OPTIMIZATION OF MICROPROPAGATION FOR *Curcuma longa*

by

RAHAYU BINTI MOHAMAD YUSOFF

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DECLARATION

I declare that this entitled Optimization of Micropropagation for *Curcuma longa* 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.

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"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 Science (Natural Resources Science) with Honors"

Signature : …….....
Name of Supervisor I : …….
Date : ……..

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Optimization of Micropropagation for *Curcuma longa*

ABSTRACT

Curcuma longa is also known as turmeric and commonly as *kunyit* among the local Malay people. It belongs to Zingiberaceae family and is one of the highly commercialised and sought after ginger member. The production in field for this ginger species is growing by year owing to its use in cooking, as traditional medicine, cloth dye industry and in food processing industry. However, the growth of this plant species is very much dependent on environmental factor which influences the production of the secondary metabolites especially for medical research. Hence, micropropagation offers an alternative for massive plant production under controlled environment for homogenous secondary metabolite production. Previous studies done was not focused on optimization of surface sterilization and use of growth hormone to promote growth of turmeric in *in vitro* which this study has reported.The rhizome buds were immersed in 100% (v/v) Clorox® solution for 20 minutes and were then rinsed thrice with sterilised distilled water. Rhizome buds were immersed in 95% ethanol for two minutes and then were rinsed again to remove the ethanol completely. Aseptic rhizome buds were cultured on the MS medium with three different growth hormone which are IAA, BAP and 2,4-D with various concentration (0.1 mg/L, 0.2 mg/L, 0.3 mg/L, 0.4 mg/L, 0.5 mg/L). The growth of each hormone with each concentration was observed after two weeks. MS media with 0.2 mg/L and 0.3 mg/L of IAA and BAP showed rapid shooting growth while plantlets cultured in 2,4-D were observed to be free of contaminants. Throughout the research, it was observed that IAA with concentration 0.2 mg/L and 0.3 mg/L has a significant growth compared to other concentration. Surface sterilization with 95% ethanol for two minutes and 100% (v/v) Clorox® solution for 20 minutes had provided aseptic explants to form new shoots. Hence, the determination on effect of three hormone concentration on shoot growth in *C. longa* has been achieved and fast surface sterilization and micropropagation protocol was established for *C. longa* through this study.

Pengoptimuman Mikropropagasi untuk *Curcuma longa*

ABSTRAK

Curcuma longa juga dikenali sebagai kunyit di kalangan orang Melayu tempatan. Ia tergolong dalam keluarga Zingiberaceae dan salah satu spesies yang mempunyai nilai komersial yang sangat tinggi selepas pokok halia. Pengeluaran produk spesies halia ini berkembang dari tahun ke tahun kerana penggunaannya dalam masakan, sebagai ubat tradisional, industri pewarna kain dan dalam industri pemprosesan makanan. Walau bagaimanapun, pertumbuhan spesies tumbuhan ini sangat bergantung kepada faktor persekitaran yang mempengaruhi pengeluaran metabolit sekunder terutamanya untuk penyelidikan perubatan. Oleh itu, mikropropagasi menawarkan alternatif untuk pengeluaran tumbuhan secara besar-besaran di bawah persekitaran terkawal untuk pengeluaran metabolit sekunder yang homogen. Kajian terdahulu yang dilakukan tidak berfokus pada pengoptimuman kaedah pensterilan dan penggunaan hormon pertumbuhan untuk menggalakkan pertumbuhan kunyit secara *in vitro* seperti yang dilaporkan oleh kajian ini. Tunas kunyit direndam dalam 100% (v / v) Clorox® selama 20 minit dan kemudiannya dibasuh tiga kali dengan air suling yang disterilkan. Kemudian tunas direndam dalam 95% ethanol selama dua minit dan kemudian dibilas semula untuk menghilangkan ethanol sepenuhnya. Tunas kunyit aseptik diinokulasi dalam MS medium yang disediakan dengan tiga hormon pertumbuhan berbeza iaitu IAA, BAP dan 2,4-D dengan kepekatan yang pelbagai (0.1 mg/L, 0.2 mg/L, 0.3 mg/L, 0.4 mg/L, 0.5 mg/L). Pertumbuhan pucuk pada setiap hormon dan kepekatan diperhatikan selepas dua minggu. Media MS dengan 0.2 mg/L dan 0.3 mg/L IAA dan BAP didapati menunjukkan pertumbuhan yang pesat manakala tunas kunyit dalam 2,4-D didapati bebas dari pencemaran kulat. Sepanjang penyelidikan, didapati bahawa tunas kunyit yang diinokulasi dalam hormon IAA, BAP dan 2,4-D dengan kepekatan 0.2 mg/L dan 0.3 mg/L mempunyai pertumbuhan ketara berbanding kepekatan yang lain. Kaedah pensterilan menggunakan 95% ethanol selama dua minit dan 100% (v/v) Clorox® selama 20 minit didapati merupakan kaedah pensterilan permukaan tunas rizom terbaik untuk membentuk pucuk baru di persekitaran *in vitro*. Oleh itu, penentuan kesan tiga hormon dengan pelbagai kepekatan ke atas pertumbuhan tunas rizom kunyit telah dicapai dan pensterilan yang optimum dan protokol mikropropagasi yang cepat untuk *C. longa* juga telah berhasil melalui kajian ini.

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LIST OF ABBREVIATIONS AND SYMBOLS

MS	Murashige & Skoog
BAP	6-Benzylaminopurine, benzyl adenine
IAA	Indole-3-Acetic Acid
2,4-D	Dichlorophenoxyacetic acid
NaOH	Sodium Hydrochloride
HCl	Hydrochloric Acid
$HgCl_2$	Mercuric chloride
g/L	gram per Liter
mg/L	milligram per Liter
v/v	volume per volume
mm	Millimeter
cm	Centimeter
m	Meter
µm	Micro millimeter
ANOVA	One-Way Analysis Of Variance
SD	Standard Deviation
%	Percentage
<	Less Than
° C	Degree Celsius

CHAPTER 1

INTRODUCTION

1.1 Background of Study

The Zingiberaceae has approximately 50 genera and over 1,000 species in this world. It is considered as one of the largest families in Zingiberales order. In the region of Penisular Malaysia and Borneo, they have abundant species of Zingiberaceae and it is estimated to be around 150 species that belong to 23 genera of ginger in Penisular Malaysia (Holttum, 1950) while 100 species belong to 19 genera are representing the Borneo (Lam *et al*., 2013).

