Effects of Plant Growth Regulators on Callus Induction of *Clitorea ternatea*

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THESIS DECLARATION

I admit this report is fully on my own originality except for the literature review that had been cited.

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LIST OF ABBREVATION

	Meaning
PGR	Plant Growth Regulator
TCL	Thin Cell Layer
2,4-D	2,4-Dichlorophenoxyacetic acid
KN	Kinetin
NAA	Naphthalene Acetic Acid
IBA	Indole-3-butyric Acid
BA	6-Benzylaldenine
tTCL	Transversally TCL
lTCL	Longitudinally TCL
IAA	Indole-3-acetic acid
MS	Murashige and Skoog
HCl	Hydro Chloric acid
NaOH	Sodium Hydroxide

Kesan Pengawal Pertumbuhan Tanaman Terhadap Induksi Kalus *Clitorea ternatea*

ABSTRAK

Clitoria ternatea adalah tumbuhan herba yang mempunyai nilai perubatan yang berharga. Ia mempunyai ciri-ciri yang mampu yang menyembuhkan penyakit dan tumbuhan ini juga menunjukkan potensi untuk dieksploitasi secara berlebihan disebabkan oleh perubahan gaya hidup dan penjagaan kesihatan masyarakat. *C. ternatea* biasa digunakan dalam perubatan tradisional dan juga perubatan moden. Dalam projek ini, terdapat dua Pengawal Pertumbuhan Tanaman (PPT) yang berbeza iaitu 6-benzylaminopurine (BAP) dan Indole-3-acetic acid (IAA) pada beberapa kepekatan dengan padanan yang berbeza. Eksperimen ini mengaplikasikan kaedah Lapisan Sel Nipis (LSN) iaitu melintang, membujur dan persegi $1cm^2$ sebagai pembolehubah untuk induksi kalus. Rawatan induksi kalus yang terbaik dalam eksperimen ini adalah 2 mg/L BAP + 0.1 mg/L IAA dengan nilai 56.95 mg dan kaedah LSN terbaik digunakan ialah cara melintang dengan nilai 18.52mg. Oleh itu tumbuhan ini mempunyai potensi untuk menghasilkan metabolik sekunder melalui kaedah kultur tisu.

Kata kunci: Kalus, *Clitoria ternatea*, Lapisan Sel Nipis, Pengawal Pertumbuhan Tanaman

Effects of Plant Growth Regulators on Callus Induction of *Clitorea ternatea*

ABSTRACT

Clitoria ternatea is an herb plant that have valuable medicinal value. The properties of the plant that able to cure diseases shows the potential of this plant being over exploit due to the changing trend in life style and health. *C. ternatea* commonly used in traditional medicine and had also been used in modern treatment. In this project, the used of the two different Plant Growth Regulator (PGR) 6-benzylaminopurine (BAP) and Indole-3-acetic acid (IAA) at several different combination concentration together with the application of thin cell layer (TCL) method transverse TCL (tTCL), longitudinal TCL (lTCL) and Square 1cm^2 are applied as the variable to bring out the callus. The best callus induction treatment in this experiment is 2 mg/L BAP + 0.1 mg/L IAA with value 56.95 mg and the best TCL method used is tTCL with value 18.52 mg. Thus, this plant have a potential to produce secondary metabolite through tissue culture method.

Keyword: Callus, *Clitoria ternatea*, Thin Cell Layer, Plant Growth Regulator

CHAPTER 1

INTRODUCTION

1.1 Research Background

Asian pigeon wings or *Clitoria ternatea* is an herbal plant that usually found at Southern East Asia for example Malaysia, Indonesia and Thailand. As in Malaysia, it can be found at East Coast of Malaysia. This plant is a wild type but some people likes to plant it as for aesthetic value to their garden. It commonly use as natural food coloring as it give the shade blue or violet. However, this plant is actually has other benefits from any part of the plant that is not just as a food coloring but also can cure certain disease (Chauhan, Rajvaidhya, & Dubey, 2012). Looking forward to future, the use of traditional method in healing using Asian pigeon wings plant might be commercialize as the alternative way to treat illness. As for now it's already use as a tea and supplement.

Medically, this plant is not well known for its herbaceous function which is environmentally good because *C. ternatea* is not undergo extinction unlike the other extinct plant. However, the idea of protecting this species is due to the future trend that is developing as the human populations are more concern for health and would

like to rely on natural resources. Based on a report about dietary supplement market that include botanical ingredients, the market is globally at USD 132.8 billion in 2016 and it is expected to increase about 8.8% by 2022 (Zion Market Research, 2017).

Based on the previous research, most were done to propagate *C. ternatea* to replenish the species as the process of extraction of the plant need a huge amount of the whole plant to produce a small amount extracted molecule. Commonly extracted compound is the blue dye coloration of the flower and the other part of the plant as it contain medicinal benefits. Propagation of plant using field planting practice is time consuming and might not suitable for certain climate. Plus, field planting could expose the plant to viruses and diseases which would cause foliar diseases that will affect the production of secondary metabolite (Somashekhara Achar & Shivanna, 2013). However, callus induction would be one of the good ways to propagate since it is an *in vitro* methods that prevent outside diseases with intensive care.

1.2 Problem Statement

In order to overcome the potential over exploitation of *C. ternatea* based on the statistical expectation by Zion Market Reasearch, the method of *in vitro* callus induction is chosen to increase the production of plant. By this method also, it help in further study to produce secondary metabolite from *C. ternatea*. Thus, tissue culture can be used to extract compound from the callus tissue instead of taking the whole plant cultivation.

1.3 Hypothesis

H_0 : The different type of plant growth regulator would not affect the callus growth of *C. ternatea*

H_1: The different type of plant growth regulator would affect the callus growth of *C. ternatea.*

1.4 Scope of Study

There are a lot of plants that have organic substance which available for extraction in an appropriate quantities. This organic substance includes plant chemical compound that are useful in various field for example pharmaceutical field. Plant with pharmaceutical value often being a subject of study by researcher. These plant chemical are classified into primary and secondary metabolite.

