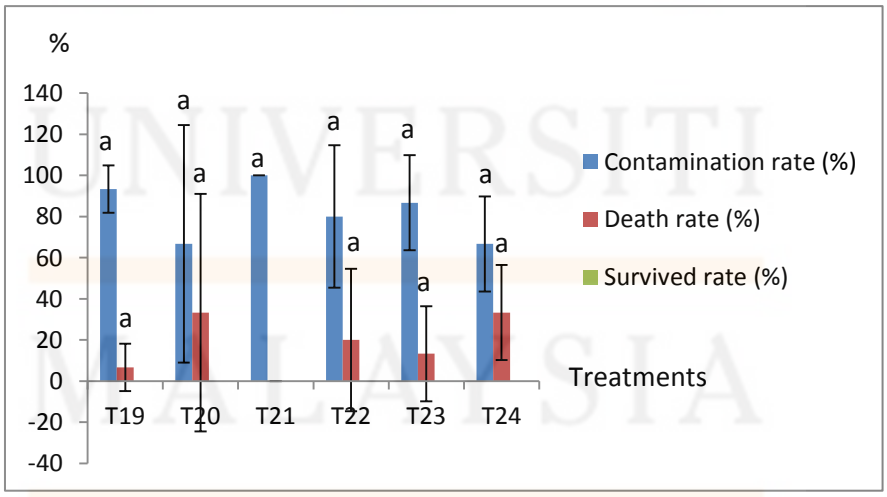

Figure 4.7 The contamination, and survived rate in the fourth sterilization method (H_2O_2 with fungicide)

Table 4.4 and Figure 4.7 show the mean ± SD of contamination, death and survived rate in the fourth sterilization method (H_2O_2 with fungicide). Based on the result, T21 indicates highest contamination rate which is 100.00^a ± 11.55 and the lowest contamination rate which is 66.67^a ± 11.55 indicated by T20 and T24. As for the death rate, the highest rate is 33.33^a ± 23.09 which indicated by T20 and T24 and lowest rate is 0 indicated by T21. There is none of the explants were survived in this sterilization method.

Since all of the treatments indicate significance value ≥ 0.05; this presumed that there are no significant differences between all of those treatments. This sterilization method does not give positive effect in controlling the contamination of the *in vitro* establishment of BTM6 explants.

4.4 Factors That Contribute to the Contamination

Based on the results obtained from this research, it indicates that all of the sterilization method; (1) Sodium hypochlorite without fungicide treatment, (2) Sodium hypochlorite with fungicide treatment, (3) Hydrogen peroxide without fungicide treatment, (4) Hydrogen peroxide with fungicide treatment do not give positive result in controlling the contamination of the *Ficus carica* cv BTM6. explants *in vitro* culture. Although, there was one explant that fortunately survived during the micropropagation, it still cannot prove the effectiveness of the sterilization methods that were used in this study.

There are several factors that might be the cause of contamination. The first factor, fig tree is one of woody plant type. Woody plants are naturally exposed to the environment surrounding. Hence, it was also easily exposed to the contaminants at the outside environment such as bacteria, dust, fungus and others. It is difficult to

establish a proper sterilization method for a woody plant because the contaminants from its surrounding environment might infect on the exogenous and endogenous of the plant materials (Mihaljevic, et al., 2013).

Next factor is the figs explant surface contained small and fine hair (epidermal hair). The fine hair could trap dust, spores and others contaminant and inhibit the microbial activity during the micropropagation (Smith, 2013). These fine hairs are hard to remove although the explants were rinsed thoroughly under the running tap water. If the explant were washed too harsh in order to remove the hair, the plant cell tissue could be damage and might cause the plant to die during the *in vitro* propagation. Besides, the latex produced from the explant also might cause contamination (Smith, 2013). Based observation during this study were conducted, after the explants were cultured it still producing the latex liquid which flow out from the cut explants and store inside the culture media. After several days the bacterial and contaminants started to clump and grow on the media.