Members of Zingiberaceae grow in a damp and shaded area of low-land. They can be identified when the rhizome are crushed or through the smell of pungent leaves (Holttum, 1950). Figure 1 shows a picture of *Curcuma longa* plant which is commonly known as tumeric belonging to Zingiberaceae family.

In Asian region, many species of Zingiberaceae are used as ointment for pain alleviation, flavoring food and as the source of cloth dye agents (Larsen, Ibrahim, Khaw & Saw, 1999). *Curcuma longa* is one of the species that have many benefit towards human health that can be consumed via various routes of application including by inhalation, topically and orally (Chainani-Wu, 2003).

Some of the medicinal properties of turmeric such as antiinflammatory, antibacterial and anti– protozoal activities have been demonstrated in experimental animal (Chandra & Gupta, 1972).

Figure 1: *Curcuma longa* plant at Universiti Malaysia Kelantan, Jeli campus

Rhizomes of turmeric posses a dormancy cycle and only germinate during monsoon season. The rhizomes are usualy stored yearly for use throughout the year. Yearly planting of turmeric plant were reported to have high cost of germ plasm maintenance and requires a large amount of work (Nayak & Naik, 2006). Moreover, tumeric sometimes can be infected by some diseases such as rhizome rot and leaf spot that are caused by *Taphrina*, *Collectrichum* and *Pythium* species which results in planting material destruction and inconsistent supply to local markets (Abdelmageed, Faridah, Norhana, Julia, & Kadir, 2011).

Micropropagation have been done for several medicinal plant species and it have shown promises for the conservation of the plant species, especially of vegetatively propagated plants (Hoffman & Smith, 2018). There are early report on induction of storage organs such as tubers and bulbs in potato (Hussey & Stacey, 1984), yams (Jean & Cappacodia, 1991), tulip (Taeb & Anderson, 1990) and ginger (Bhat, Chandal & Kackae, 1994). These organs serves as planting material and tubers and bulbs are easy to transport.

A studies on propagation of *Hypoxis hemerocallidea* by inducing corm bud has been reported because of the rapid decline of its wild population due to the large scale harvesting (Mofokeng, Kleynhans, Sediane, Morey & Araya, 2018). Through *in vitro* culture technique it is believed to have higher potential to conserve wild populations. In the previous study, rapid shoot multiplication rhizome of *Alpinia galanga* has been reported (Shamsudheen, Mehaboob, Thiagu & Shajahan, 2018). However, tissue culture raised plants need special treatment for field establishment and it is one of the major hindrance to use this technique for commercial develop (Sunitibala, Damayanti, & Sharma, 2001).

The present study attempts to determine the effect of hormone *i.e* Indole-3-acetic acid (IAA), 6-Benzylaminopurine (BAP) and Dichlorophenoxyacetic acid (2,4-D) with various concentration on growth of *Curcuma longa* and to establish optimum micropropagation protocol for the species. Micropropagation offers an alternative technique to produce large numbers of planting materials with homogenous growth and disease free to increase the productivity of turmeric plants.

1.2 Problem Statement

Previous study have been done on establishment of tissue culture for selected medicinal *C. longa* using cytokinin BAP for shoot multiplication (Yusuf, Khalid & Ibrahim, 2007). According to this study, the optimum concentration of BAP for in vitro micropropagation were 3.0 mg/L for all species studied. However, this study does not establish the optimization of surface sterilization for *Curcuma longa.* Other than that, the growth of this plant species is very much dependent on environmental factor which influences the production of the secondary metabolites especially for medical research and the rhizomes only germinate during monsoon season. Hence, micropropagation offers an alternative for massive plant production under controlled environment for homogenous secondary metabolite production. Tumeric sometimes can be infected by some diseases such as rhizome rot and leaf spot which results in planting material destruction and inconsistent supply to local markets (Abdelmageed *et al.*, 2011). Thus, this study want to explore the best hormones to produce a homogenus production and establish optimum surface sterilization for *C. longa*.

1.3 Objective

1. To determine the effect of various growth hormone IAA, BAP and 24-D concentration on growth of *C. longa in vitro*.
2. To establish optimum surface sterilization and micropropagation protocol for *C. longa*.

1.4 Scope of Study

This study focused on sampling *C. longa* plant around Jeli, Kelantan. The sample used to established micropropagation and the process followed until the growth of the plant is optimize.

1.5 Significance of Study

Curcuma longa is a well known spice around the global. However, rot disease that attacks the rhizome and short season for harvest often reduces quality supply in market. In order to fulfill the market demand, micropropagation technique can be used as an alternative method which provides an suitable and disease free environment for better growth of the plant and with this method, rapid *in vitro* propagation of the plants can be achieved. Therefore, a free disease and good quality of turmeric plant can be produced in large scale via propagation method once the protocol for micropropation is optimized which is reported in this study.

CHAPTER 2

LITERATURE REVIEW

2.1 Historical Background (Zingiberaceae)

In Asia, Zingiberaceae family is a perennial herb that are widely raised in tropical regions (Araujo & Leon, 2001). *Curcuma longa* mainly contribute to colour and flavour for food.

Curcuma longa or commonly known as turmeric can easily be found in the form of powder and also used for medicinal purposes. In the 19th century, the rhizome has been used for extraction and isolated and the yellow pigment then was named as curcumin. It is claimed that the anti- inflammatory effects comes from the curcumin (Sood & Nagpal, 2013). Tumeric is described for its aromatic, stimulant and carminative properties (Yadav, Khar, Mujeeb, Akhtar, & Yadav, 2013). Other solution such as slaked lime also can be added to create another medicinal solution that locally can been apply over the wound area which is believed can treat twisting and swelling caused by injury. According to Sigrist (2010), some diseases such as anorexia, cough, diabetic wounds and sinusitis can also be cured using tumeric powder.

2.2 Medicinal Value of *Curcuma longa*

For the past few decades plants, fungi and bacteria are the examples of natural resources that have been used by many physicans from different ethnic as natural medicine or drugs. These natural resources are beneficial to human for healing and curing purpose. The usage of these natural medicine are still been used continuosly either in pure compound or extracts in pharmaceutical industry. For instance, opium poppy contained morphine compound that could act as analgesic medicine has been used for decades yet it still being used up until today in medicine industry (Araujo & Leon, 2001). Thus, it is proven that massive production of substances and compound can be retrieved from these natural resources especially from plants.

