OPTIMIZATION OF SURFACE STERILIZATION FOR RHIZOME AND SEEDS OF *Etlingera megalocheilos* USING INDOLE-3-BUTYRIC HORMONE AT DIFFERENT CONCENTRATION

by

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A report submitted in fulfillment of the requirement for the degree of Bachelor of Applied Science (Natural Resources Science) with Honours

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DECLARATION

I declare that this entitled Optimization of Surface Sterilization for Rhizome and Seeds of *Etlingera megalocheilos* using Indole-3-Butyric Hormone at Different Concentration is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature : ……

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Date : ……

APPROVAL

"I hereby declare that I have read this thesis and in our opinion this thesis is sufficient in terms of scope and quality for the award of the degree of Bachelor of Applied Sciences (Natural Resources Science) with Honours"

Signature : ……...

Name of Supervisor : …….

Date : …….

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OPTIMIZATION OF SURFACE STERILIZATION FOR RHIZOME AND SEEDS OF *Etlingera megalocheilos* USING INDOLE-3-BUTYRIC HORMONE AT DIFFERENT CONCENTRATION

ABSTRACT

'*Tepus*' or *Etlingera megalocheilos* belongs to family of *Zingerberaceae*. It is a wild ginger found mostly distributed in the tropical rainforest. The *in vitro* propagation of *Etlingera megalocheilos* was carried out as to mass propagate the potentially medicinal sources for conservation purposes. Conventional propagation of *Etlingera megalocheilos* is not yet established and limited studies were conducted on the medicinal properties of *Etlingera megalocheilos*. There is no report available on tissue culture studies of this species. Therefore, the establishment of appropriate surface sterilization protocols is important to initiate a successful *in vitro Etlingera megalocheilos*. The rhizome buds and seeds of *Etlingera megalocheilos* were surface sterilized and tested with different concentration and exposure of time of clorox and ethanol as sterilizing agent and incorporation with Indole-3-Butyric hormone to produce a sterile explant. The most effective protocol to produce aseptic explants was by using seed as the explant and treated with 95% of ethanol for 20 minutes with 15% of clorox for 3 minutes. It produced the longest *in vitro* explant survived. However, contamination could not be fully eliminated due to the contaminants located within the plant tissue. Future studies on *in vitro* initiation of *Etlingera megalocheilos* could focus on determination of optimum concentration of plant growth regulator in promoting shoot and rooting for this wild ginger species.

OPTIMISASI PENSTERILAN PERMUKAAN UNTUK TUNAS DAN BIJI *Etlingera megalocheilos* MENGGUNAKAN HORMON INDOLE-3-BUTYRIC PADA KEPEKATAN YANG BERBEZA

ABSTRAK

'*Tepus*' atau *Etlingera megalocheilos* adalah milik keluarga *Zingerberaceae*. Ia adalah halia liar yang banyak dijumpai di hutan hujan tropika. Propagasi *Etlingera megalocheilos* secara *in vitro* telah dijalankan untuk menghasilkan sumber yang berpotensi mempunyai nilai perubatan untuk tujuan pemuliharaan dalam kuantiti yang banyak. Propagasi *Etlingera megalocheilos* secara konvensional belum pernah diterbitkan dan kajian yang dijalankan tentang nilai-nilai perubatan *Etlingera megalocheilos* adalah terhad. Tiada laporan yang tersedia mengenai kajian tisu kultur mengenai spesies ini. Oleh itu, penubuhan protokol pensterilan permukaan yang sesuai adalah penting untuk memulakan kejayaan propagasi *Etlingera megalocheilos* secara *in vitro*. Tunas dan biji *Etlingera megalocheilos* adalah eksplan yang disterilkan dan diuji dengan kepekatan yang berbeza dan pendedahan masa clorox, ethanol, sebagai ajen pensteril dan digabungkan dengan hormon Indole-3-Butyric untuk menghasilkan pensterilan yang berkesan. Protokol yang paling berkesan untuk menghasilkan eksplan yang aseptik adalah dengan menggunakan biji sebagai eksplan dan dirawat dengan 95% ethanol selama 20 minit dengan 15% clorox selama 3 minit. Protokol ini telah menunjukkan eksplan *in vitro* yang paling lama bertahan. Walau bagaimanapun, isu pencemaran tidak dapat dikurangkan 100% disebabkan oleh bahan pencemar yang berada di dalam tisu tumbuhan.

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LIST OF ABBREVIATIONS

HgCl2	Mercuric chloride
NaOH	Sodium hydroxide
HCI	Hydrochloric acid
IBA	Indole-3-Butyric Acid

LIST OF SYMBOLS

±	Plus-minus
℃	Degree Celcius

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Ginger belongs to the family *Zingiberaceae*. Bartley & Jacobs (2000) stated that it is a valueable medicinal crops which over 2000 years, it has been used as a spice. Its origin is still unknown, probably in tropical Asia and China (Purseglove *et al.*, 1981). It is cultivated in many tropical and subtropical countries in which China and India are the world's leading producers (Blumenthal *et al.*, 2000). *Zingiber officinale Rosc.* (Ginger) is an important tropical horticultural plant, values all over the world as an influential spices for its medicinal properties (Kambaska & Santilata, 2009).

Micropropagation is a technology that was established within the past 30 years and it is the true-to-type propagation of a selected genotype using *in vitro* culture techniques (Debergh & Zimmerman, 1991). It is a vegetative method for multiplying plants (Scoggins & Bridgen, 2013). When plants are multiplied vegetatively, all the offspring from a single parent can be classified as clone. This means that the genetic make-up of each offspring is identical to that of parent (Scoggins & Bridgen, 2013). Castellar *et al.* (2011) state that the vegetative propagation methods are recognized as excellent tools for the propagation of medicinal plants that allow the production of

contamination-free plants under controlled conditions that independence from climatic factors.

Etlingera megalocheilos belongs to the family of *Zingiberaceae* and distributed in the tropical and subtropical dense rain forests under the very shady lush big trees as in Java, Sumatra, Kalimantan, Sulawesi and Malaysia. The local name of *Etlingera megalocheilos* is commonly called '*Tepus*' used by many tribes in Borneo (Heyne, 1927; Poulsen, 2006; Poulsen 2007). It has such a strong smelling volatiles or pungent smell (aroma/scents). Ginger roots, inner stems and flower are used as herbs in traditional healing remedies especially traditional practitioner in India and China as it contain many medicinal properties. Tissue culture protocols and *in vitro* study for others *Etlingera* species have been reported by few researchers such as for *in vitro* multiplication (da Silva Júnior 2015), embryogenic callus induction (Gomes-Dias *et al.* 2014), *in vitro* mutagenesis (Yunus *et al.* 2013), *in vitro* propagation (Yunus *et al.* 2012), micropropagation using axillary buds (Abdelmageed *et al.* 2011) and shoot tip culture (Mendez *et al.* 2004). However, there is no propagation studies done on E. *megalocheilos*. Therefore, this study was conducted to investigate the most effect sterilization protocol to produce a successful *in vitro E. megalocheilos*.