In plant tissue culture, callus induction is a method of developing undifferentiated cell, callus or tumors due to the stress responses like wounding (Ikeuchi, Sugimoto, & Iwase, 2013). It also a method that derive callus from plant tissues such as leaves, stems and seed. Callus induction often related to the secondary metabolite production. In callus induction method, there was a theory that undifferentiated cell cannot produce secondary compound differ from differentiated cell or specialized organ which can produce secondary compound (Krikorian & Steward, 1969; Bourgaud, Gravot, Milesi, & Gontier, 2001). However, this theory was proved mistaken when an experiment conducted by Zenk

and co-worker that happened to observed dedifferentiated cell of *Morinda citrifolia* producing about 2.5g of anthraquinones (Zenk, 1991; Bourgaud et. al, 2001).

1.5 Significance of Study

The extraction of *C. ternatea* to produce herbal supplement or as other purpose can bring an issue of potential over-exploitation. Furthermore, Malaysian is unaware about the goodness of this herb and often mistreat this species as other wild flowering plant while manufacturer keep on extracting. According to the herbal medicine trend, *C. ternatea* could increase in demand especially among manufacturers in order to extract the flavonoids out of the plant parts. Thus, callus induction help to protect this species from being over-exploit in the future.

1.6 Objectives

The objectives of the study are :

a) To identify plant growth regulator (PGR) that able to produce denser callus production on *C. ternatea.*

b) To identify method of Thin Cell Layer (TCL) that able to produce denser callus production on *C. ternatea*.

CHAPTER 2

LITERATURE REVIEW

2.1 *Clitoria ternatea*

Clitoria ternatea (Figure 2.1) is originally a wild herbal plant in which is very useful to many industries.

Figure 2.1 *Clitoria ternatea* flower

Traditionally in Malaysia, this plant is often use as natural food colouring in many traditional dishes. The natural colour are extract by soak the blue flower

petal in an amount of boiling water. The flower petal is said to be a natural food colour that are free from toxic disorder and carsinogenic that cause cancer (Praja, 2015). On top of that, other parts of *C. ternatea* are also been used in traditional medicinal method. According to Mukherjee et al. (2008), Cubans would mixed a handful of cleaned roots in a bottle of water to encourage menstruation and induce uterine contraction. Interestingly, the flower gives the same effect against the problem. The plant is a legume and a climbing plant that has short and soft hair at the stem of the plant. It has ornamental flowers that are pollinated by insects. The shape of flower is the funnel shape which around 4 cm by 3 cm. The leaves are alternately arrange on the stalk and the leaflets is thin. The fruits are the liner-oblong pods which usually 5-11 cm long (National Parks, 2013).

2.2 Medicinal Herb

Human being relied a lot on herbs due to its healing function and some people value it due to the belief that plants are created as for food, healing sources and others. World Health Organization estimated in less developed countries, approximately 80% of population depends on traditional method of healing rather than modern method since medicinal plants are the strength of traditional medicine (Davidson-Hunt, 2000; Ahvazi et al., 2012). Thus medicinal herbs are synonym with traditional medicinal method. One of the most famous traditional medicine is known as traditional Chinese medicine which show the historical growth of medical into modern days (Wachtel-Galor & Benzie, 2011).

In modern days herb used as a treatment to chronic and critical diseases. For example in 2003, China, in order to treat severe acute respiratory syndrome (SARS), the traditional Chinese medicine take a big part as a strategy to contain the disease (De Smet, 2005; Tilburt & Kaptchuk, 2008; Wachtel-Galor & Benzie, 2011). In the other hand, with all the technologies existed, herbal plant function can be extracted out and processed into essential oil, ointment, capsules, tablets, and more and this happened because the various of compound stored in the plant that valuable for it to be called medicine (Wachtel-Galor & Benzie, 2011).

2.3 *In vitro* Method of *Clitoria ternatea*

In previous research of *C. ternatea*, there are numbers of *in vitro* method that successfully propagate this species. Pandeya et.al. (2010), micro-propagated *C. ternatea* from the nodal, shoot tip and cotyledonary node of explant. Other than that, there are also a research on *in vitro* propagation of *C.ternatea* for identification of antibacterial activity, but the method shows the best two PGRs that initiate maximum callus production that are 1.0 mg/L of 2,4-Dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/L of Kinetin (KN) while, achieving callus differentiation by adding 2,4-D (0.3 mg/L) and KN (0.3 mg/L) (Arumugam & Panneerselvam, 2012). Thus, this mean not only different type of PGRs but different concentration also gives different result of callus production.

Callus are usually apply to produce virus-free plantlet that will benefit grower in controlling viral diseases. It also apply for the production of useful secondary metabolite as proved by Zenk (1991) as in the journal of Bourgaud et

al.(2001) which are not produce by the parent plants. Thus, this also help in the selection of line that have precious plant properties for example the resistance towards disease, the attraction of pollinator and more. Moreover, it helps in vary of studies such as biotransformation and mutagenic studies

2.4 Plant Growth Regulator (PGR) Used in Callus Induction

Plant growth regulator is a substance that is either natural or synthetic, that regulate the growth of the plant which are small molecule derived from many kind of important metabolic pathways (Santner, Calderon-Villalobos, & Estelle, 2009). From the previous researches, most common PGR use for callus induction are 2,4-Dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), kinetin (KN), 6-benzylaldenine (BA) and indole-3-butyric acid (IBA). As stated by Arumugam and Panneerselvam (2012), a test is done on *C. ternatea* from different plant parts which were node, leaf and petiole with same two PGR but different concentration showed a slight different in the response of the culture. This shows that it is not necessarily the same PGR would produce the same outcome as ever tested on different plant parts or other variable. Thus, in this study the effects of plant growth regulator on callus induction of *C. ternatea* should probably having the same response as in the previous research with the other two PGR.

2.5 Callus Induction in Other Plant

Callus induction are widely applied on medicinal plant as to produce secondary metabolite. Thus, there are a lot application of callus induction in other medicinal plant.