2.3 Activities of *Curcuma longa*

2.3.1 Anti Inflammatory

Any substances or treatment of reducing inflammation can be refered as anti-inflammatory, or antiinflammatory. According to Chainani-Wu (2003) research on the identification of antiinflammatory activity involved a number of different molecules that are inhibited by curcumin including phospholipase, lipooxygenase, cyclooxygenase 2, leukotrienes, thromboxane, prostaglandins, nitric oxide, collagenase, elastase, hyaluronidase, monocyte chemoattractant protein-1 (MCP-1), interferon-inducible protein, tumor necrosis factor (TNF), and interleukin-12 (IL-12). Each one of the molecules play its own role in curcumin and mostly of them are enzymes.

For example, according to Rao (2007), the role of lipooxygenase (LOX) and cyclooxygenase (COX) isoforms, particularly cyclooxygenase 2 (COX-2) in the inflammation has been well established. The study is carried out at cellular and molecular levels and curcumin has shown to regulate a number of signaling pathways, including the eicosanoid pathway involving COX and LOX. A number of studies have been conducted to support curcumin-mediated regulation of COX and LOX pathways, which is an important mechanism by which curcumin prevents a number of disease processes, including cancer. Thus, this proves that curcumin is advantageous in the prevention and treatment of inflammatory diseases due to its anti-inflammatory activity.

2.3.2 Antioxidant Activity

According to Unnikrishnan and Rao (1995) studies on antioxidative properties of curcumin and its three derivatives (demethoxy curcumin, bisdemethoxy curcumin and diacethyl curcumin) have been carried out. The demonstration of these three substances studies shows a protection of hemoglobin from oxidation at a concentration as low as 0.08 mM. Exception for diacethyl curcumin which has a little effect in inhibition of nitrite induced oxidation of hemoglobin. While according to Reddy and Lokesh (1994), the lipid peroxidation contribute mainly in the inflammation, in heart diseases, and in cancer. Curcumin also inhibit lipid peroxidation in rat liver microsomes, erythrocyte membranes and brain homogenates. It has been studied in various models to see the different effect on the different models.

2.4 Morphology of *Curcuma longa* plant

The plant morphology represent study on development, form and structure of plants. Determination of a species within a species requires the morpholgy to be identified to distinguish the difference and similarity of each species (Sarangthem & Haokip, 2010). The different structure within the species or different are believed to appear and developed as inherited genetic pathways. Some basic structure of plant can be similar as other plant, but somehow the physical appearances might be differ. As for instance, the leaves of cabbage and oak look very different, however it does share certain basic arrangement and structure at some part of the plants. Example of herbarium is shown in Figure 2.

Figure 2: Herbarium of *Curcuma longa* (Source: Eugen, 1887)

Curcuma longa is a sturdy and seasoning herbal plant that can reach 1m tall with leafy stems appear in a clump from the rhizomes and then slowly form to a larger clump. Shoots (leafy part) can appear to several arrangement in the opposite vertical rows. The very light green leaves is under the darker one, densely studded with pellucid dots while dark green leaves with a green midrib. The rhizome's colour

inside and outside is orange colour, and a pungent smell (Figure 3) can be smelled when the rhizome is cut (Lam *et al*., 2013). The flowers are tubular, several colours such as white, purple and yellow appear between the leaf sheaths (Figure 4). Fruits are never produced in *Curcuma longa*.

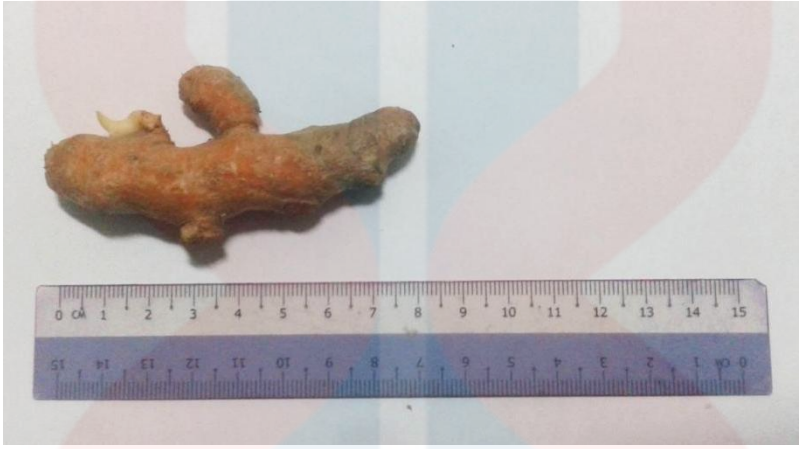

Figure 3: Picture of tumeric's rhizome with scale

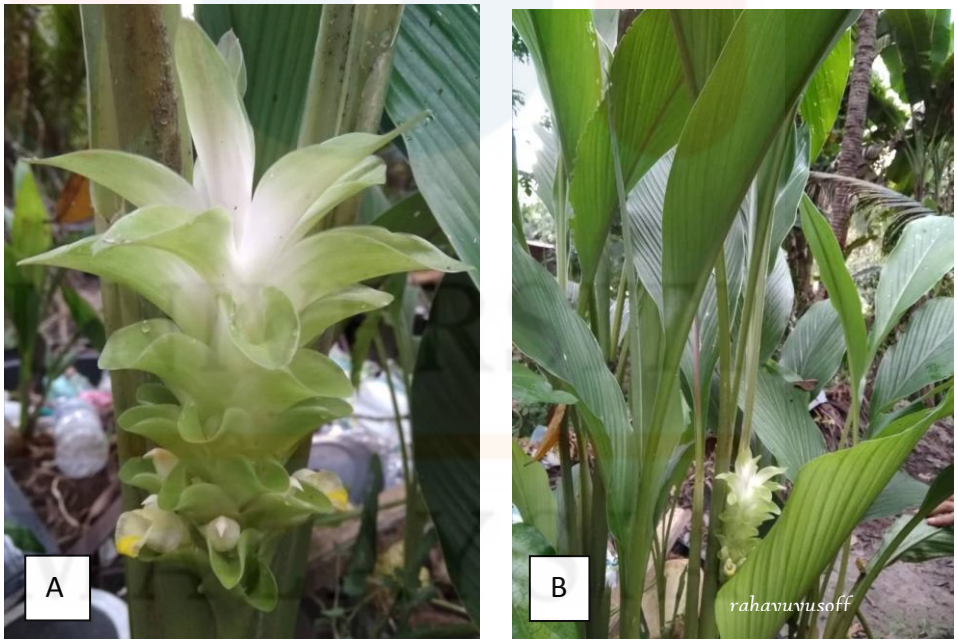

Figure 4 A: Flower of tumeric **B:** The flower located between the leaf sheaths

2.5 Ecology of *Curcuma longa* plant

Turmeric requires warm and moist conditions. It can be cultivated in most areas of the tropics and subtropics provided rainfall is adequate (1000-2000 mm) or facilities for irrigation are available. As reported by Dahal and Idris (2016) a well-distributed rainfall of 1200-1400 mm in 100-120 days is ideal. Cultivation has been extended into areas with over 2000 mm rainfall. It is grown up to altitudes of 1200 m in the Himalayan foothills but it performs better at altitudes of 450-900 m. Temperature ranges of 30-35 °C during sprouting, 25-30 °C during tillering, 20-25 °C during rhizome initiation and 18-2O °C during bulking stage have been identified as optimal. Though turmeric is grown in various soil types, well-drained, loose and friable, fertile loam or clay loam, with good organic matter status, in the pH range of 5-7.5 is preferred. It cannot stand waterlogging and alkaline soils. Gravelly, stony and heavy soils are unsuitable for the development of rhizomes. As a sciophyte it does well in partial shade and can be cropped under fruit trees.