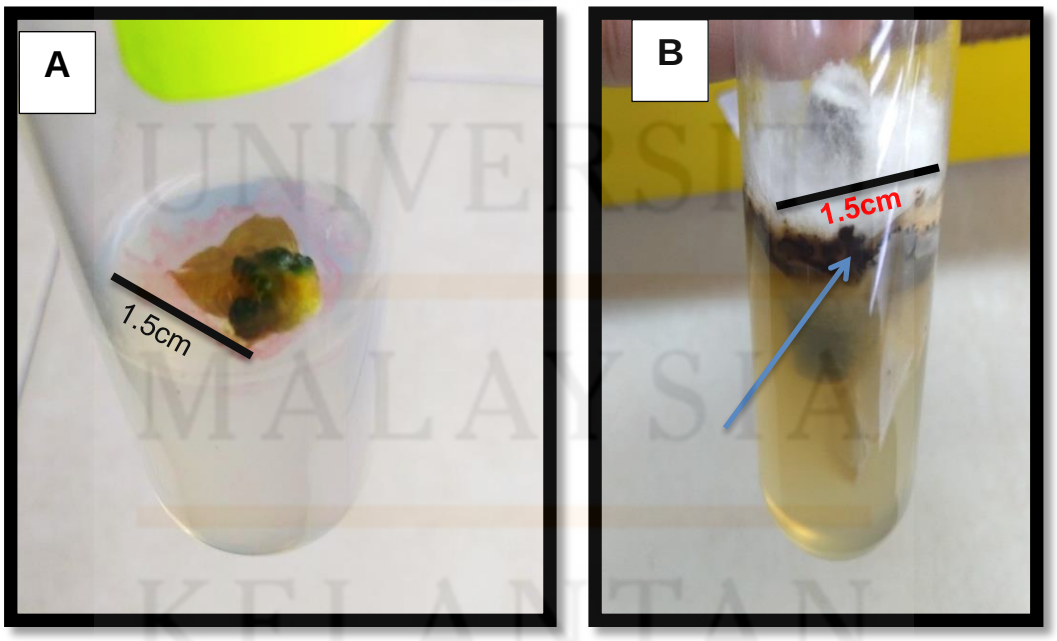

Figure 4.8: A) Bacteria infection on the BTM6 explant; B) Fungus start to clump after several days

Instead of microbes contamination caused by the plants, bacteria that were associated by human also might be one of the factors that cause contamination. The bacteria or fungal spore could be spread through the movement while working with the *in vitro* culture. The air-borne contaminant spores available in the laboratory also could infect the plant materials while *in vitro* propagation was conducted (Govil, Aggarwal, & Sharma, 2017).

Last but not least, the sterilizing agents used during the sterilization method of *Ficus carica* cv. BTM6 was conducted also might not effective. The concentration used may not be effective in removing the bacterial contaminants on the plant materials. The concentration of Hydrogen peroxide may be increased to higher concentration, 10% - 30%. This is because, hydrogen peroxide with concentration of 30% were commonly used in sterilization process (Michigan State University, 2015). Other stronger sterilizing agent such as calcium chloride ($CaCl_2$), mercuric chloride ($HgCl_2$), and Silver nitrate ($AgNO_3$) also could be used during the sterilization process (Mihaljevic et al., 2013).

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Based on this study, the relation between different concentrations of sterilizing agents and time exposure are not significant. Based on the analysis variance (ANOVA) , the P value ≥ 0.05, which indicates that null hypothesis of this study is accepted, $H_0 = \mu_1 = \mu_2$. All of those sterilization treatment methods do not give any effect on the *Ficus carica* cv. BTM6 micropropagation. This study did not give positive effects on the surface sterilization for the *Ficus carica* cv. BTM6. Thus, further study is needed to establish *in vitro* propagation of BTM6.

5.2 Recommendations

Some prevention methods can be used in controlling the contamination of the *Ficus carica* cv. BTM6 and might be useful and helpful for further study of this research. The most important step is by controlling the contamination on the plants. This is because most of bacterial contaminations were carried by the plants itself. The explants taken from outside especially from woody plant must be sterilized properly. In order to remove the contaminants on the surface, the plant can be washed in a warm water and soapy water to remove any contaminants such ad dust, spore and soil that was attached on the plant materials. Wetting agents like Tween – 20 or any detergents can be used as the surfactant agent to enhance the disinfestation process.