1.2 Problem Statement

According to the previous study by Vairappan *et al*. (2012) *Etlingera megalocheilos* has a great potential as an element to treat cancer because it shows a good inhibition towards human cancer cell. From this finding, the properties of this species has a great potential that may one day form the basis of drugs to fight cancer diseases as cancer has become the fourth leading cause of death among medically

certified death in Malaysia (Lim, 2002). Therefore, more future research or work is needed to preserve the endangered, vulnerable and rare plant species for future pharmacological demands. According to Ramakrishnan (2017), *in vitro* regeneration and conservation of endangered medicinal plants has pave the way for production of pharmacologically active substances. However, the conventional propagation of *Etlingera megalocheilos* is not yet established and there is no information about this medicinal plant tissue culture method for future studies. There is no report available on tissue culture studies of this species. Furthermore, only limited studies were conducted on the medicinal properties of *Etlingera megalocheilos*.

1.3 Objective

The aim of this study is to establish *in vitro E. megalocheilos*. The objective of this study was:

1. To identify an appropriate sterilization protocols to initiate *in vitro* propagation of *Etlingera megalocheilos*

1.4 Scope of Study

The sampling of *E. megalocheilos* had been done around Agro Park, Universiti Malaysia Kelantan, Jeli Campus. This study was focused on the optimization of *E. megalocheilos* through *in vitro* propagation method to obtain high quality of homogenous plants growth for future pharmacological research. This study was also been done to determine the effects of different concentration of indole-3-butyric hormone on the growth of this species *in vitro*.

1.5 Significance of the Study

Plant *in vitro* propagation act as the alternative techniques to conserve wild ginger population for commercial propagation and conservation of potential endangered medicinal plant resources. Plant tissues and organs are grown *in vitro* on artificial media, which supply the nutrients necessary for growth (George *et al*., 2008). Ginger are vegetatively propagated using rhizome buds because it cannot be propagated by seed. *In vitro* propagation of *E. megalocheilos* was carried out to produce high-quality of homogenous plants in a short period of time through the optimization of propagation and thereby facilitate future downstream researches for this ginger species. This study also was carried out to identify the most suitable concentration of plant growth regulator (IBA hormone) towards the optimum scale of growth performance of *E. megalocheilos*.

CHAPTER 2

LITERATURE REVIEW

2.1 Morphology of *Etlingera megalocheilos*

Yeats (2013) stated that *Zingerberaceae* is a pan-tropical ginger family composed of 51 genera and 1, 200 species predominantly found in Asia and the Pacific regions. One of the largest genus in *Zingeberaceae is* Etlingera which consist of more than 1,200 species (Yeats, 2013). *Etlingera* species, family *Zingiberaceae*, tribe Alpineae, produce colorful inflorescences, flowers and fruits (Vairappan *et al*., 2012). The flowering shoot is a cone-like spike comprise of bracts and flower that are held on a various length of peduncle. The roots are creeping close to the ground via the leaf-litter zone where it give stability and anchorage on steep slopes in montane habitats and elevate the rhizome from wet ground in lowland forest (Figure 2.1). The inflorescence is partly sunk in the ground, with white or pale pink bracts underground.

Figure 2.1 The habitat of *Etlingera megalocheilos* at Agro Park of Universiti Malaysia Kelantan. © Fatien Nur Syazwanie, 2018

Figure 2.2 Flower of *Etlingera megalocheilos* found at Agro Park, Universiti Malaysia Kelantan, Jeli Campus. © Fatien Nur Syazwanie, 2018

2.2 Origin and Geographic Distribution

Griffith (1851) were first to described *Etlingera megalocheilos* from Peninsular Malaysia. While Valeton (1904) documented its presence in Java. In Asia, it is distributed in the tropical and subtropical dense rain forests under the lush big trees and very shady trees such as in Java, Sumatra, Kalimantan, Sulawesi and Malaysia. *Etlingera* species are native to Bangladesh, Laos, Burma, China, Vietnam, Cambodia, Thailand, Malaysia, Brunei, Indonesia, Singapore, Papua New Guinea, Philippines, Queensland, India and several Pacific Islands, predominantly close to the equator between sea level and 2500 metre.

2.3 Botanical of *Etlingera megalocheilos*

Ginger has been traditionally used from time immemorial for varied human ailments in different parts of the globe (Shukla & Singh, 2007). Vairappan *et al*. (2012) state that the traditional practitioners, they use the oils of *E. megalocheilos* as a wound cleanser and remedy for ear infections.

Etlingera megalocheilos contain highly antibacterial properties that makes it an excellent herbal remedy to curb an ear infection. The ear infection diseases that commonly occur are the acute otitis media (AOM) and otitis media with effusion (OME) that commonly affect young children and infrequently in adults. This often causes pain, inflammation and fluid buildup within internal spaces of the ear. According to Ruohola *et al*. (2006), acute middle ear infection is a co-infection with bacteria and viruses. Grönroos *et al*. (1964) and Ruohola *et al*. (2006) found that the types of bacteria that have became the primary causes of AOM are the *Diplococcus*

pneumoniae, Haemophilus influenzae, Staphylococcus aureus and *Streptococcus pyrogene*. Thus, this has become a great remedy of ear infection in the past decades because it can combat the presence of *Staphylococcus aureus* and *Streptococcus pyrogene*.

Other than that, previous practitioner used *E. megalocheilos* to cleanse wounds, to reduce infection and swelling. It is full of curcumin, a spice that is a derivative of a ginger. It has natural anti-inflammatory properties where it contains antioxidant properties that helps to alleviate pain and other major illnesses symptoms. According to Rhoads *et al*. (2012), out of 168 wounds tested, the most common organism identified was *Staphylococcus aureus*. Similarly, Kirketerp-Møller (2008) found that the majority of the detected bacteria in his studies were *Staphylococcus aureus*. This data complement the traditional use of *E. megalocheilos* as a wound cleanser. This medicinal plant effectively used by our ancestor as it combat the main bacteria in the wounds.

2.4 Antibacterial Activity and Anticancer Activity of *Etlingera megalocheilos*

Etlingera megalocheilos are scientifically proven to be a potent antibacterial oils because Vairappan *et al*. (2012) found that the essential oil of *E. megalocheilos* shows strong inhibition against four strains of clinical bacteria that is *Staphylococccus aureus* and *Streptococcus*. The data from Vairappan *et al*. (2012) complement with the discussion in the section of 2.1.2 whereas the antibacterial properties of *E. megalocheilos* combat the existence of the *Staphylococcus aureus* and *Streptococcus pyrogene as* the basis to cure an ear infection.