C. longa is found to naturalize mainly in teak forest, but also in sunny places, on clayey to sandy soils, up to 2000 m altitude.

2.6 Distribution of *Curcuma longa* plant

The country that is believed to be the origin region for *C. longa* to grow is in South Asia, India. However because of this species is suitable with tropical climate regions, *Curcuma longa* nowadays is in the cultivated state, in many tropical or subtropical areas, particularly in Asia and Africa (Guido Bissanti, 2017). Figure 5 shows the distribution of *C. longa* cultivation area.

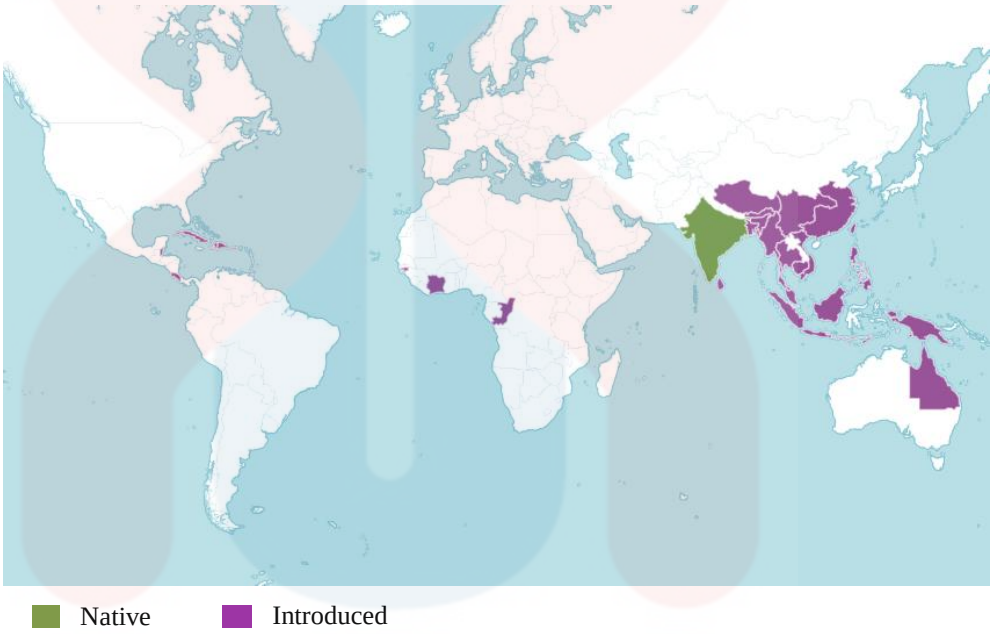

Figure 5: World Map shows the distribution of *Curcuma longa*

Sources: Retrieved 13 Nov, 2018 from http://apps.kew.org/wcsp/

Native to:India

Introduced into:Andaman Is., Assam, Bangladesh, Belize, Borneo, Cambodia, Caroline Is., China South-Central, China Southeast, Congo, Cook Is., Costa Rica, Cuba, Dominican Republic, East Himalaya, Easter Is., Fiji, Gilbert Is., Guinea-Bissau, Gulf of Guinea Is., Haiti, Hawaii, Ivory Coast, Jawa, Leeward Is., Lesser Sunda Is., Malaya, Mauritius, Myanmar, New Caledonia, New Guinea, Nicobar Is., Philippines, Pitcairn Is., Puerto Rico, Queensland, Réunion, Samoa, Society Is., Sri Lanka, Sumatera, Taiwan, Thailand, Tibet, Tonga, Trinidad-Tobago, Vietnam, Windward Is.

2.7 Advantages of Micropropagation

Micropropagation technique have a lot of advantages that are beneficial to human especially for tumeric growers. Micropropagation requires only small

growing area (Figure 6). The objective of micropropagation application is to enhance the rate of multiplication. *In vitro* tissue culture technique of gingers usually use minitubers or microcorms for plant multiplication throughout the season (Nayak, 2000). Other than that, by applying this technique, the plants that free from diseases can be raised and maintained domestically and economically also the germplasm can be stored for few years.

However, there are also limitation with the micropropagation method which required sophisticated facilities with sterilization equipments and it need a qualified laboratory technician in managing and maintaining the micropropagation techniques than the conventional field planting techniques. The history of plant tissue culture and its applications according to Leong-Skornickova, Šída, Wijesundara, & Marhold (2008) have been reviewed and discussed from time to time (Gautheret, 1983, 1985; Krikorian, 1988; Thorpe, 1990; Gamborg, 2002; Vasil, 2008).

Figure 6: The figure shown the example of successful micropropagation in a tissue culture laboratory, Faculty of Agro Based Industry, UMK Jeli.

2.8 6-Benzylaminopurine, benzyl adenine (BAP) hormone

6-Benzylaminopurine, benzyl adenine (BAP) is a synthetic cytokinin which together with auxins elicits plant growth and development responses. BAP is a first-generation synthetic cytokinin that elicits plant growth and development responses, setting blossoms and stimulating fruit richness by stimulating cell division (Buah, Danso, Taeh, Abole, Bediako & Baidoo, 2010). BAP can raise the harvesting life cycle of vegetables and inhibit kinase in plant's respiratory. As an example, BAP affect shoot and root formation, and a study on the comparison of height of *Musa* spp. between BAP and other hormone have been done and it was observed that the explant with BAP have a higher height of shoot . Thus, BAP has been proven to have higher shoot inducing ability than Kinetin (Bhattacharjee & Islam, 2014).