Since the fig explants contain epidermal hairs, it can trap air bubble which might contain or trap the microbes' contaminants. It is possible to evacuate the air

bubble by using specific vacuum. Sterilizing agent such as calcium chloride ($CaCl_2$), hydrogen peroxide (H_2O_2), mercuric chloride ($HgCl_2$), Silver nitrate ($AgNO_3$) and Sodium hypochlorite can be used to disinfect the microbes' contamination (Mihaljevic, et al., 2013). By using various sterilizing agents, the differences of effect on the explant sterilizations could be seen and the best sterilizing agents could be chosen for further study. Moreover, the disinfecting agents also must be properly rinsed by rinsing the explant in sterilized distilled water three to five times (Smith, 2013).

Besides, some of the explant also might carry the microbial contaminant in its internal surface. In order to remove these contaminants, a broad spectrum of fungicide such as Benzimidazole (Benomyl) and Carbendazim (Bavistin) and antibiotics such as Ampicillin, Streptomycin and Tetracycline might be useful in reducing the internal bacterial infection. However, the fungicide and antibiotics must be used properly or otherwise it will suppress back the explants and eventually kill the explants (Smith, 2013).

Finally, the contamination in the plant tissue culture also can be carried by human while working with the *in vitro* propagation and surrounding lab environment. The air – born fungal spores from human such from clothes and human hair and also surrounding laboratory might spread on the plants material and caused it to contaminate. Hence, aseptic technique and good laboratory practices must be strictly applied in other to minimize the bacterial and fungal contamination (Cassells, 2001). Personnel aseptic technique must be applied by every person before performing any *in vitro* propagation. Proper attire such as lab coat, sterile latex gloves and face mask must be wear by each individual to minimize the contamination risks. The hands and arms always needed to be sterilized by using 70% of alcohol to remove any germicide on the hands (Smith, 2013). All of these factors must be strictly considered in order to reduce all the contamination risk that might occur.

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APPENDICES

APPENDIX A

Table A 1: Two – way ANOVA for the first sterilization method

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	CONTAMINATION RATE	1511.111[a]	5	302.222	3.400	.038
	DEATH RATE	1444.444[b]	5	288.889	3.250	.044
	SURVIVED RATE	111.111[c]	5	22.222	1.000	.458
Intercept	CONTAMINATION RATE	149422.222	1	149422.222	1.681E3	.000
	DEATH RATE	1088.889	1	1088.889	12.250	.004
	SURVIVED RATE	22.222	1	22.222	1.000	.337
CONC	CONTAMINATION RATE	355.556	1	355.556	4.000	.069
	DEATH RATE	200.000	1	200.000	2.250	.159
	SURVIVED RATE	22.222	1	22.222	1.000	.337
TIME	CONTAMINATION RATE	844.444	2	422.222	4.750	.030
	DEATH RATE	844.444	2	422.222	4.750	.030
	SURVIVED RATE	44.444	2	22.222	1.000	.397
CONC * TIME	CONTAMINATION RATE	311.111	2	155.556	1.750	.215
	DEATH RATE	400.000	2	200.000	2.250	.148
	SURVIVED RATE	44.444	2	22.222	1.000	.397
Error	CONTAMINATION RATE	1066.667	12	88.889		
	DEATH RATE	1066.667	12	88.889		
	SURVIVED RATE	266.667	12	22.222		
Total	CONTAMINATION RATE	152000.000	18			
	DEATH RATE	3600.000	18			
	SURVIVED RATE	400.000	18			
Corrected Total	CONTAMINATION RATE	2577.778	17			
	DEATH RATE	2511.111	17			
	SURVIVED RATE	377.778	17			