2.5.1 Callus Induction of Corm of *Gloriosa superba* Linn.

Gloriosa superba Linn. is an endangered medicinal plant species that is one of seven Upanishads in the Indian medicine and the *in vitro* culture has successfully rapid the propagation of the plant (Singh, Mishra, & Yadav, 2012). The micro propagation allow the valuable metabolite to be collected in larger amount due to the rapid propagation of selected genotype (George, Hall, & Klerk, 2008; Singh et al., 2012). 1 mg/L of IAA + 2.5 mg/L of BAP + 0.5 mg/L KN shows the best outcome of callus growth from the corm. This shows, the *in vitro* technique could save the endangered plant species due to the rapid multiplication process at the same time producing larger amount of useful metabolite.

2.5.2 Callus Induction of *Lantana camara* L.

A medicinal plant that have bioactive compound and the production of callus of this research, could be used for extraction of bioactive ingredient (Veraplakorn, 2016). The experiment began with the preparation of single shoot tip that was cultured in MS medium with combination of PGR of NAA and BA with three different concentration. The result shows positive outcome in the media with

NAA and BA but not in media without NAA but only BA (Veraplakorn, 2016). Also the finding shows that different size of callus found in different combination of NAA and BA concentration (Veraplakorn, 2016). This show that different combination of PGR with different concentration would affect the outcome in various aspect.

2.5.3 Callus Induction of *Ephedra*

The research is about callus induction and the shoot regeneration from the nodal explant. In last few decades, this medicinal plant species undergo extinction (Ignacimuthu, Ayyanar, & Sivaraman K., 2006; Mamta et. al., 2011). The nodal explant are culture in MS medium with NAA, BAP, KN, IBA and 2,4-D either in combination or alone (Mamta et al., 2011). The outcome shows that the different combination are differ in callus response.

2.6 Thin Cell Layer (TCL) Technique

TCL technique were proposed about 30 years ago by introducing method of cutting out explant into small size from different part of the plant such as leaves, roots, stems, the flower parts, cotyledons, epical zone or embryo. The explant can be cut into either transversally (tTCL) or longitudinally (lTCL) (Teixeira, 2003). TCL are mostly committed on smaller size plants that are derived from a restricted number of cell of homogenous tissue (Nhut, Huy, Chien, Luan, Vinh, Thao, 2012). Currently, the TCL culturing method of different explant has appear to be beneficial tool in study area of cellular, molecular mechanisms on controlling *in vitro*

morphogenesis of plants and bio and biochemical (Nhut, Hai, Don, Silva, & Van, 2006). The study of *in vitro* propagation in *Brasilidium forbesii* with the use of TCL technique shows that the lTCL method was more efficient for protocorm-like bodies (PLBs) and the shoot regeneration than the tTCL technique (Lucas Roberto, Cristina do Rosário, & Luciana, 2015). Next, the study on the Vietnamese ginseng showed that various morphogenesis pattern that could be induced when the petiole lTCL of the explant were cultured on supplemented media with PGRs either separately or in combination, in darkness and under light. The various morphogenesis pattern of the culture such as shoot, root, callus and somatic embryo (Nhut, et al., 2012). These studies show that the outcome of the callus are possible in use of technique of TCL.

CHAPTER 3

MATERIALS AND METHOD

3.1 Plant Materials

This research was used *C. ternatea* that were propagate *in vitro* in the laboratory of Plant Tissue Culture of Universiti Malaysia Kelantan, Jeli Campus. The seeds were collected at nearby Tunnel Garden of University Malaysia Kelantan, Jeli Campus.

3.2 Apparatus and Materials

Experiment were done at Plant Tissue Culture (PTC) Laboratory in Universiti Malaysia Kelantan, Jeli Campus. All equipment involved were apparatus that include in preparation of stock such as jars, measuring cylinder, magnetic stirrer and other apparatus that involved direct or indirectly while preparing the solution. It also included microwave oven, autoclave, refrigerator, and pH meter. This experiment were carried out with full strength Murashige and Skoog (MS)

stock solution and PGR that were Indole-3-acetic acid and 6-benzylaminopurine (BAP).

3.3 Stock Preparation

3.3.1 Preparation of MS Stock Solution

MS Stock solution will be prepared as it frequently used in tissue culture and callus culture. The MS solution must include all the element below before mixing them together:

Table 3.1: Ingredient of MS stock media

Macronutrient	Micronutrient	Iron	Vitamin
NH_4NO_3	$MnSO_4.4H_2O$	$FeSO_4.7H_2O$	Myo-inositol
KNO_3	$ZnSO_4.7H_2O$	$Na_2EDTA.2H_2O$	Glycine
$CaCl_2.2H_2O$	H_3BO_3		Thiamine-HCL
$MgSO_4.7H_2O$	KI		Nicotinic acid
KH_2PO_4	$NaMoO_4.2H_2O$		Pyridoxine-HCL
	$CuSO_4.5H_2O$		
	$CoCl_2.6H_2O$		

All chemicals required were prepared for 500 mL of MS stock medium for callus induction of *C. ternatea*.

3.3.2 Preparation of Macronutrient Stock

All five chemicals were required for the 500 mL stock preparation. Those chemical were weight according to calculation. Required amount are as shown in Table 3.2 below:

Table 3.2: Weight of chemical for macronutrient stock for concentration (20X)

Chemical	1X (g/L)	20X (g/500 mL)
Ammonium nitrate, NH_4NO_3	1.65	16.5
Potassium nitrate, KNO_3	1.90	19.0
Calcium chloride, $CaCl_2.2H_2O$	0.44	4.4
Magnesium sulphate, $MgSO_4.7H_2O$	0.37	3.7
Potassium dihydrogen phosphate, KH_2PO_4	0.17	1.7

Each chemical were weight referring to the worksheet of MS medium stock preparation on the weighing machine with a piece of aluminium foil and spatula. The weighed chemical were dissolved with an amount distilled water in 500 ml beaker with the help of magnetic stirrer. Once the chemical was solubilize, the solution were made up to the final volume. The prepared stock solution was labelled and stored at 4 degree Celcius.