According to Vairappan *et al*. (2012) the toxicity properties of *E. megalocheilos* essential oil showed a good cancer cell inhibition against human breast adenocarcinoma (MCF-7), cervix carcinoma (HeLa), lymphocytic leukemia (P388) and promyelocytic leukemia cell line. This means that the cytotoxicity activities of the essential oil possessed a potent antibacterial oil in fighting human cancer cell excellently that makes it a good anticancer element.

These findings illustrate the potential of this plant as a probable source of medicinal element and provide a scientific basis of uses for infectious diseases and cancers. *E. megacheilos* have the potential of serving a novel therapeutic agents to the modern pharmacopeia to counteracting with various diseases. FAO (Food and Agricultural Organization) state that the human population will be reach around 9.1 billion at the end of 2050 and 80% of this population solely depends upon the medicinal plants for their health care (Ramakrishnan *et al*., 2017). Therefore *in vitro* propagation method is needed to provide or multiply vast amount of this plant so that it can facilitate more research in the future to help in the emergence of new cure.

2.5 Advantages of Plant *In Vitro* Propagation

According to Ramakrishnan *et al*. (2017), human beings relies on medicinal plants for their primary health needs as medicine and natural preservatives in various traditional medicinal practice. The demand of herbal remedies in the global market is increasing every year. However, Ramakrishnan *et al*. (2017) pointed that the seed indequacy or unavailability, seed dormancy, rare seed set production, viability loss and endophytic bacterial and contaminations of fungal in explants are the uppermost inhibitions in the conventional propagation. This has greatly become a difficult task to

yield a massive amount of plants via conventional propagation as demanded by pharmaceutical industries or traditional practitioners. At present, *in vitro* propagation has captivate many scientific community to preserve and yield a massive volume of plant material, secondary metabolites, synthetic seeds and transgenic plants.

In vitro propagation has various prons compared to the conventional vegetative propagation. Its commercial use in horticulture, agriculture and forestry is currently expanding worldwide (Kozai, 1991). According to Kambaska & Santilata (2009), tissue culture is important to sustain the evolutionary process and the genetic variation in viable populations of ecologically and commercially viable genotypes. Other than that, Miguelez-Sierr *et al*. (2017) state that the techniques provide an alternative to produce a high-quality of plants in a short duration of time and minimise the usage of space.

Ginger is vegetatively propagated using rhizome buds as the seeds are hard to found because it is seasonal. Balladin *et al*. (1998) state that the rhizome is treasured for its essential oils and oleoresins. The previous tissue culture study of ginger that used rhizome as their explant was reported by many researcher (Balachandran *et al*., 1989; Kambaska & Santilata, 2009; Barthakur & Bordoloi, 1992). The other previous *in vitro* propagation plant study that used rhizome as their explant was turmeric (*Curcuma* sp.) (Nasirujjaman *et al*., 2005; Balachandran *et al*., 1989).

2.6 Indole-3-Butyric Acid (IBA) as Plant Growth Regulator

Indole-3-butyric acid hormone, $C_{12}H_{13}NO_2$ is a crystalline solid with white to light-yellow features. IBA is now used commercially worldwide to root many plant species (Hartmann *et al*., 1990). IBA usually used in innumerable commercial

horticultural plants rooting products because Epstein & Ludwig-Müller (1993) and Zimmerman & Wilcoxon (1935) state that it induces plants adventitious rooting of many species and was even more effective than IAA. IBA are classified as an endogenous constituent of various plants (Epstein & Ludwig-Müller, 1993). IBA induces the formation of masses of undifferentiated cells which then form other tissues such as roots.

It has a variety of different effects on plant growth and development when applied exogenously (Ludwig-Müller, 2000). According to Hartmann *et al*., (1990) the exogenous application of auxin may be promotive, ineffective or even inhibitory to the rooting of cutting. There are some species that show positive change, some shows negative change and some show no changes at all. This shows the differences of reactions on their sensitivity towards IBA treatment in rooting induction. According to Shiembo *et al*. (1996), tree species that are insensitive to exogenous auxins are usually well supplied with endogenous auxins. Different species shows different scenario such as Shiembo *et al*. (1996) reported that the *Gnetum africanumin* rooting was insensitive to IBA treatment but the number of roots per rooted was positively related to IBA treatment. According to the study conducted by Tchoundjeu *et al.* (2002) on *Prunus africana* stem cuttings, IBA significantly increase the rooting percentage but not the mean number of roots. Nevertheless, another tropical tree species such as *Albizia guachepele* and *Cordia alliodora* (Mesen, 1993), *Nauclea diderrichii* (Leakey, 1990) and *Bobgunnia madagascariensis* (Amri, 2011) show that IBA application resulted in sharp increase of number of roots formed. However, Akwatulira *et al*., (2012) reported that there was significant positive change of the roots sizes formed with the highest concentration of IBA concentration (0.8% w/w) in *Warburgia ugandensis*.

CHAPTER 3

MATERIAL & METHOD

3.1 MATERIAL

3.1.1 Apparatus

The apparatus that were used is beaker, glass rod, conical flask, Erlenmeyer flask, petri dishes, measuring cylinder and different sizes of jam jar bottles. These apparatus was sterilized using liquid detergent and rinsed using distilled water and finally autoclave (TOMY SX-500, 120-121 ℃, 15 Ib/inch^2). The instruments that were used for culture works is scalpel, forceps, scissors, statistical balance, spatula, disposable blades and autoclave. All the autoclaved and sterilized materials was dried in oven at temperature 50°C.

3.1.2 Chemicals and Reagants

The basal medium that was used for initiation and establishment of *in vitro E. megacheilos* is Murashige and Skoog (1962) medium with 0.8 % of agar and 3 % of sucrose. The MS basal medium composition of macronutrients, micronutrients, and

organic supplement was prepared. All the chemical had been purchased from Sigma Aldrich (Malaysia).

3.2 METHOD

3.2.1 Murashige and Skoog (1962) Basal Medium Preparation

a. MS Stock Preparation

The stock solution of macronutrient, micronutrient, growth regulators and vitamins was prepared separately. All of the solutions was kept in a clean cabinet and checked visually to check if any ingredients precipitations or microorganisms contamination occur before each use. Plant growth regulator that was being used are IBA hormone at five different concentrations (1.0, 2.0, 3.0, 4.0, and 5.0 mg/L).

b. MS Medium Preparation

Eight hundred ml of distilled water was filled into 1L beaker. After that, thirty gram of sucrose was weighed. The sucrose was added and dissolved in the flask. Then, macronutrient, micronutrient, MS iron, Myoinositol, vitamin stocks solution was mixed together following the method by Murashige and Skoog (1962).