Table A 2: Two – way ANOVA for the second sterilization

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	CONTAMINATION RATE	1711.111[a]	5	342.222	1.400	.292
	DEATH RATE	1711.111[b]	5	342.222	1.400	.292
	SURVIVED RATE	.000[c]	5	.000	.	.
Intercept	CONTAMINATION RATE	99755.556	1	99755.556	408.091	.000
	DEATH RATE	11755.556	1	11755.556	48.091	.000
	SURVIVED RATE	.000	1	.000	.	.
CONC	CONTAMINATION RATE	200.000	1	200.000	.818	.384
	DEATH RATE	200.000	1	200.000	.818	.384
	SURVIVED RATE	.000	1	.000	.	.
TIME	CONTAMINATION RATE	577.778	2	288.889	1.182	.340
	DEATH RATE	577.778	2	288.889	1.182	.340
	SURVIVED RATE	.000	2	.000	.	.
CONC * TIME	CONTAMINATION RATE	933.333	2	466.667	1.909	.191
	DEATH RATE	933.333	2	466.667	1.909	.191
	SURVIVED RATE	.000	2	.000	.	.
Error	CONTAMINATION RATE	2933.333	12	244.444		
	DEATH RATE	2933.333	12	244.444		
	SURVIVED RATE	.000	12	.000		
Total	CONTAMINATION RATE	104400.000	18			
	DEATH RATE	16400.000	18			
	SURVIVED RATE	.000	18			
Corrected Total	CONTAMINATION RATE	4644.444	17			
	DEATH RATE	4644.444	17			
	SURVIVED RATE	.000	17			

Table A 3: Two – way ANOVA for third sterilization method

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	CONTAMINATION RATE	3577.778[a]	5	715.556	.619	.688
	DEATH RATE	3577.778[b]	5	715.556	.619	.688
	SURVIVED RATE	.000[c]	5	.000	.	.
Intercept	CONTAMINATION RATE	131755.556	1	131755.556	114.019	.000
	DEATH RATE	3755.556	1	3755.556	3.250	.097
	SURVIVED RATE	.000	1	.000	.	.
CONC	CONTAMINATION RATE	2688.889	1	2688.889	2.327	.153
	DEATH RATE	2688.889	1	2688.889	2.327	.153
	SURVIVED RATE	.000	1	.000	.	.
TIME	CONTAMINATION RATE	577.778	2	288.889	.250	.783
	DEATH RATE	577.778	2	288.889	.250	.783
	SURVIVED RATE	.000	2	.000	.	.
CONC * TIME	CONTAMINATION RATE	311.111	2	155.556	.135	.875
	DEATH RATE	311.111	2	155.556	.135	.875
	SURVIVED RATE	.000	2	.000	.	.
Error	CONTAMINATION RATE	13866.667	12	1155.556		
	DEATH RATE	13866.667	12	1155.556		
	SURVIVED RATE	.000	12	.000		
Total	CONTAMINATION RATE	149200.000	18			
	DEATH RATE	21200.000	18			
	SURVIVED RATE	.000	18			
Corrected Total	CONTAMINATION RATE	17444.444	17			
	DEATH RATE	17444.444	17			
	SURVIVED RATE	.000	17			

Table A 4: Two – way ANOVA for the fourth sterilization method

Source	Dependent Variable	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	SURVIVED RATE	.000[a]	5	.000	.	.
	DEATH RATE	2844.444[b]	5	568.889	.595	.704
	CONTAMINATION RATE	2844.444[c]	5	568.889	.595	.704
Intercept	SURVIVED RATE	.000	1	.000	.	.
	DEATH RATE	5688.889	1	5688.889	5.953	.031
	CONTAMINATION RATE	121688.889	1	121688.889	127.349	.000
CONC	SURVIVED RATE	.000	1	.000	.	.
	DEATH RATE	355.556	1	355.556	.372	.553
	CONTAMINATION RATE	355.556	1	355.556	.372	.553
TIME	SURVIVED RATE	.000	2	.000	.	.
	DEATH RATE	311.111	2	155.556	.163	.852
	CONTAMINATION RATE	311.111	2	155.556	.163	.852
CONC * TIME	SURVIVED RATE	.000	2	.000	.	.
	DEATH RATE	2177.778	2	1088.889	1.140	.352
	CONTAMINATION RATE	2177.778	2	1088.889	1.140	.352
Error	SURVIVED RATE	.000	12	.000		
	DEATH RATE	11466.667	12	955.556		
	CONTAMINATION RATE	11466.667	12	955.556		
Total	SURVIVED RATE	.000	18			
	DEATH RATE	20000.000	18			
	CONTAMINATION RATE	136000.000	18			
Corrected Total	SURVIVED RATE	.000	17			
	DEATH RATE	14311.111	17			
	CONTAMINATION RATE	14311.111	17			