3.3.3 Preparation of Micronutrient Stock

All seven chemical are required for the 500 mL stock preparation. Those chemical are weight according to calculation. Require amount are as shown in Table 3.3 below:

Table 3.3: Weight of chemical for micronutrient stock for concentration (200X)

Chemical	1X (g/L)	200X (g/500 mL)
Manganese sulphate, $MnSO_4.4H_2O$	0.02230	2.33
Zinc sulphate, $ZnSO_4.7H_2O$	0.00860	0.86
Boric acid, H_3BO_3	0.00620	0.62
Potassium iodide, KI	0.00083	0.083
Sodium molybate, $NaMoO_4.2H_2O$	0.00025	0.025
Copper sulphate, $CuSO_4.5H_2O$	0.000025	0.0025
Cobalt chloride, $CoCl_2.6H_2O$	0.000025	0.0025

Each chemical must be weight referring to the worksheet of MS medium stock preparation on the weighing machine with a piece of aluminium foil and spatula. The weighed chemical must be dissolve with an amount distilled water in 500 ml beaker with the help of magnetic stirrer. After all chemical is solubilize, the solution made up to the final volume. The prepared stock solution is label and stored at 4 degree Celcius.

3.3.4 Preparation of Iron Stock

Table 3.4 below is the list of chemical that needed for iron source preparation in 500 mL for 200X concentration.

Table 3.4: Weight of chemical for iron source for concentration (200X)

Chemical	1X (g/L)	200X (g/500 mL)
Iron (II) sulfate heptahydrate, $FeSO_4.7H_2O$	0.0278	2.78
Disodium ethylenediaminetetraacetate dihydrate, $Na_2EDTA.2H_2O$	0.0373	3.73

In a volumetric flask, $FeSO_4.7H_2O$ and $Na_2EDTA.2H_2O$ were dissolved by using distilled water. When the chemical solubilize, the solution were mix together and made up to the final volume, 500 mL. The prepared stock solution was label and stored at 4 degree Celcius.

3.3.5 Preparation of Vitamin Solution

The 0.01g of thiamine was dissolved in hydrochloric acid (HCL). Distilled water were added up to 100 ml in volumetric flask. The solution were dissolved with 0.2g of glycine, 0.5g of nicotinic acid, and 0.05g of Pyridoxine acid. When the

chemical solubilize, the solution was made up to the final volume, 1 litre. The prepared stock solution was label and stored at 4 degree Celcius.

3.3.6 Preparation and Preservation of PGR

a. Preparation of 1mg/ml PGR for Indole-3-acetic acid (IAA), 100mg of IAA was weight accurately and slowly dissolve in 1N NaOH and heat gently if required. When it dissolve, dilute it with 100ml of distilled water in measuring flask. The prepared stock solution is label and stored at 4 degree Celcius.

b. Preparation of 1.0 mg/ml of 6-benzylaminopurine (BAP), 100 mg of BAP was precisely weight and dissolve in 10-20ml of warm double distilled water and add few drops of 1N NaOH. The solution was shaken and made up to 100ml, label and stored.

The prepared PGR stock solution were stored in refrigerator to avoid deterioration of light and temperature

3.3 Preparation of MS Medium

3.4.1 Full Strength of Murashige and Skoog (MS) Medium for Callus Induction

About 200ml of distilled water were added to 1 litre jar. The magnetic stirrer were placed in the jar and 50 ml of macronutrient, 10 ml of micronutrient, 2 ml of vitamin, and 5 ml of iron were added in to the water one at a time. 30 g/L of sucrose were dissolve in the solution. Then, the pH of the solution needed to be around 5.7-5.8. The water level were added up to 1 litre with distilled water. 8g/L of agar were added and dissolved. The solution were heated up in the microwave until agar is fully dissolve. The dissolved solution were poured into the culture jars and autoclaved at 121 degrees of Celcius for 15 minutes.

3.4.2 Preparation of Plant Material

The seeds were collected from the Universiti Malaysia Kelantan, Jeli Campus. The seed were grown *in vitro* in the laboratory of Plant Tissue Culture, University Malaysia Kelantan, Jeli Campus. The seed collected must be dried enough to obtain the seed as in Figure 3.1

Figure 3.1 seed pods of *Clitoria ternatea*

3.4.3 Preparation of Leaves Part for Callus Induction

The sterile young explant were ready for callus induction from the leaves part. The young explant can be directly use for culture. The leaf part were taken and the Thin Cell Layer technique were applied. The leaves were cut into strips of 0.2 mm with the help of graph paper vertically and horizontally. Others, the leaves were cut in square shape of 1.0 cm^2.