The pH was adjusted to 5.8 by using 0.1M NaOH or HCl. After that, distilled water was added into the beaker to make up the volume up to 1L. Ten gram of agar was added into the beaker. Then, the solution in the beaker was boiled in the microwave oven until the agar melts completely. After that, the medium was aliquoted into tall jam jar vessels and being closed with transparent screw caps. The

culture bottles with the MS basal media was sterilized by autoclaving for 15 minutes at 120-121^0 C at 15 Ib/inch^2. Then, the culture bottles was kept in the room temperature (26^0 C $\pm$ 2^0 C) to cool and solidify the agar and stored in the clean storage room in the Laboratory of Tissue Culture, Universiti Malaysia Kelantan, Jeli Campus.

3.2.2 Sampling of *Etlingera megalocheilos*

Etlingera megalocheilos was sampled randomly in the wild around Agro Park, Universiti Malaysia Kelantan, Jeli Campus. The plant was planted in the Green House at Universiti Malaysia Kelantan, Jeli Campus for growth and each of the plants are being labeled with the date and collector's name. The plant were maintained in a small pots and will be watered daily. The sample identification was confirmed by referring to pictorial book entitled *Etlingera* of Borneo by Poulsen (2006).

3.2.3 Surface Sterilization and Initiation of *In Vitro* Culture of *Etlingera megalocheilos* using Protocol by Sarmila (2018) with Different Concentration and Exposure Duration

The surface sterilization of *E. megalocheilos* was done following the protocol unpublished by Sarmila (2017) who reported sterilization method for various *Zingiber* species. The rhizomes of *E. megalocheilos* was obtained from Agro Park of Universiti Malaysia Kelantan, Jeli Campus. The rhizome with emerging new buds (5 to 6 cm) was chosen and trimmed using sterile scalpel.

a) Treatment with 95% of Ethanol for 15 minutes and 100% of Clorox for one minute

The explant was first treated for surface sterilization with commercial liquid detergent for 15 minutes. The explant was rinsed with running tap water for 20 minutes and followed by washing it with distilled water to remove all of the surface contaminants. The explant then was transferred quickly to laminar air flow cabinet and the explant was soaked in 95% of ethanol for 15 minutes. Then the explant were immersed into the 100% of Clorox for another one minutes. Lastly, the explant was rinsed 3 to 4 times thoroughly with sterile distilled water to remove any chlorine traces (Kinabalu, 2015) and soaked with sterile blotted paper to remove any water traces from the surface of the rhizome. The sterile rhizome was inoculated in sterilized MS basal media and the culture vessels was incubated in 16 h light/8 h dark photoperiod at 25 ± 2 °C under white fluorescent white light at 2000-2500 lux (Appalasamy *et al.*, 2014) under 60 to 70% relative humidity on the culture bench in growth room of Tissue Culture Laboratory, Universiti Malaysia Kelantan, Jeli Campus.

b) Treatment with 95% of Ethanol for 20 minutes and 15 % of Clorox for 3 minutes

The experiment was carried out to using method described by Sarmila (2017) as in section 3.2.3(a) but with some modification. Rhizomes and seeds of *E. megalocheilos* were used for establishment of a sterile culture of *Etlingera megalocheilos*. The modification involves usage of 95% ethanol to soak the rhizomes

for 20 minutes and then washing with 15% of Clorox for 3 minutes.

The explants were sterilized in the laminar flow chamber by soaking it in a liquid detergent for 20 minutes, 95% of ethanol for 20 minutes and 15% (v/v) of Clorox for one minutes. Finally, the explants were rinsed with sterile distilled water thrice and the washings was discarded. Then, the rhizomes were inoculated into the culture medium as stated in section 3.2.4.

3.2.4 Surface Sterilization and Aseptic Establishment of *Etlingera megalocheilos* using Shalini *et al.* (2018) Protocol

The protocol used 95% ethanol and the rhizomes were dipped and flamed over burning flame for 15 seconds. The explants were washed under running tap water for 50 minutes and soaked in liquid detergent for 20 minutes. Then, the rhizomes were soaked in distilled water for 10 minutes to wash the detergent.

In the laminar flow chamber, the explants were treated with 95% of ethanol for 60 seconds followed by 15% of Clorox for 30 seconds and washed in sterile distilled water by swirling for 10 seconds and quickly flamed for 5 seconds over the Bunsen burner. Then, the aseptic explants were inoculated in the prepared sterile MS media and placed it at the culture bench at 25 ± 2 ℃ under white fluorescent white light at 2000-2500 lux (Appalasamy *et al.*, 2014) under 60 - 70% relative humidity in the growth room of Tissue Culture Laboratory of Universiti Malaysia Kelantan, Jeli Campus.

3.2.5 Surface Sterilization and Initiation of *In Vitro* Culture of *Etlingera megalocheilos* using Iliev *et al.* (2010) Protocol

The experiment was carried out to identify the effect of using Tween 20 as sterilizing agent in eliminating microbial contaminants. The rhizome buds were washed under running tap water for 5 minutes. Then, it was quickly transferred to laminar flow cabinet. The explants was washed and stirred with magnetic stirrer in 70% (v/v) ethanol for 2 minutes and 5% (w/v) NaCIO, containing 20 drops per litre of Tween 20 for 20 minutes. After immersion in each solution, the explants was washed thrice with sterile distilled water to remove any adhering chemicals or contaminants.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Sampling of *Etlingera megalocheilos*

The rhizome buds and the seeds of *E. megalocheilos* were used as the explant sources for *in vitro* propagation. Both plant materials are collected from wildings from the forest of Agro Park, Universiti Malaysia Kelantan, Jeli Campus and brought to the Tissue Culture Laboratory of Universiti Malaysia Kelantan. Odutayo *et al.* (2007) found that the problems are aggravated when the plant material are sourced directly from field grown because Naz *et al.* (2009) state that it is difficult to terminate infection from *Zingiberaceae* explants of underground origin. Therefore a few protocol has been tested and discussed to establish an effective disinfecting protocol of the field grown explants. According to Mihaljevic *et al.* (2013) each plant material has a different level of surface contaminant that depends on the age, growth environment and type of plant part.

Figure 4.1 **(A-D)** *Etlingera megalocheilos*. **(A)** Rhizome in underground habitat in Agro Park of Universiti Malaysia Kelantan. **(B)** Cleaned rhizome to be used as explant for propagation. **(C)** The fruits of *E. megalocheilos* (Chongkraijak *et al.*, 2013). **(D)** The seeds of *E. megalocheilos*.

4.1.1 Surface Sterilization and Initiation of *In Vitro* Culture of *Etlingera megalocheilos* using Protocol by Sarmila (2018) with Different Concentration and Exposure Duration

In this experiment, the protocol tested yielded no surviving plantlets as shown in the Figure of 4.2. This protocol are tested on both explants; rhizome buds and seeds. It uses the Clorox and Ethanol as the sterilizing agent or sterilants that were tested on all explants types.