Table A 5: Duncan's test for first sterilization method

CONTAMINATION RATE

Duncan

TIME EXPOSURE	N	Subset	
		1	2
30 MINUTES	6	83.33	
20 MINUTES	6	90.00	90.00
10 MINUTES	6		100.00
Sig.		.244	.091

(A)

DEATH RATE

Duncan

TIME EXPOSURE	N	Subset	
		1	2
10 MINUTES	6	.00	
20 MINUTES	6	6.67	6.67
30 MINUTES	6		16.67
Sig.		.244	.091

(B)

SURVIVED RATE

Duncan

TIME EXPOSURE	N	Subset
		1
10 MINUTES	6	.00
30 MINUTES	6	.00
20 MINUTES	6	3.33
Sig.		.266

(C)

Table A 6: Duncan's test for second sterilization method

CONTAMINATION RATE

Duncan

TIME EXPOSURE	N	Subset 1
30 MINUTES	6	66.67
20 MINUTES	6	76.67
10 MINUTES	6	80.00
Sig.		.185

(A)

DEATH RATE

Duncan

TIME EXPOSURE	N	Subset 1
10 MINUTES	6	20.00
20 MINUTES	6	23.33
30 MINUTES	6	33.33
Sig.		.185

(B)

Table A 7: Duncan's test for third sterilization method

CONTAMINATION RATE

Duncan

TIME EXPOSURE	N	Subset 1
30 MINUTES	6	80.00
10 MINUTES	6	83.33
20 MINUTES	6	93.33
Sig.		.531

(A)

DEATH RATE

Duncan

TIME EXPOSURE	N	Subset 1
20 MINUTES	6	6.67
10 MINUTES	6	16.67
30 MINUTES	6	20.00
Sig.		.531

(B)

Table A 8: Duncan's test for fourth sterilization method

CONTAMINATION RATE

Duncan

TIME EXPOSURE	N	Subset 1
20 MINUTES	6	76.67
30 MINUTES	6	83.33
10 MINUTES	6	86.67
Sig.		.604

(A)

DEATH RATE

Duncan

TIME EXPOSURE	N	Subset 1
10 MINUTES	6	13.33
30 MINUTES	6	16.67
20 MINUTES	6	23.33
Sig.		.604

(B)