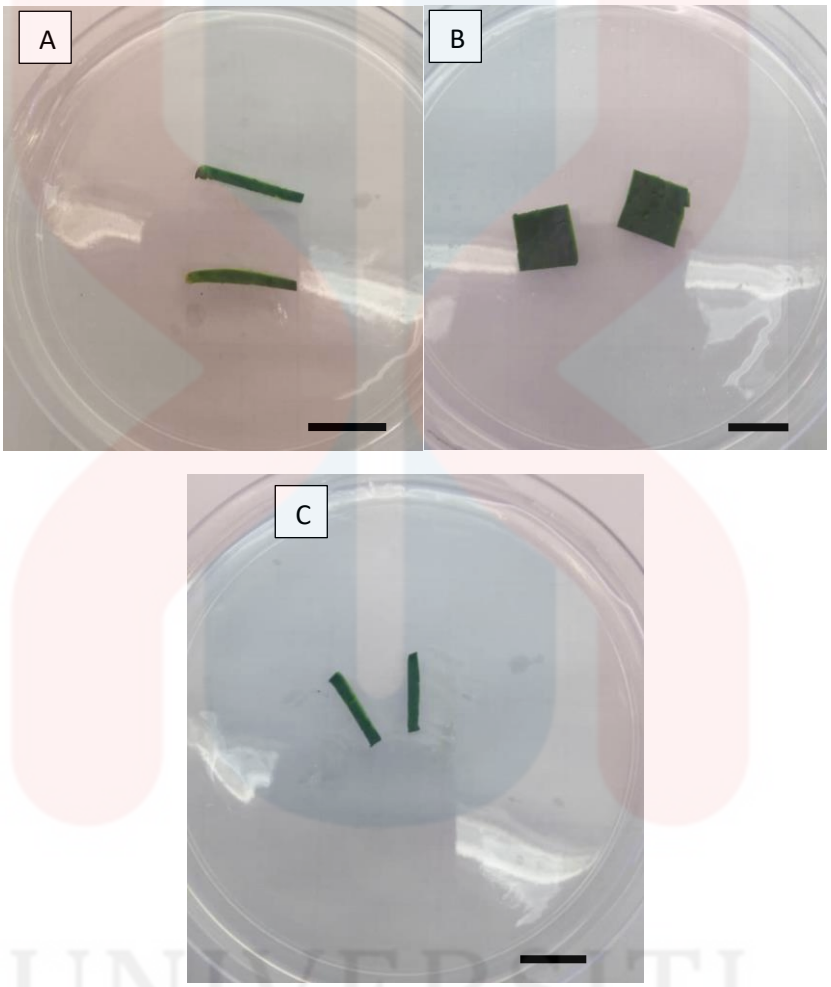

Figure 3.2 Thin Cell Layer technique A = The vertical cut of leaf, lTCL (0.2 cm width),B = The 1 cm^2 square cut of leaf, C = The horizontal cut of leaf, tTCL (0.2 cm width).

1 Bar = 1 cm

3.4.4 Callus Induction of *Clitoria ternatea*

Leaves explant were cut into preferable cutting and placed on the media of each treatment. While placing the leaves part, the dorsal surface must had contact with the medium with 9 different treatment (Table 3.4). After that, the culture were placed into sterile room with suitable light intensity and temperature.

Table 3.5 Treatment of PGRs with concentration

Treatments	**BAP (mg/L)**	**IAA (mg/L)**
Treatment 1	0	0
Treatment 2	0	0.1
Treatment 3	0	0.2
Treatment 4	1	0
Treatment 5	1	0.1
Treatment 6	1	0.2
Treatment 7	2	0
Treatment 8	2	0.1
Treatment 9	2	0.2

3.4 Data collection

The observation were done weekly and data based on the observation were recorded after four weeks for statistical analysis using software Statistical Package for the Social Science (SPSS). The data collected were analysed using the one way ANOVA procedure. The replication of the callus induction were two replication for each treatment with two sample on each replication. The weight were record at the time of subculture using the equation (1) below.

Fresh weight = Final weight of sample – initial weight of sample (1)

CHAPTER 4

RESULTS AND DISCUSSION

4.1 *In Vitro* Seed Germination

The *in vitro* germination of *C. ternatea* seed was started with the collection of the dried seed pod. The average number of seed in one seed pod were around three to four seeds. The total seeds used for the germination are 51 seeds and around three to four seeds per jar depends on the size of seeds. There were total of 15 jars of media used for germination with hormone of 1mg/L BAP + 0.5 mg/L KN. The data collected on the germination progress are the average number of the leaves per seed. Table 4.1 shows the collection on average number of leaves per seed.

Table 4.1 Average number of leaves of *C. ternatea*

Jars	Num. of seed per jar	Num. of germination after 2 weeks	Num. of germination after 4 weeks	Average Num. of leaves per seed after four weeks
1	3	2	3	13.67
2	3	2	3	16.00
3	3	2	3	11.33
4	3	3	con	0
5	3	3	3	11.67
6	3	1	3	10.00
7	3	2	3	9.33
8	3	1	con	0
9	3	1	con	0
10	4	1	4	10.25
11	4	2	4	3.50
12	4	2	4	8.50
13	4	2	4	6.50
14	4	1	4	7.50
15	4	3	4	8.00

*con=contaminated culture

The Table 4.1 above shows the number of germination of the seed and the average number of leaves per seed. The minimum number of leaves on the plants were 4 while the highest number of leaves was 18 leaves. The PGR that present in the media was kinetin which are cytokinin. Cytokinin is a class of hormone that initiate the growth of shoots. It stimulate the division of cell and in higher

concentration it help in adventitious shoot growth (Machado, Silva, & Biasi, 2011). The explant of the callus induction are leaves thus, the number of leaves per plant are important to proceed the experiment. However, the size of a leaf are smaller than expected makes it not suitable for explant since the targeted size of a leaf must exceed $1cm^2$. Most of the seeds were germinated while there were 3 jars that were contaminated that need to be discard immediately. There are 3 classes of contamination in cell culture. First is minor annoyance which the contamination lost happen only on several plates or jars. Second, the serious problem which the contamination lost increases or happens to the entire experiments. The third class is the major catastrophes which the discovered contaminant bring doubts to the past or current work (Ryan, 2008). However, in this experiment the contaminant are classify into minor annoyance. There are many factor that causes contamination such as the aseptic technique during culturing, the use of sterilized apparatus, the water used for making media or rinsing explant and many more.

Figure 4.1. Germinated seed. A = growth of explant after 4 weeks, B= growth of individual explant after 4 weeks, C= the measurement of an individual plant and cotyledon leaves.

1 bar= 1 cm

4.2 Subculture of explant

After four weeks, the germinated plant were subcultured into a new medium with the same concentration of hormone (1mg/L BAP + 0.5 mg/L KN) to enhance better growth of the plant and the leaves size. Every culture must be subculture after 4 weeks due to the nutrient deficiency in the medium. The data of the plant per jar are as below.

Table 4.2 Percentage of plant survive per jar after subculture

Jars	Number of seeds per jar	Percentage of plant survived (%)
1	5	100
2	4	100
3	5	100
4	4	100
5	4	100
6	4	0
7	5	0
8	5	0
9	5	0
10	5	0

The total of plant survived and able to be the explant for callus induction were 22 plants, Meanwhile, the other culture of the other jars had contaminated due to possible slacks in technique which causing minor annoyance. Minor

annoyance is the total number of contamination that happened on several culture jar or plates (Ryan, 2008). The leaf part were then used for callus induction.