2.9 Dichlorophenoxyacetic acid (2,4-D) hormone

The chlorophenoxy herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is widely used throughout the United States to controls broadleaf weeds in residential, agricultural, and aquatic environments. Similar to other chlorophenoxy herbicides, it acts as a plant growth hormone. At low levels, these herbicides can enhance plant growth, but at higher levels are herbicidal. According to (Tahir, Victor & Abdulkadir, 2011) research project on the effect of (2,4-D) concentration on callus induction in sugarcane (*Saccharum officinarum)*, the lower 2,4-D concentration is proved to give a better embryonic callus and callus growth among the two genotypes.

CHAPTER 3

MATERIAL AND METHOD

3.1 Materials and Apparatus

Beakers, conical flask, culture test tube, petri dishes, Erlenmeyer flasks, aluminium foil, measuring cylinder, Schott bottles and jam jar bottles were washed using soap and rinsed with distilled water then were autoclaved (TOMY SX 500) to sterilize all instruments and materials that have been used. Other than that, various sizes of scalpel, forceps, spatula, scissors and disposable blades were used for tissue culture work. All the sterilized items were dried in oven at 50℃.

3.2 Chemicals and reagents

Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) containing 3% sucrose and 0.8% agar were used for basal medium preparation. Stock solution of macronutrient, micronutrient, and vitamin were included in the composition of MS basal medium. All the chemicals were obtained from Sigma-Aldrich, Malaysia.

3.3 Methodology

3.3.1 Preparation of Murashige and Skoog Basal Media

Murashige and Skoog (1962) medium containing macroelements, micro elements, vitamins, iron source, sucrose and plant growth regulator were prepared with different concentration throughout this study. The medium were attuned to pH 5.5 by using NaCl and HCl and medium were autoclaved (TOMY SX 500) for 19 min at 120-121˚C at 15 Ib/inch² (Naz, Ilyas, Javad, & Ali, 2009) and were poured into the petri dish. The sterile MS media were left to solidify and stored in the clean cabinet in laboratory BAP 1.1, Universiti Malaysia Kelantan, Jeli Campus.

3.3.2 Collection of *Curcuma longa*

The sampling for *C. longa* plants together with its rhizome was done randomly around Jeli, Kelantan and the fresh plants were immediately planted and maintained at greenhouse in Universiti Malaysia Kelantan, Jeli campus. Each of the pots was labelled with the date of sampling with the name of collectors. The sample identification was confirmed by referring to A Guide To Gingers Of Borneo - Natural History Publications (Borneo).

3.3.3 Optimization of Surface Sterilisation and Initiation of *In Vitro* Culture of *Curcuma longa* plant

The surface sterilization of *C. longa* were done following the method reported by Naz *et al*. (2009). The sprouted buds (1-2 cm) was cut using sterile

scalpel and washed with commercial liquid detergent for 5 minutes. Then it was followed by throughly washing under flowing tap water for 15-20 min to remove the surface pathogens and traces of detergent. The final surface sterilization were carried out in laminar air-flow cabinet by treating them in 100% clorox for 20 minutes. Then, the explants were washed with sterilized double distilled water for a few times to remove traces of clorox and followed with immersion of explant in 95% of ethanol for 2 minutes and then were washed five times with sterilized distilled water to remove ethanol completely (Nayak & Naik, 2006). The sterile rhizomes were inoculated in sterilized MS basal media. All the cultures then were kept in a sterile growth room at Tissue Culture Laboratory, Universiti Malaysia Kelantan Jeli campus at 25±2˚C under 16 h light photoperiod provided by white fluorescent tubes (Singh, Chakpram & Devi, 2014). The cultures were maintained by regular subcultures at 4 weeks intervals on fresh MS medium (Behera & Sahoo, 2010).

3.3.4 Effect of Different Hormone on the Growth of *In Vitro Curcuma longa*

The excised shoot tips were inoculated onto solidified MS medium supplemented with different types of hormones and varying concentration (Sharma *et al*., 1991), *i.e* 2-4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP) and 3-indoleacetic acid (IAA). The concentration range of the hormones were 0.0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/L respectively. Thirty culture bottles with MS basal media were prepared for each concentration. Ginger plants with green and healthy leaves were used for this study. The cultures then were incubated at 25±2˚C under 16 h photoperiod and be maintained for four to six weeks in the culture room. The

growth data were obtained from the height of plant, the shoot and root initiation in centimeter (cm) and the number of leaves were recorded once every two days.

3.3.5 Statistical Analysis

The data that had been obtained from section 3.3.4 on the effect of the different hormone on the growth of *Curcuma longa* were recorded and analysed using a one-way analysis of variance (ANOVA). The means were compared by Tukey test at 5% probability level.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Establishment of Aseptic Explants of *Curcuma longa*

The success of plant tissue culture protocol depends on explant sterilization (Dodds & Roberts, 1985). According to Sarmila (2018), the highest percentage of aseptic buds (80%) was obtained when it was surface sterilized thrice with 100% (v/v) Clorox® solution. The study on micropropagation *Zingiber spectabile* and *Zingiber zerumbet* showed that the aseptic bud explants turned green after 2 weeks of culture when it were surface sterilized for 15 second with 95% ethanol and followed with 100% (v/v) Clorox® solution.

However, when the same treatment was applied in this study showed no explant survived in the MS media without hormone. Hence a trial treatment was conducted to optimize the surface sterilization protocol for *C. longa* used in this study. The buds were immersed in 100% (v/v) Clorox® solution for 20 minutes and then it were rinsed thrice with sterilised distilled water. Then the buds were immersed in 95% ethanol for two minutes and then it were rinsed again to remove the ethanol completely.

Ethanol acts as powerful sterilizing agent but also extremely phytotoxic. Therefore very short exposure of few minutes is enough for the explants (Tiwari, Arya, & Kumar, 2012). The buds then were innoculated in MS media with three different hormone (IAA, BAP, 2,4-D) with various concentration (0.1 mg/L, 0.2 mg/L, 0.3 mg/L, 0.4 mg/L, 0.5 mg/L) respectively.

4.2 Effect of IAA on Aseptic Explants Growth of *Curcuma longa*

The bud explants were treated with the trial treatment and nine jam jar were innoculated with sterile explants. After seven days, seven out of nine jam jar were contaminated and two survived. The MS media was contaminated with faint pink pigmented yeast as shown in Figure 7. According to Nagy, Sule, & Sampaio (2005), some fungicides inhibited growth of yeast identified as *Rhodotorula slooffiae*. However, fungicides was excluded from the preparation of MS media and in the contamination occur owing to the improper handling of the medium.

Figure 7: The MS media was infected with three spot of faint pink pigmented yeast

The survived explants were propagated in MS media with IAA hormone concentration of 0.2 mg/L and 0.3 mg/L. After 2 weeks, the explant with concentration 0.2 mg/L showed shoot initiation and growth and the explant with concentration 0.3 mg/L while was free from fungus infection, no sign of shooting growth was observed. Table 1 shows the data collection between the days taken for shoot and root initiation and growth of shoot and root for five concentration of IAA hormone.