APPENDIX B

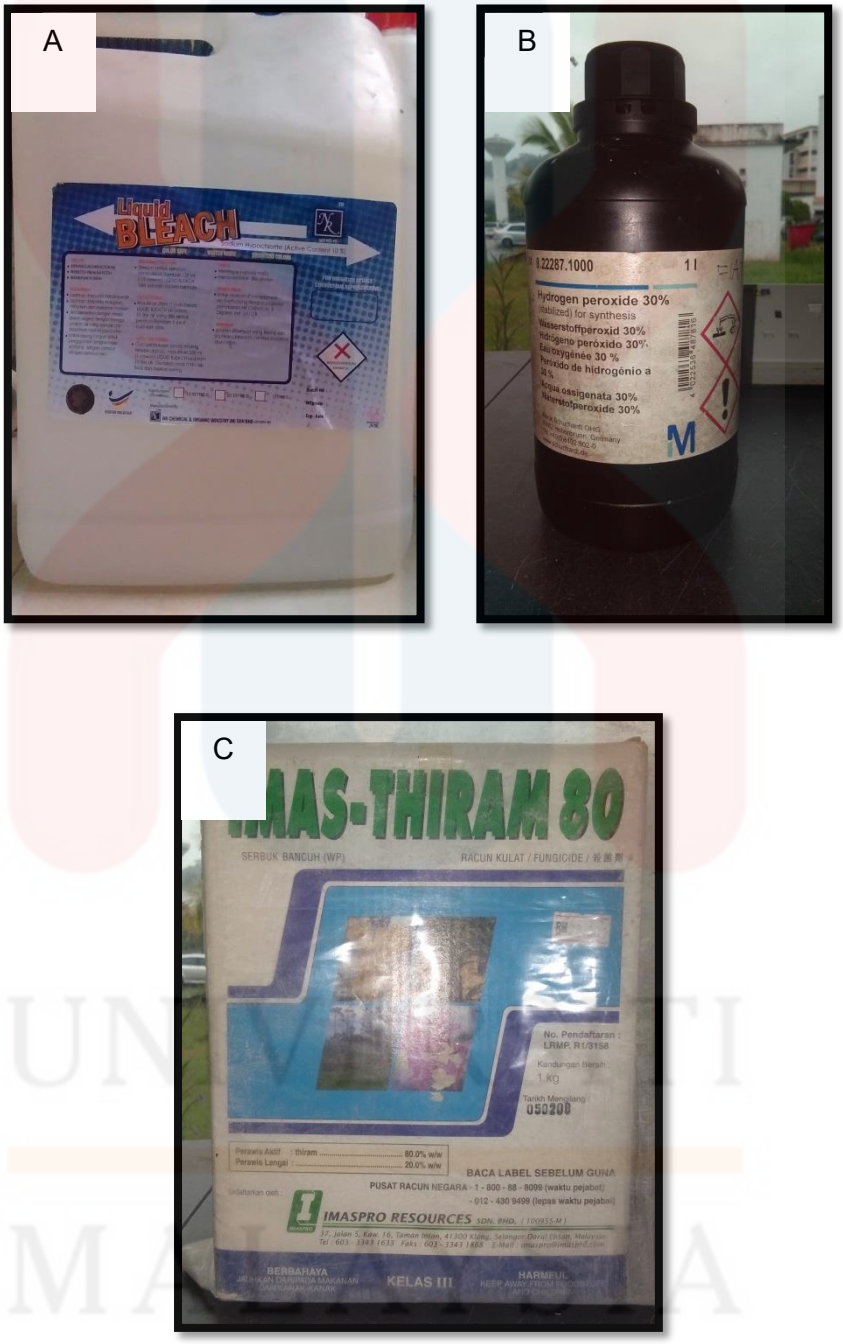

Figure B 1: A. Commercial bleach (NaOCl); B. Hydrogen peroxide (H_2O_2); C. Fungicide used for the steriliztion method