4.3 Callus induction

On fourth week of callus induction, the percentage of the callus induction on every treatment of every method of TCL were taken.

Table 4.3 The percentage of the callus induction on 9 treatment of every method of tTCL

Treatment	Average of callus growth (%)
T1	0
T2	0
T3	0
T4	0
T5	100
T6	100
T7	0
T8	25
T9	25

On the TCL method of tTCL cut it showed that there were 100% growth of callus on the T5 and T6 which mean the callus induced on both strips on both jar. Meanwhile, on T8 and T9 the callus induce only on one strips of one jar which brought the average growth percentage to on 25%.

Table 4.4 The percentage of the callus induction on 9 treatment of every method of lTCL

Treatment	Average of callus growth %
T1	0
T2	25
T3	25
T4	0
T5	0
T6	25
T7	25
T8	100
T9	75

On the TCL method of vertical cut, T2, T3, T6, T7, T8 and T9 showed responds as the culture shows the callus growth. On average, 25% of callus growth are present at T2, T3, T6 and T7 while 100% and 75% on T8 and T9 respectively.

Table 4.5 The percentage of the callus induction on 9 treatment of every method of TCL square ($1cm^2$)

Treatment	Average of callus growth %
T1	0
T2	0
T3	50
T4	0
T5	50
T6	0
T7	25
T8	25
T9	0

Based on the table above, the callus induction on every treatment of the method of square $1cm^2$ were present on the T3, T5, T7 and T8. The callus induced in T3 and T5 were 50% as it only present on one sample of each jar. On the T7 & T8, callus were present only in one jar on one sample. Thus, it gave the average percentage of growth of callus 25%.

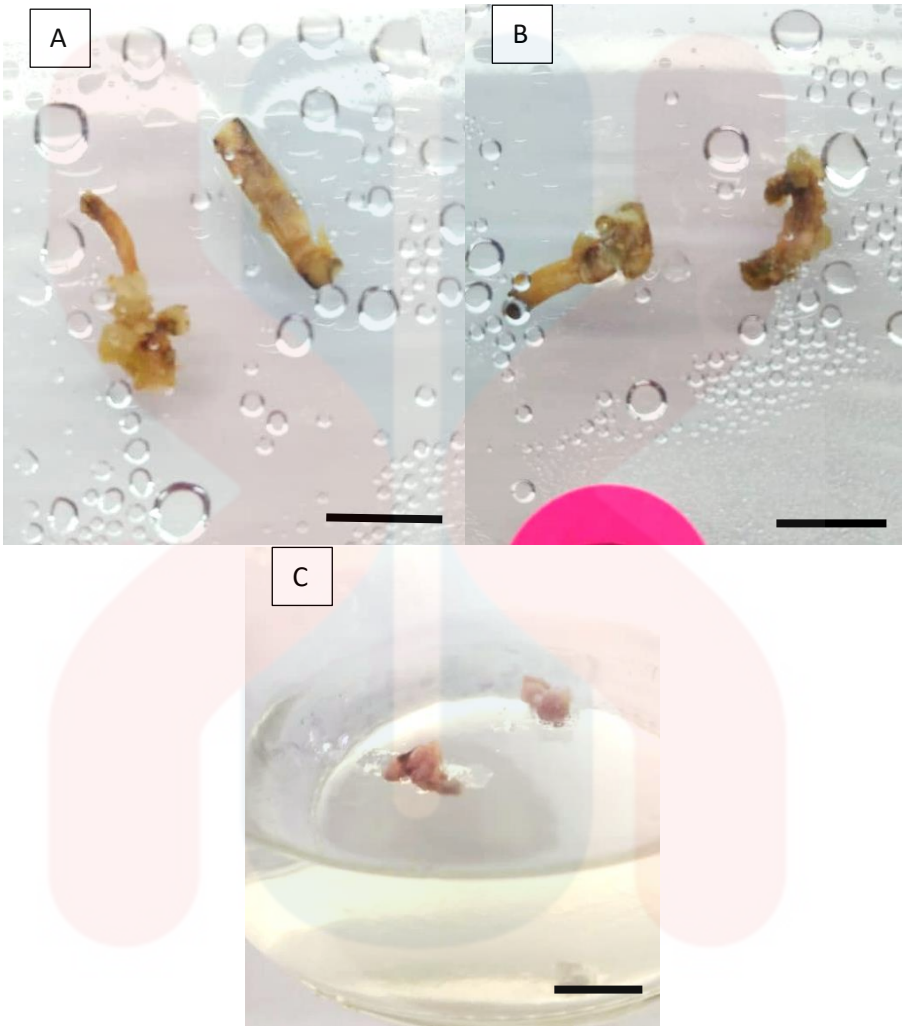

Figure 4.2 The callus growth. A = the callus induction on the second week, B = the callus induction on the fourth week, C = the callus after subculture on the fourth week

1 Bar = 1 cm

4.4 Weight of Callus

After four weeks, the data of weight of callus are recorded using the equation (1). The data were then analysed and Table 4.6 below show the mean and standard error of weight of callus with the different method of TCL.

Table 4.6 The effect of different method of TCL technique on weight of callus.

Method of TCL technique	**Weight of callus (mg) M ± S.E**
tTCL	18.52 ± 8.81^{a}
lTCL	12.93 ± 8.81^{c}
Square ($1cm^2$)	15.47 ± 8.81^{b}

M (Mean) ± S.E (Standard Error). The label a, b, c are based on significance difference from Tukey' test.

For a clear view, Figure 4.3 shows the bar graph of mean for weight of callus with three method of TCL.