In many plant tissue culture studies, sodium hypochlorite (bleach) and ethanol at variant concentrations was employed in many research studies due to their availability and economic merits (Ndakidemi *et al.* 2013). The successfulness of these sterilants have been recorded in many studies such as *Solanum tuberosum* (Badoni & Chauhan, 2010); *Magnifera indica* L. (Nower, 2013); *Parkia biglobosa* (Oluwaseun & Erhinmeyoma, 2004) and *Latropha curcas* (Siang *et al.*, 2012). Bleach has the capability to be an effective sterilants because sodium hypochlorite is known to be effective bacteria killer, whereby even micromolar concentrations are sufficient to significantly repress the bacterial population (Oyebanji *et al.*, 2009). Ethanol is a strong and extremely phytotoxic sterilant. Ethanol can eliminated numerous epiphytic fungi but not the endophytes. This aligned with the statement by Bloomfield (1978) that ethanol does not destroy bacterial spores. According to Manzanilla (n. d.), subsequently high concentrations of ethanol are required to destroy endophytic culture contaminants. Sen *et al.* (2013) stated that it is used prior to treatment with other compounds because it is considered to be rapidly bactericidal rather than bacteriostatic against vegetative forms of bacteria. Ethanol are also toxic to the plant tissues, hence appropriate concentration, duration of exposure and the sequences of using the sterilant has to be standardized to reduce the plantlet injury and achieve a superior survival (Central Potato Research Institute, 1992). Alcohols are one of the most effective and widely utilized as bacterial disinfectants that act through protein denaturation and increased membrane leakage (Ingram & Buttke 1985; Wistreich & Lechtman 1988; Ingram 1990).

a) Treatment with 95% of Ethanol for 15 minutes and 100% Clorox for One Minute

Based on the previous research by Sarmila (2018) on micropropagation of *Zingiber zerumbet* and *Zingiber spectabile,* it produces 80% of aseptic explants. However, when this protocol were applied to sterilize *E. megalocheilos*, it does not successfully produce a clean and survive explant. This might due to the origin and the morphological character of *E. megalocheilos*. The rhizome of *E. megalocheilos* are collected from wildings and the size are much bigger, harder and tougher compared to *Zingiber zerumbet* and *Zingiber spectabile*. The use of plants originally wildings from the forest as direct sources of explants to produce 'clean' *in vitro* plantlets commenced a major challenge (Webster *et al.*, 2003). Naz *et al.* (2009) reported that it is difficult to terminate infection from *Zingiberaceae* explants of underground origin. This is due to the plants surface carries a wide range of microbial contaminants and with the much bigger and harder structure that makes the sterilization process are more difficult as it need to be trimmed using a cleaver and trimmed outside the laminar flow. This handling of this explant require a lot transfer to unaseptical surrounding that might attract other new contaminant to the explants and makes the sterilization more troublesome.

According to Sen *et al.* (2013), the sterilizing agents should show a maximal effect against microbiological contamination at higher concentration but the survival rate is low. However, when the protocol were tested, it recorded the highest contamination rate among all protocols tested. It cannot last longer where it finally contaminated after 5 days of culturing. The results showed that the explants were contaminated by day 5, giving a 100% contamination rate. This may due to the

shorter exposure of time that cause the death of the explants where it does not provide enough duration for the sterilants to combat any microbial survival. Therefore, even though the concentrations are high, the duration of time did not compliment to the effectiveness of the sterilants to fight contaminants. A research done by Oyebanji *et al.* (2009) concluded that sodium hypochlorite with time intervals between 20 to 45 minutes produced the zenithal reduction in fungal and bacterial contamination towards *Brachylaena huillensis*. The fact from these research provide an insight that by extending the duration of the sterilizing agent towards the plantlet could suppress the growth of contaminants. Ndakidemi *et al.* (2013) state that the probable reasons for the weak response in high concentration of sterilizing agent could be due to the fact that these sterilants are becoming toxic to the plant tissues. A balance between concentration and time must be determined empirically for each type of explant because of its phytotoxicity (Srivastava *et al.*, 2010). Another plausible reason recorded by Wlodkowski & Rosenkranz (1975) and Dukan *et al.* (1999) that the sodium hypochlorite will form HClO when diluted with water, which is negatively correlated with bactericidal activity, might in part due to lethal DNA damage. In this study, the effect can be seen through the dense growth of fungal as shown in Figure 4.2.

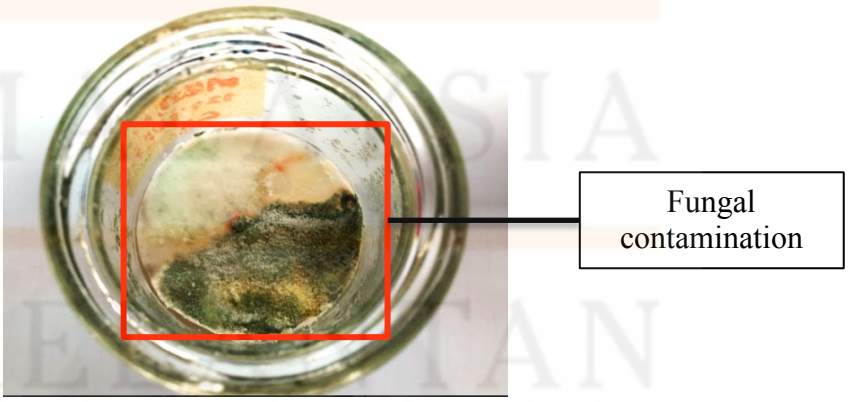

Figure 4.2 Fungal growth were observed surrounding of *in vitro* explants of *E. megalocheilos* on basal media after 5 days of culturing.

b) Treatment with 95% of Ethanol for 20 minutes and 15 % of Clorox for Three Minutes

The different exposure of time tested for ethanol and clorox (20 minutes and 3 minutes) and different concentration of clorox (15%) used. The increasing exposure of times of the sterilants significantly increased the number of 'clean' plantlets produced compared to when the shorter time of sterilants used on both buds and seeds. According to Oyebanji *et al.* (2009) explants such as dormants buds or seeds are allowable to be treated for a longer time frame because the tissue that are going to be established is actually within the structure that is being sanitized. This has proved the statement from Oyebanji *et al.* (2009) that true that seeds and buds needed to be exposed for a longer time frame because Çölgeçen *et al.* (2011) state that the concentration and duration of exposure contradict from one to another depending on the tissue tenderness/hardness. The result also proved the statement from Rezadost *et al.*, (2013) that surface sterility depends on the explants and Ndakidemi *et al.* (2013) and Sharma *et al.* (2014) that the duration of sterilization is reliant on the type of tissue.