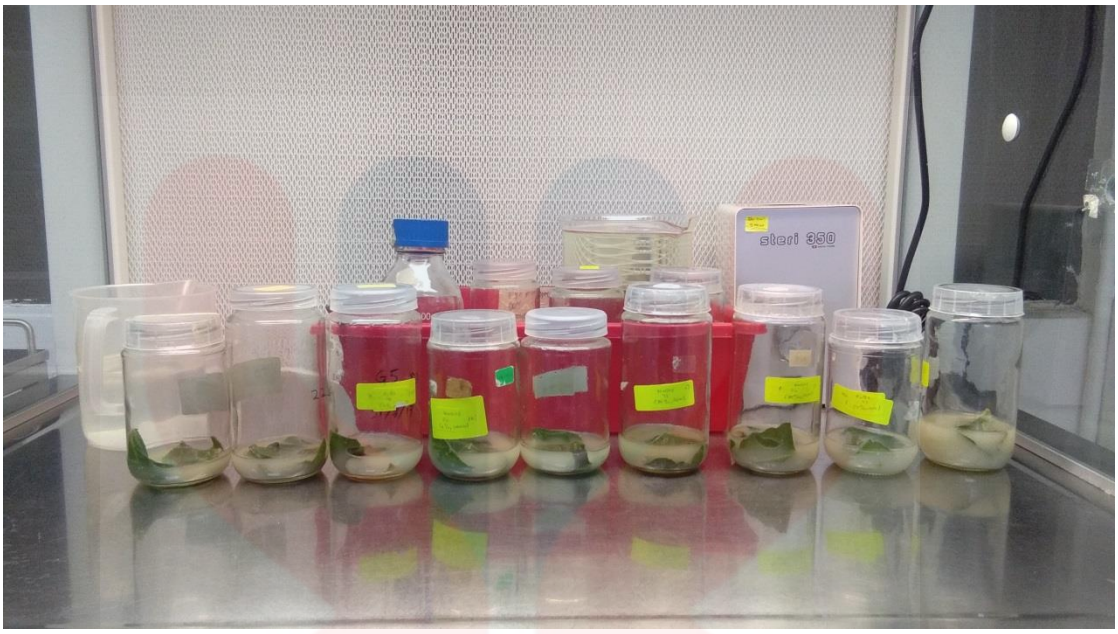

Figure B 2: Treatments treated during sterilization

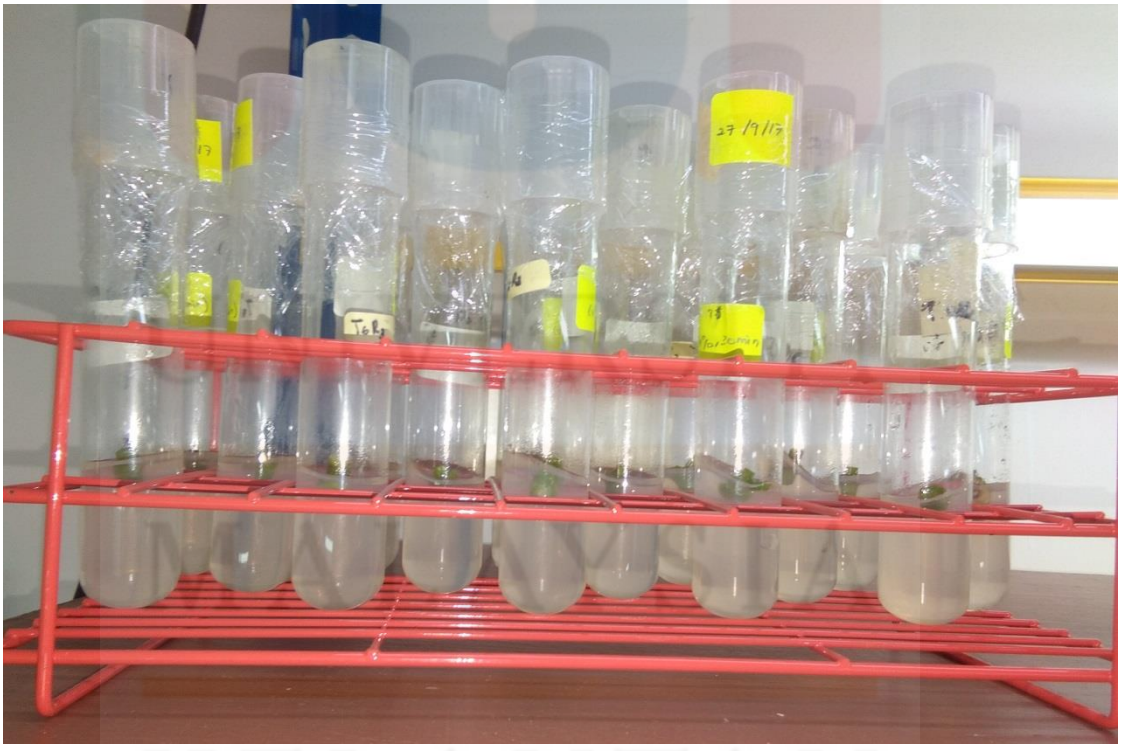

Figure B 3: Cultured media that were stored in growth room with 16 hours of photoperiod