After 21 days, *C. longa* plantlets inoculated in IAA 0.2 mg/L showed rapid growth with green shoot and the rhizomes in IAA 0.3 mg/L has grown a white bud that turned to a green shoot. Figure 8 and 9 showed the picture of the growing explants in the stated concentration. On the 32 days, rhizomes in IAA 0.3 mg/L has started root initiation while no rooting was observed in plantlets inoculated in concentrations ie. 0.2 mg/L of IAA hormone. Figure 10 shows the rooting initiation in IAA 0.3 mg/L.

Figure 8: The explant with green shoot (orange circle) in IAA 0.2 mg/L

Figure 9: The explant grown a white bud (orange circle) in IAA 0.3 mg/L

Figure 10: Rooting initiation in MS media with IAA 0.3 mg/L after 32 days of inocculation.

(orange circle)

4.3 Effect of BAP on Aseptic Explants Growth of *Curcuma longa*

MS media with five different of BAP concentration (0.1 mg/L, 0.2 mg/L, 0.3 mg/L, 0.4 mg/L, 0.5 mg/L) were prepared. The bud explants were treated with the same trial treatment and 14 jam jar were innoculated with sterile explants. After four days, 10 out of 14 jam jar were found to be contaminated as shown in Figure 11 and four showed aseptic growth *in vitro*. MS media with BAP concentration of 0.3 mg/L was found to initiate shoot growth as shown in Figure 12. Table 2 shows the data collection between the days taken for shoot and root initiation and growth of shoot and root for five concentration of BAP hormone.

After 21 days, the explants with the shoot were infected with bacteria. The explant was transferred to new media with the same BAP hormone concentration but it was not a successful transfer. The shoot did not survive as shown in Figure 13. According to Yen and Hain (2018) the culture medium is also an excellent resource for the growth of contaminates such as fungus and bacteria.

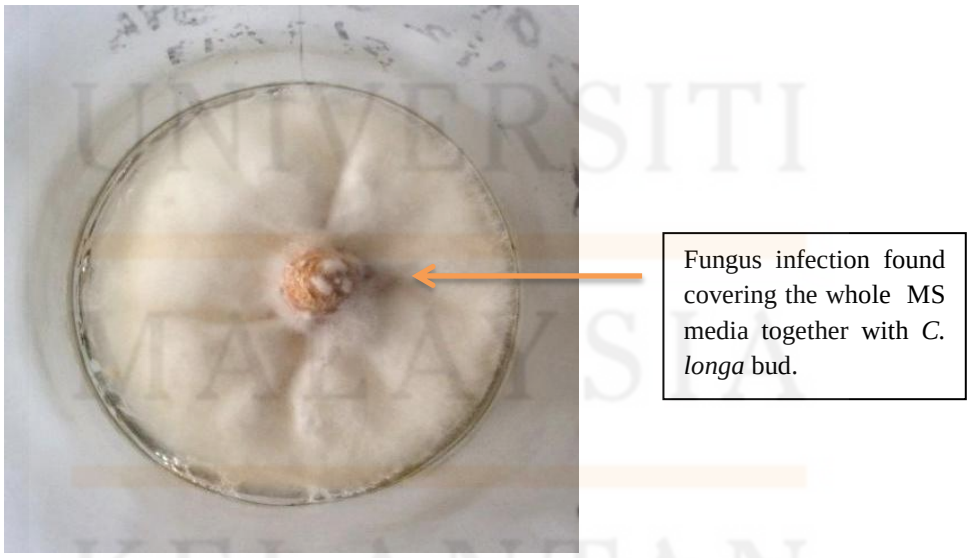

Figure 11: The explant was infected with fungus created the cloudy cotton inside the jam jar

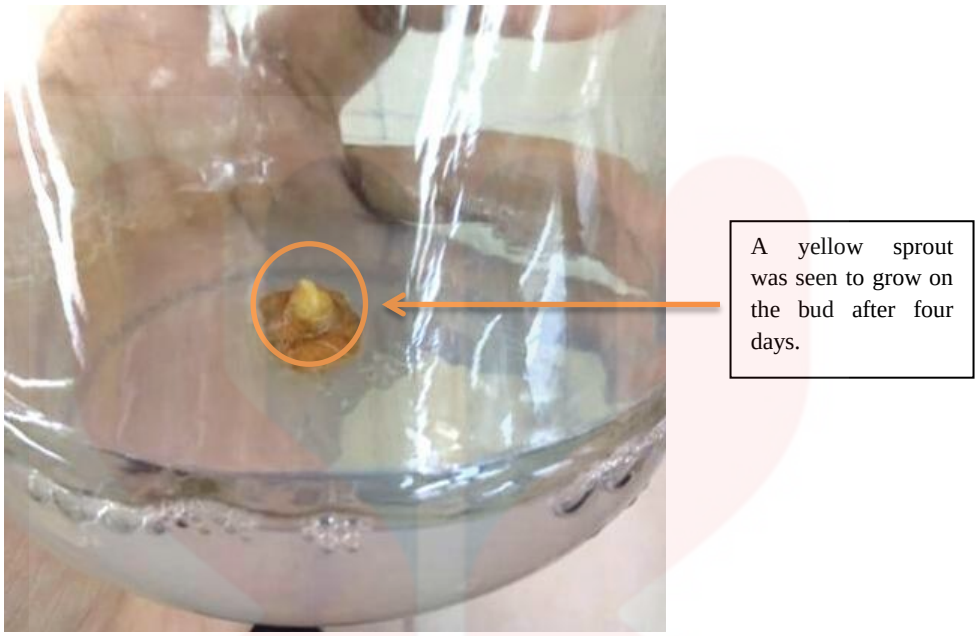

Figure 12: *Curcuma longa* explant in MS media with 0.3 mg/L concentration of BAP hormone. After four days of innoculation a yellow sprout was seen to grow on the bud.

Figure 13: The picture shown the explant with yellow sprout have been infected by bacteria and fungus (orange circle)

4.4 Effect of 2,4-D on Aseptic Explants Growth of *Curcuma longa*

Lastly, explants with MS media containing hormone 2-4-dichlorophenoxyacetic acid (2,4-D) was tested using the same trial treatment. 13 explants were innoculated. On eighth day all explants were contaminated. No explants were survived in MS media containing hormone 2,4-D. Table 3 shows no data collection for the days taken for shoot and root initiation between five concentration for 2,4-D hormone.