The results showed that the rhizome buds of *E. megalocheilos* have shown distinct sterility response for different concentration of clorox and ethanol at different exposure of time. This can be seen by the density of the appearance of contaminants in cultured medium where in the Figure 4.3 shows a very much lower density compared to Figure 4.2 and this protocol yielded the most highest and abundant clean explants compared to other protocols and also the longest explant survive using this protocol. The result from studies done by Thokchom & Maitra (2016), surface sterilization on *Anthurium andraeanum* Lind recorded the highest percentage of

contamination and lowest percentage in survivability when the explants are treated only with 15% of sodium hypochlorite for 5 minutes. Therefore, based on the surface sterilization by Thokchom & Maitra (2016), the additional sterilization applied on *E. megalocheilos* by immersion in 95% of ethanol enhances the effectiveness of 15% of sodium hypochlorite or reduces the percentage of contamination and increases the percentage of survivability. After one week of culturing, the explants cannot be considered survive because there was growth of bacteria on the plantlet surface after 2 weeks of culturing. This might be due to the tissues are overexposed that makes it scorched by the decontaminating chemicals that could kill tissues, so Qin *et al.* (2012) and Olowe *et al.* (2014) recommended to balance the execution between sterilizing explants and killing the explants themselves. From the observation made, the Thokchom & Maitra (2016) protocol combined with immersion in 95% of ethanol for 20 minutes produce the least contamination out of all protocols.

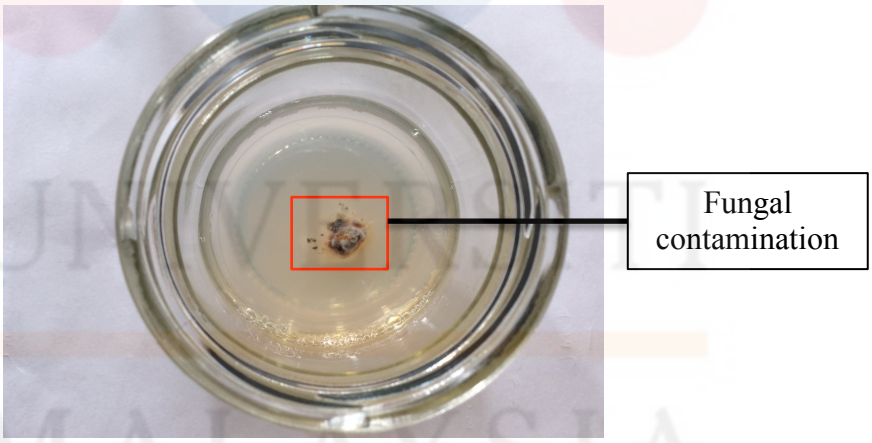

Figure 4.3 Least fungal contamination on the explant surface of *E. megalocheilos* when the buds are treated with 95% of Ethanol for 20 minutes and 15 % of Clorox for 3 minutes.

Nevertheless, when seed are used, it shows different effect towards the treatment. When this sterilization protocol were applied to seeds of *E. megalocheilos*,

it has successfully produce the most abundant of clean explant without any contamination occur as shown in Figure 4.4 that has been survived for 2 months and up until now. However, there was no growth rate observed because there was no germination or development seen from the seed for 2 months of culturing. The situation occur can be related by the statement from Mendez *et al.* (2004) that seeds of *Etlingera* sp. has a slow growth rate to accommodate demand for commercial production.

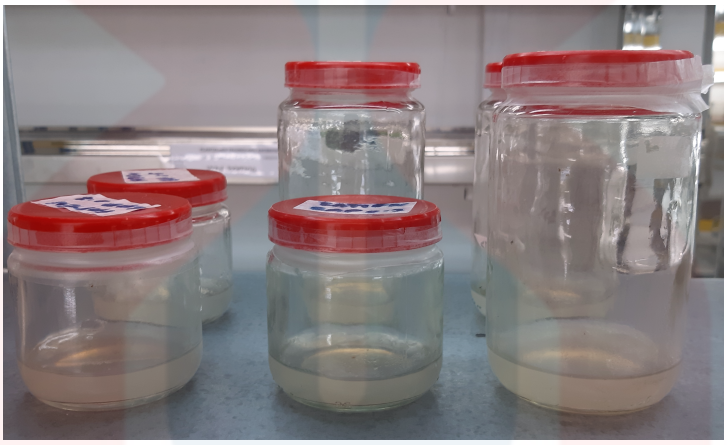

Figure 4.4 A clean explant was produced when the seeds are treated with 95% of ethanol for 20 minutes and 15 % of clorox for 3 minutes that have been survived for 2 months.

4.1.2 Surface Sterilization and Aseptic Establishment of *Etlingera megalocheilos* using Shalini *et al.* (2018) Protocol.

This experiment was conducted by adding additional steps of flaming the rhizomes for 4 seconds after dipping it in 95% of ethanol for 60 seconds. However, the growth of bacteria and fungal were observed after one week of culturing. The result shown can be concluded that flaming over heat does not reduce or suppress bacterial contamination. Anish *et al.* (2008) found that multilevel decontamination

procedures were beneficial to improve disinfection of underground origin explants. However, the explant are not considered as clean and survive explant due to contamination occur after one weeks of inoculation as shown in Figure 4.5. The immersion of instruments in ethanol with the concentration of 70% or higher and followed by flaming is a frequent sterilization protocol employed by many tissue culture laboratories (Wetherell 1982; Singha *et al.* 1987). It is to kill the bacteria inhibiting the instruments to prevent the widespread of contaminants throughout the process. This concept can be applied to kill the rhizome-inhibiting microorganism by flaming over the heat. Wistreich & Lechtman (1988) and Nester *et al.* (1995) state that the bacterial endospores are tough to hydrate and they require a protracted exposure. So additional step of sterilizing (flaming) could lessen the contaminants populations on the explant surface. Ethanol is comparatively less effective as a sterilizing agent. Therefore, the addition of water will increase the alcohol wetability with the maximal disinfection activity ranging 70–80% v/v (Wistreich & Lechtman 1988; Presscott *et al.* 1990; Nester *et al.* 1995).

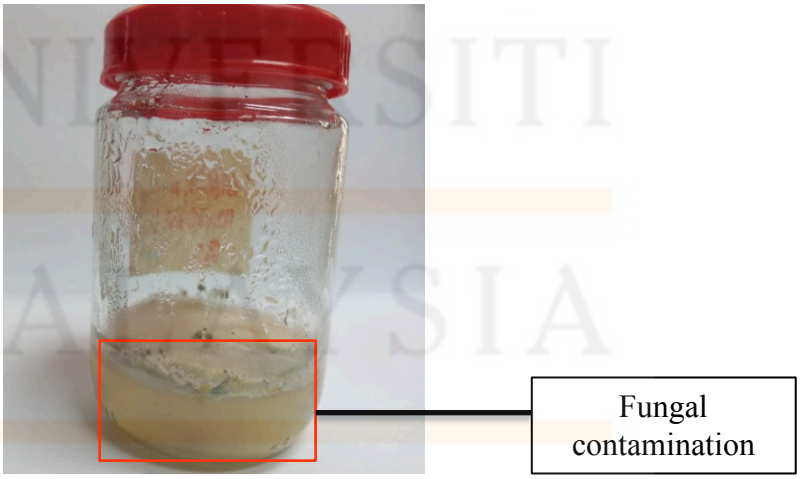

Figure 4.5 The dense growth of fungal on the explant surface and the surface of MS basal media after one week of inoculations when using dipping and flaming method.