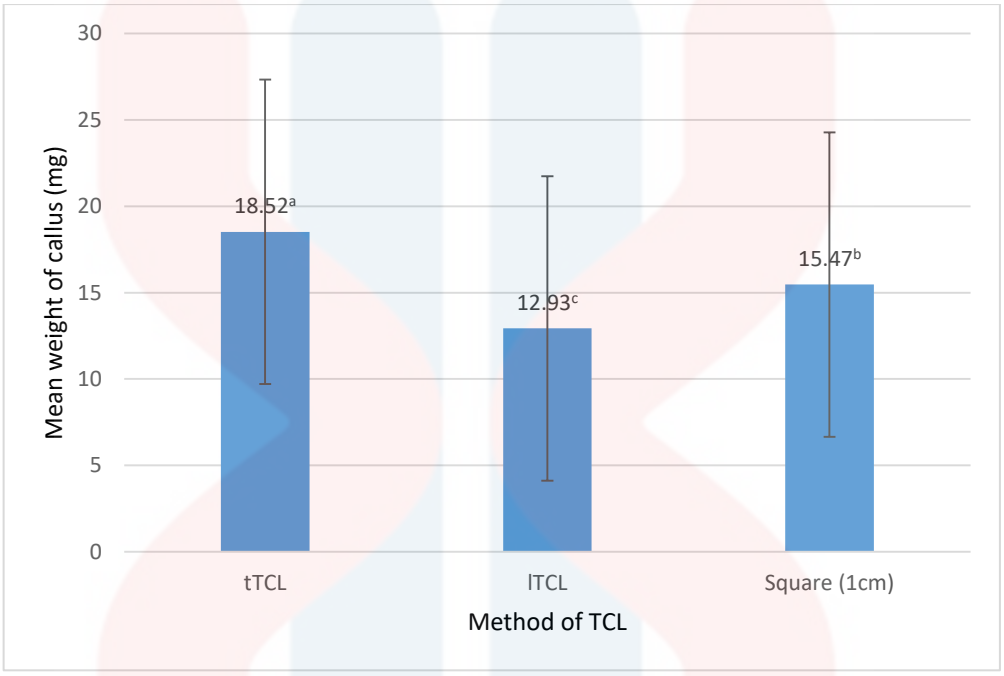

Figure 4.3 Weight of callus towards different method of TCL

Based on the Table 4.6 and Figure 4.3, the highest mean are the method tTCL that is 18.52. According to a study the response of callus on TCL are differ based on the donor organ (Nhut, Silva, Le, & Van, 2003). In tTCL, it usually contain a small quantity of cell from distinct tissue-type such as cortical, medullar tissue and perivascular, epidermal, cambium and parenchyma cells (Van, 1980;Teixeira da Silva, Tran Thanh Van, Stefania, Nhut, & Altamura, 2007). Meanwhile, lTCL consist only one tissue type like the monolayer of epidermis cell, sub-epidermal chlorenchyma and the composed epidermal cells (Altamura, Torrigiani, Falasca, Rossini, & Bagni, 1993; Teixeira da Silva et al., 2007).

Table 4.7 The effect of concentration treatment combination on weight of callus (mg).

Concentration combination of BAP + IAA (treatment)	**Weight of callus (mg) M ± S.E**
T1	0 ± 0.00^{c}
T2	0.48 ± 15.26^{c}
T3	11.55 ± 15.26^{b}
T4	0 ± 0.00^{c}
T5	40.57 ± 15.26^{a}
T6	16.75 ± 15.26^{b}
T7	0 ± 0.00^{c}
T8	56.95 ± 15.26^{a}
T9	14.43 ± 15.26^{b}

M (Mean) ± S.E (Standard Error). The label a, b, c are based on significance difference from Tukey' test.

For a clear view, Figure 4.4 shows the bar graph for mean of callus with the nine treatments.

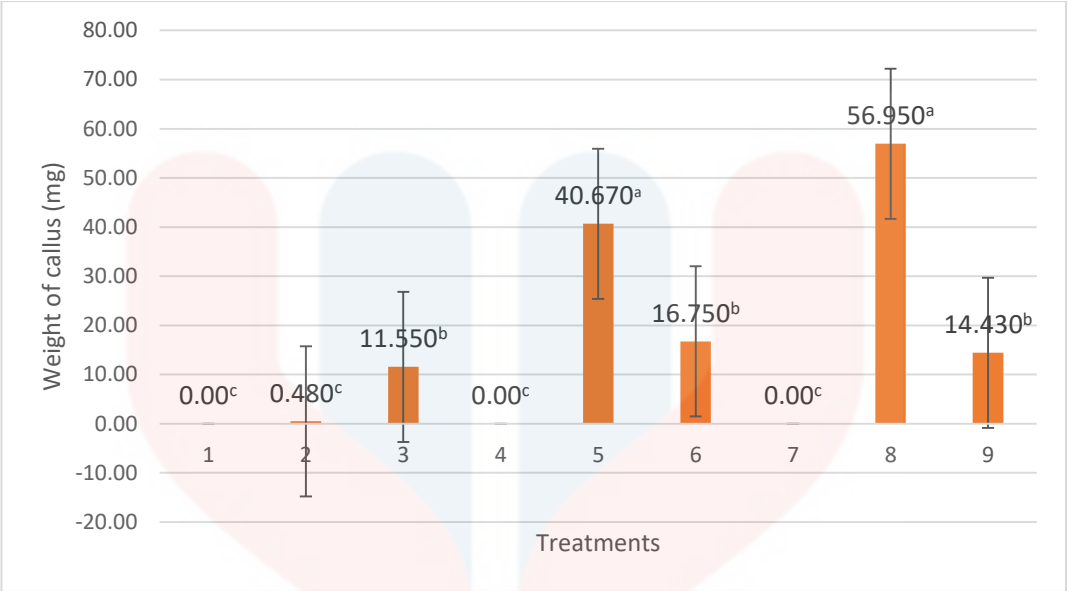

Figure 4.4 Effects of different treatment on weight on callus

Based on the Table 4.7 and Figure 4.4 shows the effect of concentration treatment combination on weight of callus. Treatment 8 (T8) had the highest mean among all which mean T8 is the best PGR concentration combination out of the 9 treatments in callus induction. Meanwhile, T1, T4 and T7 had no callus growth as the mean value is zero. This is because to induce callus the auxin and cytokinin must present and balance in the media which in this experiment IAA act as the auxin and BAP as cytokinin (Skoog & Miller, 1957; Ikeuchi et al., 2013). As in those three treatments, there were neither BAP, KN nor both present in the media. Thus, the callus failed to be induced.