4.5 Determination of Growth Hormone IAA, BAP and 2,4-D Various Concentration Effect on Growth of *C. longa In Vitro*

Throughout the study, IAA hormone marked as the best hormone for having the higher ability in inducing shoot and root formation compared to BAP and 2,4-D. On contrary, Buah *et al.*, (2000) in their study for *Musa* spp. showed that BAP induced the highest shoot induction after eight weeks of culture which disagree with the findings of this study. Each of the hormones tended to have its own ability to achieve shooting and rooting responses. However, in this study IAA 0.3 mg/L has the better performances of inducing shoot and root formation compared to IAA 0.2 mg/L.

IAA is the main auxin that are usually used for easier to root herbaceous plants (Sharma, 2006). IAA also stimulates shoot growth but inhibits bud and roots according to Thimann & Skoog (1934) that were the first suggested a correlation between IAA and apical dominance. This idea was further developed by Cholodny-Went who proposed that plant tissues responded to different concentrations of IAA. Thus, IAA is produced at the tip of the plant and is transported downward.

The high concentrations near the apex inhibit lateral buds. As the concentration decreases it frees the buds from the inhibition and they develop (Stephen, 2009). This proved the reason for IAA 0.3 mg/L that has showed rapid growth of shoot and root after three to four weeks of culture.

From observation, BAP hormone have the fastest shooting growth which it only takes four days to initiate the shoot while IAA take seven days to initiate shoot growth and 2,4-D did not show any activity of shoot formation. This showed that BAP is the most suitable hormone for shooting growth compared to IAA and 2,4-D hormone. However, in this study due to contamination of fungus, the plantlets in BAP did not survived. 2,4-D hormone did not showed any shooting growth due to higher rate of contamination and improper handling innoculation method.

4.6 Optimum Surface Sterilization and Micropropagation Protocol for *C. longa*

Many study regarding surface sterilization for tumeric have been using mercury chloride ($HgCl_2$). This is because $HgCl_2$ can reduce the rate of contamination for underground rhizomes. According to Yusuf, Khalid, & Ibrahim (2007) in their study, once the contamination-free cultures of the shoot bud were established by surface sterilize using $HgCl_2$ axillary buds developed from the base of the main shoot grew rapidly. However, mercuric chloride is corrosive to mucous membranes and it also can be toxic by inhalation, ingestion, and skin absorption. Due to high toxicity, this study did not used $HgCl_2$ as surface sterilization.

The optimization of surface sterilization can still be achieved by using 100% (v/v) Clorox® solution for 20 minutes and then buds were immersed in 95% ethanol for two minutes. The rate of explants surviving were lower by using this method but to avoid any dangerous circumstances, it was safe to only use clorox and ethanol.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

As a conclusion, the determination on effect of various concentration for three hormone can be achieved. Throughout the research, it was observed that IAA, with concentration 0.2 mg/L and 0.3 mg/L has a significant growth rather than 0.1 mg/L, 0.4 mg/L and 0.5 mg/L. IAA also had provided the best hormone for shoot and root initiation among the two other BAP and 2,4-D. Besides that, surface sterilization with 95% ethanol for 2 minutes and 100% (v/v) Clorox® solution for 20 minutes had provided aseptic explants to forms new shoots.

It was difficult to achieve optimization of micropropagation. It was because as the underground rhizomes were used as explants, establishment of aseptic cultures were more challenging and the complications faced in preventing contamination in the rhizomatous explants of *Curcuma longa* was higher than other part of the plant (Naz *et al*., 2009). However, through this study, fast surface sterilization and micropropagation protocol was established for *C. longa*.

5.2 Recommendation

There are many reasons on the source of contamination can be happened. If contamination is seen in medium but not on explant, its means, the source of contamination is medium or improper handelling. If contamination is seen on explant but not on medium its means, the source of contamination is because the explants are not proper surface steriliszed.

In order to free from contamination it is importance to have a sterile environment for tissue culture. The laboratory which is used to carry out micropropagation of tumeric is tissue culture laboratory however, it is exposed to many peoples such as it open for learning session, final year project, master and PhD project and common laboratory pathogens. The glassware that had been autoclaved should be kept in an oven which is specialized only for tissue culture purposes. The hardest part in *in vitro* propagation is the constant exposure towards the bacteria and fungi and to maintain the aseptic conditions. Thus, a culture room which has clean and pathogen free environment is essential for successful establishment of tumeric explants.

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APPENDIX A

FYP I & FYP II PLANNING CHART

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

Table 4.2: Effect of various concentration of IAA hormone (0 – 0.5mg/L) on number of shoot and root produced from rhizome explants of *C. longa* and mean number of the day taken for shoot and root to produced

Concentration of IAA hormone (mg/L)	Mean number of the day taken for shoot to produced	Mean number of shoot produced	Mean number of the day taken for root to produced	Mean number of root to produced
0	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$
0.1	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$
0.2	$1.167 \pm 2.858_a$	$0.333 \pm 0.082_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$
0.3	$3.500 \pm 8.573_a$	$0.017 \pm 0.041_a$	$5.333 \pm 13.064_a$	$0.215 \pm 0.531_a$
0.4	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$
0.5	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Day taken for shoot to grow	Between Groups	59.889	5	11.978	.880	.506
	Within Groups	408.333	30	13.611		
	Total	468.222	35			
Height of shoot	Between Groups	.006	5	.001	.840	.532
	Within Groups	.042	30	.001		
	Total	.048	35			
Day taken for root to grow	Between Groups	142.222	5	28.444	1.000	.435
	Within Groups	853.333	30	28.444		
	Total	995.556	35			
Length of root	Between Groups	.235	5	.047	1.000	.435
	Within Groups	1.408	30	.047		
	Total	1.643	35			

Day taken for shoot to grow

Tukey HSD

treatment	N	Subset for alpha = 0.05
		1
control	6	.0000
TR1	6	.0000
TR4	6	.0000
TR5	6	.0000
TR2	6	1.1667
TR3	6	3.5000
Sig.		.578

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Height of shoot

Tukey HSD

treatment	N	Subset for alpha = 0.05
		1
control	6	.0000
TR1	6	.0000
TR4	6	.0000
TR5	6	.0000
TR3	6	.0167
TR2	6	.0333
Sig.		.637

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Day taken for root to grow

Tukey HSD

treatment	N	Subset for alpha = 0.05
		1
control	6	.0000
TR1	6	.0000
TR2	6	.0000
TR4	6	.0000
TR5	6	.0000
TR3	6	5.3333
Sig.		.522