4.1.3 Surface Sterilization and Initiation of *In Vitro* Culture of *Etlingera megalocheilos* using Iliev *et al.* (2010) Protocol

Wetting agents such as Tween 20 added to the sterilant can improve surface contact with the tissue (Guma *et al.*, 2013). Tween 20 were added to remove adhering particles of living and non-living (Sheena & Jothi, 2015). It enhances the disinfectant competent by breaking the surface tension between the plant tissue and water (Ndakidemi *et al.*, 2013). The combination of three sterilization agent which is 70% of ethanol, 5% of NaCIO and 20 drops of Tween 20 was to improve the effectiveness of sterilization to remove contaminants. Unfortunately, after 2 weeks of culturing, there was a few colonies build up on the explant surface. Although the surface is effectively sterilized, it is very challenging to discard the contaminants that are located inside the explants tissue that might show up at a posterior stage. According to Daud *et al.* (2012) and Gunson & Spencer-Phillips (1994), the endophytic contaminants may located within the inner tissue of the plant at the cortical parenchyma intercellular spaces and at the junctions of cell. When the explant were excised, the epiphytic bacteria may come through the plant structures where the sterilants cannot reach (Gunson and Spencer-Phillips, 1994; Leifert *el at.*, 1994).

This method is similar to research studies conducted by Abdelmageed *et al.* (2011) on *Etlingera eliator* but with contradictory concentration, contrary time frame and different techniques. It sterilized the explants with 60% of Clorox with 6 to 7 drops of Tween 20 for 30 minutes and subsequently washed with distilled water. The protocol does not end here, where they dissected the outer layer of leaf sheath of explants and undergone further sterilization with 20% Clorox added with 6 to 7 drops of Tween 20 and finally the explants were dissected again to remove outer layer of

leaf sheath before inoculating into the basal media. The protocol has successfully produces many clean and survive plantlets compared to by using Iliev *et al.* (2010). This shows that this protocol are doing a deep cleansing of the explant surface to remove the traces of contaminants or rhizome-inhibiting microorganism that might not successfully kill by the previous sterilizing agent of the outer surface by removing the outer layer even though it has been sanitized. While Iliev *et al.* (2010) protocol only cleansed once to complete the whole sterilization process. The difference between the step by Abdelmageed *et al.* (2011) and Iliev *et al.* (2010) is the removal of outer layer of leaf sheath and the repetition of sterilization. Abdelmageed *et al.* (2011) shows the rationality of doing so because explant from underground origin carry large amount of contaminants, therefore a removal of outer layer and multilevel sterilization will reduce the survival of contaminants. The removal of outer layer were also done by Kinabalu (2015) on *Etlingera coccinea* where the outer scales of the buds were trimmed to remove dead tissues affected by chlorine from the sprout.

A discerning stage of the plants introduction into tissue culture is to achieve a contamination-free cultures (Daud *et al.*, 2012). Microbial contamination is a continous issues that often compromise the progress of an *in vitro* techniques (Webster *et al.*, 2003; Ogunsanwo, 2007). The microbes encounter adversely with plant tissue for nutrients, and their existence result in variable growth and increasing mortality of cultures (Oyebanji *et al.*, 2009). This can be seen by clearly observed the visible appearance of the discrete colonies in the cultured medium. Fungal contamination was observed as hyphal growth from the explants and bacterial contamination was identified by observing colonies (Daud *et al.*, 2012). The types of contaminants are hardly identified but the sources of contamination can be identified. According to Cassells (1991) contamination can originate from two sources, either

through readily exist from the sample or from the faulty procedure and unaseptic surrounding and equipment.

Contaminant may be introduced with the explant (Leifert & Cassells, 2001). This may be due to the rhizotamous-inhabiting microorganisms, surface sterilisation-resistant microorganisms or endophytic microorganisms that may invade the plant tissue through wounds, natural openings and others. Furthermore, obligate and facultative pathogens might colonise plants similarly, be vector assisted or possess host plant penetration mechanisms (Tarr, 1972; Matthews, 1981; Cassells, 1991). Thus, plant may establish endophytic 'floras' of variable species that consist of prokaryotes (bacterial and bacteria-like agents), viruses, viroids, and fungi. Even though the surface is adequately sanitized, the contaminants may emerged from the inner tissues (Daud *et al.*, 2012) and spread outside the tissue when it is inoculated into the MS media.

Other than that, faulty procedures in the laboratory such as preparation of media in unaseptic condition, non-sterilised equipment and unaseptic techniques. The wrong technique might end up allowing contamination to occur. Microbes can be reintroduced from poor aseptic handling, unhygienic conditions in the laboratory or from laboratory instruments (Daud *et al.*, 2012). The possibility of spread of heat‐resistant *Bacillus* spores through alcohol‐flamed tools has been documented in a few studies (Boxus & Terzi 1987; Singha *et al.* 1987) but not studied in detail.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

As a conclusion, a few sterilization protocols were conducted to produce a clean and survive growing plantlets via *in vitro* propagation approach. There are 4 protocols that were carried out for propagation using rhizome buds and seeds of *E. megalocheilos*. Among all the protocols, there were no protocols that produce a 100% clean and survive from contaminations issues. Out of all protocols, the most efficient protocol were the treatment of 95% of ethanol for 20 minutes and 15% of clorox for 3 minutes, where the explants types used is seed. The usage of this protocol on seeds produces the longest survived explants for 2 months. Concentration, exposure of time of sterilants and types of explants used are directly influence the growth of *in vitro* explants. When the concentration of sterilants decreases with a longer duration of exposure, the level of contaminants of explants seems to be lower. Suitable concentration of sterilants, plant growth regulator, and disinfectant with suitable exposure of time is crucial to sanitize the explants.

5.2 Recommendation

The prior situation for the accomplishment of *in vitro* propagation is asepsis (Badoni & Chauhan, 2010; Ndakidemi *et al.*, (2013). The best approach to manage contamination issues is to establish aseptic cultures and to retain good laboratory practice (Cassells, 2000) to create aseptic *in vitro* culturing. Odutayo *et al.* (2007) found that fungal contaminations were claimed to accomplice with indoor air, walls or tables and human skin. It is vital to preserve the air, surface and floor are dust free and all operations need to be operate in laminar flow sterile cabinet (Chawla, 2003). To maintain an asepsis environment, all media, culture vessels, instruments used and explant must be sterilized (Badoni & Chauhan, 2010). To get a results that are free from contamination problem, anything that may have the opportunity to make an explicit connection with the plantlet or culture medium has to be sanitized and making sure that there will be no explicit link between the culture and the non-sterile environment. Therefore, practicing a sterile environment and apparatus condition is vital to prevent contamination problem. Maintaining an aseptic situation is a perquisite for fruitful *in vitro* seed germination and proliferation (Sen *et al.*, 2013). Buckley *et al.* (1995) recommended that the poor aseptic procedure or inappropriately sterilized equipment can be amended with training.