Table 4.8 The effect of different method of TCL technique and treatment of callus induction on weight of callus

Method of cutting (TCL)	Treatment of callus induction	Weight of callus (mg) M ± S.D
	T1	0 ± 0.00[c]
	T2	0 ± 0.00[c]
	T3	0 ± 0.00[c]
	T4	0 ± 0.00[c]
tTCL	T5	92.4 ± 124.59[a]
	T6	40.80 ± 2.12[b]
	T7	0 ± 0.00[c]
	T8	31.90 ± 45.11[b]
	T9	1.65 ± 2.33[c]
	T1	0 ± 0.00[c]
	T2	1.45 ± 2.05[c]
	T3	9.80 ± 13.85[b]
	T4	0 ± 0.00[c]
lTCL	T5	9.65 ± 13.64[b]
	T6	9.45 ± 13.36[b]
	T7	0 ± 0.00[c]
	T8	44.45 ± 24.53[a]
	T9	41.65 ± 30.47[a]
	T1	0 ± 0.00[c]
	T2	0 ± 0.00[c]
	T3	24.85 ± 5.16[b]
	T4	0 ± 0.00[c]
Square ($1cm^2$)	T5	19.95 ± 14.21[b]
	T6	0 ± 0.00[c]
	T7	0 ± 0.00[c]
	T8	94.50 ±133.64[a]
	T9	0 ± 0.00[c]

M (Mean) ± S.D (Standard Deviation). The label a, b, c are based on significance difference from Tukey' test

Based on the Table 4.8 the highest callus induced was treatment 8 (T8) with the square 1cm^2 cutting method which also shows that this combination of TCL method and treatment is the best among all. Meanwhile, the least callus induced, are the combination of method tTCL, lTCL and square 1cm^2 with the treatment 1, 4 and 7 which had zero as the mean value. Basically, callus can be induce with the presence of auxin and cytokinin so does the presence of wounding area (Skoog & Miller, 1957; Ikeuchi et. al, 2013). Square shape cut are more likely to have denser callus due to the larger wounding area. Meanwhile, by comparing total mean of all treatment between tTCL and lTCL, tTCL has larger mean which indicate the callus production were denser with this method. In tTCL, it explained that the piece of leaf sample contain a small quantity of cell which are from distinct tissue-type. The cell could probably the cortical, medullar tissue and perivascular, epidermal, cambium and parenchyma cells (Van, 1980;Teixeira da Silva, Tran Thanh Van, Stefania, Nhut, & Altamura, 2007). In the other hand, there are only on tissue type consist in lTCL method such as the monolayer of epidermis cell, sub-epidermal chlorenchyma and the composed epidermal cells (Altamura, Torrigiani, Falasca, Rossini, & Bagni, 1993; Teixeira da Silva et al., 2007).

During this experiment there was some limitation that interrupt some process to be done as planned. The weight of the leaf cannot be recorded before culturing because of the need of sterile condition of the explant. Furthermore, the leaf must stay moist especially at the cutting edge. Thus, the process of culturing cannot be exposed to long. The weight of callus in the new jar can be an error as there will be water loss occur during the process. Thus, the weighing scale were brought into the laminar flow to immediately record the weight before and after subculture the

callus. The explant are from the cotyledon leaves which mean each plant only have two leaf. The true leaves are too thin that made it hard for cutting process thus replaced it with cotyledon leaves.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In the nut of shell, PGR that able to produce the best denser callus production on *C. ternatea* is the treatment 8 which 2 mg/L BAP + 0.1 mg/L IAA with the mean value 56.95 mg. Meanwhile, the best method of TCL that able to induced denser callus on *C. ternatea* is the tTCL with the mean value 18.52 mg.

5.2 Recommendation

As to improve next study about callus induction on *C. ternatea*, the process of cutting leaves must be quick and always make sure the leaves are covered to prevent water loss. During the weighing process, the weight must be determine without taking out the callus from the jar. Thus, the weighing scale would be bring into the laminar flow to immediately weight the callus along with the minimization

water loss. Next, if the experiment need the true leaves for TCL method, it must be mature enough to undergo cutting process.

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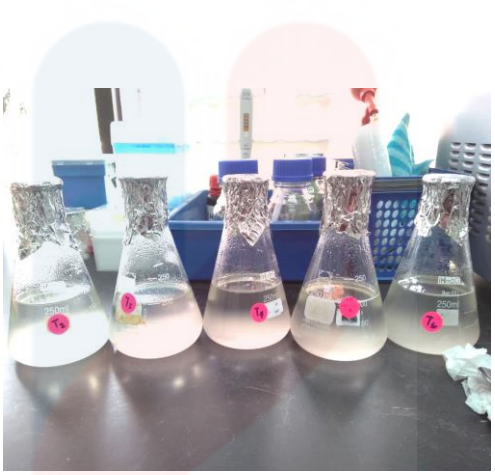
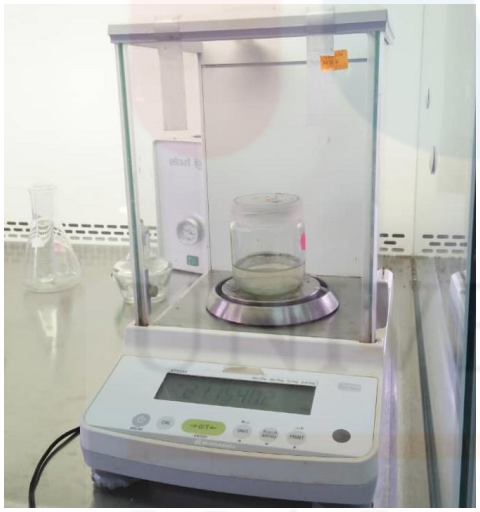
APPENDICES

Appendix A: sterilization of C. ternatea seed



Appendix B: Media preparation of callus induction treatment



Appendix D: Weighing the callus

Appendix E: performing TCL method