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Length of root

Tukey HSD

treatment	N	Subset for alpha = 0.05
		1
control	6	.0000
TR1	6	.0000
TR2	6	.0000
TR4	6	.0000
TR5	6	.0000
TR3	6	.2167
Sig.		.522

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Table 4.3: Effect of various concentration of BAP hormone (0 – 0.5mg/L) on number of shoot and root produced from rhizome explants of *C. longa* and mean number of the day taken for shoot and root to produced

Concentration of BAP hormone (mg/L)	Mean number of the day taken for shoot to produced	Mean number of shoot produced	Mean number of the day taken for root to produced	Mean number of root to produced
0	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$
0.1	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$
0.2	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$
0.3	$0.667 \pm 1.633_a$	$0.017 \pm 0.408_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$
0.4	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$
0.5	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Day taken for shoot to grow	Between Groups	2.222	5	.444	1.000	.435
	Within Groups	13.333	30	.444		
	Total	15.556	35			
Height of shoot	Between Groups	.001	5	.000	1.000	.435
	Within Groups	.008	30	.000		
	Total	.010	35			
Day taken for root to grow	Between Groups	.000	5	.000	.	.
	Within Groups	.000	30	.000		
	Total	.000	35			
Length of root	Between Groups	.000	5	.000	.	.
	Within Groups	.000	30	.000		
	Total	.000	35			

Day taken for shoot to grow

Tukey HSD

treatment	N	Subset for alpha = 0.05
		1
control	6	.0000
TR1	6	.0000
TR2	6	.0000
TR4	6	.0000
TR5	6	.0000
TR3	6	.6667
Sig.		.522

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Height of shoot

Tukey HSD

treatment	N	Subset for alpha = 0.05
		1
control	6	.0000
TR1	6	.0000
TR2	6	.0000
TR4	6	.0000
TR5	6	.0000
TR3	6	.0167
Sig.		.522

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Table 4.4: Effect of various concentration of 2,4-D hormone (0 – 0.5mg/L) on number of shoot and root produced from rhizome explants of *C. longa* and mean number of the day taken for shoot and root to produced

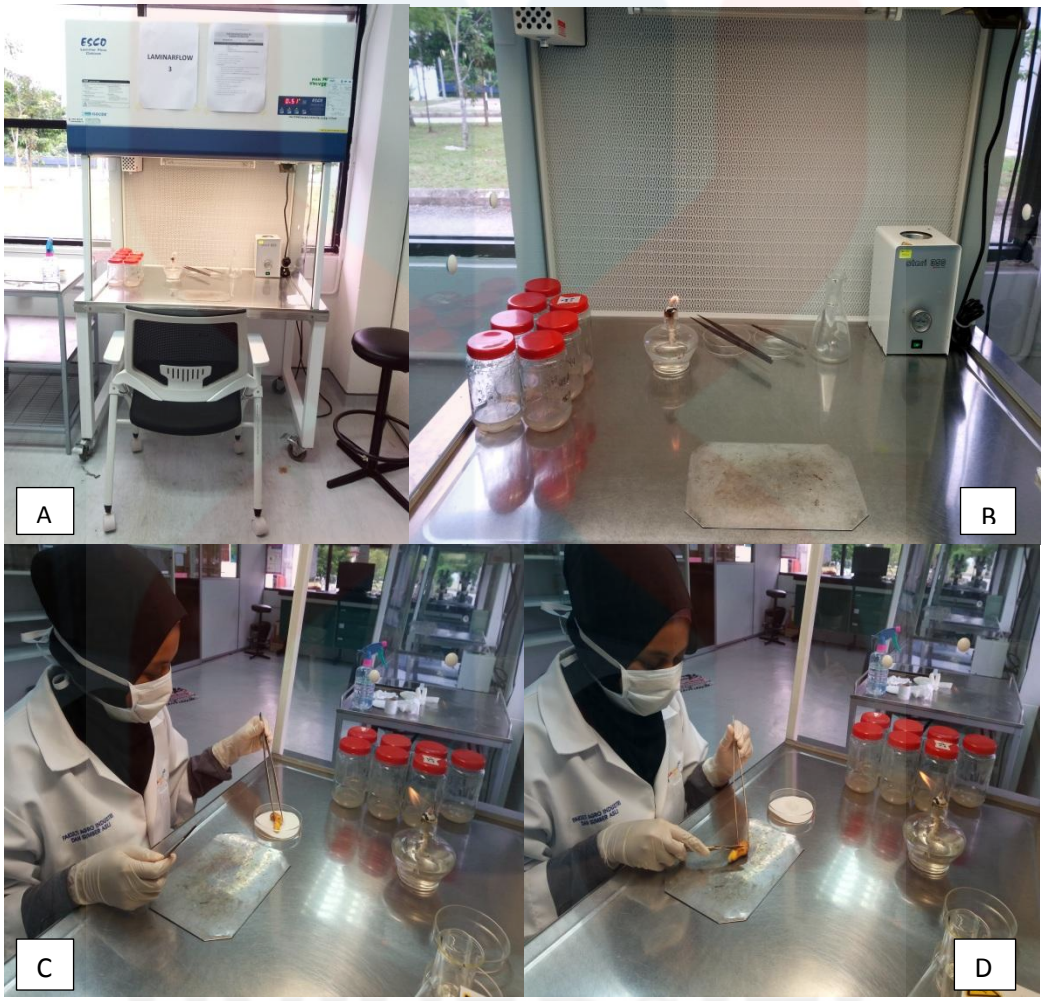
Concentration of BAP hormone (mg/L)	Mean number of the day taken for shoot to produced	Mean number of shoot produced	Mean number of the day taken for root to produced	Mean number of root to produced
0	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$
0.1	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$
0.2	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$
0.3	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$
0.4	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$
0.5	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$	$0.000 \pm 0.000_a$

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Day taken for shoot to grow	Between Groups	.000	5	.000	.	.
	Within Groups	.000	30	.000		
	Total	.000	35			
Height of shoot	Between Groups	.000	5	.000	.	.
	Within Groups	.000	30	.000		
	Total	.000	35			
Day taken for root to grow	Between Groups	.000	5	.000	.	.
	Within Groups	.000	30	.000		
	Total	.000	35			
Length of front	Between Groups	.000	5	.000	.	.
	Within Groups	.000	30	.000		
	Total	.000	35			

* No result on Tukey analysis as there was no data collected.

APPENDIX C



Micropropagation of *Curcuma longa* species being carried out at the Tissue Culture Laboratory UMK Jeli campus.

Picture A: Whole view of laminar air flow cabinet, **B**: Arrangement of equipment inside the laminar, **C&D**: Process of innoculation