Furthermore, it is important to determine the most effective concentration of sterilizing agent, the time frame of exposing the explant to the sterilizing agent, the array of using it has adverse effect, so sterilizing agent need to be standardized to downplay explants injury and achieve greater survival (Çölgeçen *et al.*, 2011). Nonetheless, the concentration and the time frame of exposure of the sterilants used in environment are not effective because contamination occurred after one to two weeks

of culturing. It might be due to the endophytic contaminants located within the plant tissue. Eliminating internal contaminants is more problematic (Buckley *et al.*, 1995; Reed & Tanprasert, 1995). Endopyhtic bacterial contaminations require antibiotic therapy (Mathias *et al.*, 1987). An analysis made by a published researched consummate that antibiotics are often integrated as prophylactics in the tissue culture medium or are used to repress or terminate bacteria (Leifert *et al.*, 1992; Reed & Tanprasert, 1995).

Last but not least, the usage of mercuric chloride to sterilize the explants has been highly raved and recommended by many researchers in sterilizing explant for *in vitro* culturing due to the effective result that have been shown. However, the use of mercuric chloride has been banded in Universiti Malaysia Kelantan due to the safety purposes of the students.

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APPENDIX A

FYP 1	
January 2018	Title Selection
February 2018 – March 2018	Proposal writing
April 2018	Draft submission
April 25, 2018	Proposal defence
FYP 2	
July 2018 – December 2018	Conducting lab work
October 2018 – December 2018	Writing thesis
December 2018	Final thesis draft submission and presentation
January 2018	Final thesis submission

APPENDIX B

CHEMICAL LIST

STOCK A (MACRONUTRIENTS)

Ammonium nitrate	NH4NO3
Potassium nitrate	KNO3
Calcium chloride dihydrate	CaCl2 2H2O
Magnesium sulphate heptahydrate	MgSO4 7H2O
Potassium dihydrogen phosphate	KH2PO4

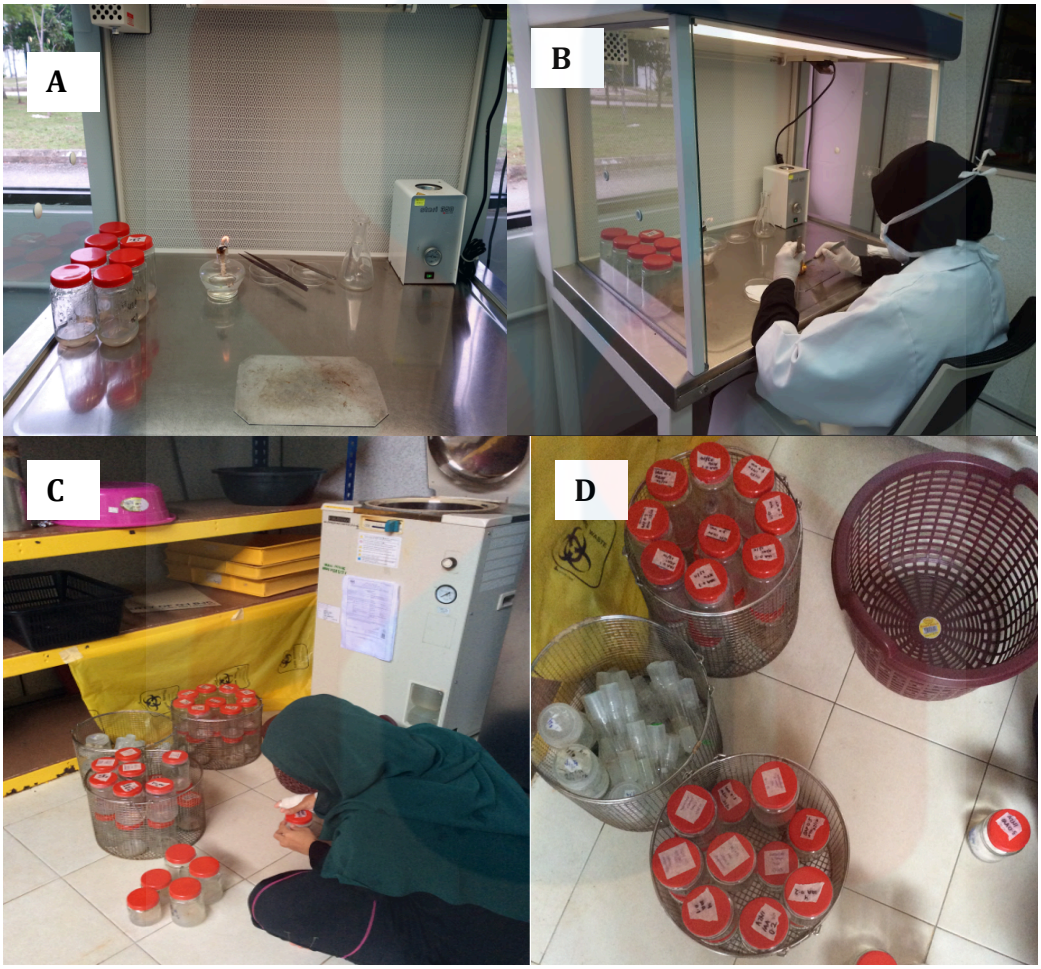
STOCK C (MICRONUTRIENTS)

Sodium molybdate dihydrate	Na2MoO4 2H2O
Copper sulphate pentahydrate	CuSO4 5H2O
Cobalt chloride hexahydrate	CoCl2 6H2O
Manganese sulphate	MnSO4 4H20
Zinc sulphate heptahydrate	ZnSO4 7H20
Boric acid	H3BO3
Potassium iodide	KI

VITAMIN

Thiamine hydrochloride	Vitamin Bl hydrochloride
Glycerine	Amino acetic acid
Nicotinic acid	Niacin, pyridine – 3 – carboxcylic acid
Pyridoxine acid	Vitamin B6 hydrochloride
MS Iron	C10H12FeN2NaO8.aq
Myo – inositol	$C_6H_{12}O_6$
6-Benzylaminopurine, benzyl adenine (BAP)	$C_{12}H_{11}N_5$
3-indoleacetic acid (IAA)	$C_{10}H_9NO_2$
2-4-dichlorophenoxyacetic acid (2,4-D)	$C_8H_6Cl_2O_3$
Sucrose	$C_{12}H_{22}O_{11}$
Sodium hydroxide	NaOH
Potassium hydroxide	KOH
Plant agar	

APPENDIX C



(A) Preparation for arrangement in the laminar airflow cabinet. **(B)** Inoculation process. **(C)** Arranging contaminated culture bottle into the autoclaved basket. **(D)** The contaminated culture bottle are ready to be autoclaved.