Micropropagation of Bentong Ginger (*Zingiber officinale*), *Black* Ginger (*Kaempferia parviflora*) and Black Turmeric (*Curcuma caesia*) Using Rhizome Buds

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A report submitted in fulfilments of the requirements for the degree of Bachelor of Applied Science (Agrotechnology) with Honours

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DECLARATION

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

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Student

Name: Jeffry Anak Tasek

Date:

I certify that the report of this final year project entitled "**Micropropagation of Bentong Ginger (*Zingiber officinale*), *Black* Ginger (*Kaempferia parviflora*) and Black Turmeric (*Curcuma caesia*) Using Rhizome Buds**" by **Jeffry Anak Tasek**, ID number **F18B0046** has been examined and all the correction recommended by examiners have been done for the degree Bachelor of Applied Science (Agrotechnology) with Honours, Faculty of Agro-Based Industry, University Malaysia Kelantan.

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_____

Supervisor

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ABSTRAK

Pada masa kini, bekalan rizom Halia Bentong, Halia Hitam, dan Kunyit Hitam di pasaran Malaysia agak rendah berbanding peningkatan permintaannya. Kebanyakan syarikat kosmetik dan farmaseutikal gemar menjadikan Halia Bentong, Halia Hitam, dan Kunyit Hitam sebagai komponen bahan dalam produk keluarannya kerana kandungan fitokimianya yang bermanfaat. HB, BG, dan BT juga kaya dengan nutrien dan antioksidan penting yang memberi manfaat kepada kesihatan manusia. Pertumbuhan yang perlahan disebabkan tempoh penanaman yang lama menyumbangkan kepada kekurangan benih Halia Bentong, Halia Hitam, dan Kunyit di pasaran. Kajian ini bertujuan untuk meningkatkan pengeluaran benih tanaman Halia Bentong, Halia Hitam, dan Kunyit Hitam yang cepat melalui kaedah mikropropagasi. Dalam kajian ini, kesan antibiotik dalam penghasilan bahan tanaman yang bebas pencemaran dan kesan PGR telah dikaji. Kajian pertama dijalankan menggunakan tunas rizom Halia Bentong, Halia Hitam dan Kunyit Hitam pada saiz yang berbeza (0.1-1.0 cm dan 1.1-2.0 cm) dengan rawatan menggunakan kepekatan ampicilin yang berbeza (20 mg/L dan 50 mg/L). Untuk kajian kedua eksplan yang terbentuk daripada kajian pertama digandakan dengan menggunakan kombinasi dan kepekatan hormon perangsang tumbuhan yang berbeza iaitu pada 1.0 mg / L BAP dengan 0.5 mg / L NAA dan 2.0 mg / L BAP dengan 1.0 mg / L NAA untuk induksi pucuk dan akar. Keputusan mendedahkan bahawa eksplan bebas pencemaran berjaya dihasilkan dengan rawatan menggunakan 50 mg/L ampicilin pada julat saiz eksplan diantara 1.1 cm sehingga 2.0 cm. Induksi pucuk tertinggi dapat diperhatikan dalam media MS yang mengandungi 2.0 BAP dan 1.0 NAA.

ABSTRACT

Nowadays, the supply of Bentong Ginger, Black Ginger, and Black Turmeric in the Malaysian market considerably low to meet the increase demand in pharmaceutical industry. Most cosmetic and pharmaceutical companies prefer Bentong Ginger (HB), Black Ginger (BG), and Black Turmeric (BT) as their component in the products due to its beneficial phytochemicals content. HB, BG, and BT are also rich in important nutrients and antioxidants that are beneficial to human health. Slow growth due to it long period to reach maturity had caused lack of Bentong Ginger, Black Ginger, and Black Turmeric to of seedling available in the market. This study was aimed to increase rapid production of Bentong Ginger, Black Ginger, and Black Turmeric through micropropagation methods. In this study, the effect of antibiotics in establishment of contamination-free explant and the effects of PGRs on the multiplication rate was investigated. First experiment was carried out using rhizome bud of Bentong Ginger, Black Ginger and Black Turmeric at different size of explant (0.1-1.0 cm and 1.1-2.0 cm) against different concentration of Ampicillin (20 mg/L and 50 mg/L). For the second experiment, plantlets form first experiment was multiplied using different PGRs combination of 1.0 mg/L BAP with 0.5 mg/L NAA and 2.0 mg/L BAP with 1.0 mg/L NAA for shoots and roots induction. The results reveal that contamination-free explant successfully establish with 50 mg/L Ampicillin for explant size range, 1.1 cm to 2.0 cm. The highest shoots induction was observed in MS media containing 2.0 BAP and 1.0 NAA.

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ANOVA	Analysis of Variance
BAP	6-benzylaminopurine
cm	centimetre
mg	milligram
MS	Murashige & Skoog
NAA	1-napthaleneacetic acid
L	Litre
UV	Ultra Violet

LIST OF SYMBOLS

ºC	Degree Celsius
%	Percent

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Zingiberaceae is a family of gingers which is herbaceous plant with flower and forming pseudo-stems by overlapping the base sheaths of the leaves; also known as distichous leaves. Generally, over 50 genera with 1600 species were recognized all over the worlds (Maarten & James, 2016). There is about 20-40 species were found in Malaysia and reported have been practised in ethnomedicine due to its pharmacologically active components (Rahmani, Al-Shabrmi, & Aly, 2014). Moreover, this aromatic herb is perennially grown and widely distributed all around the world down to its adaptation to pantropical climate. As evidence, Zingiberaceae can be found in both tropical and subtropical regions such as in Africa, Asia, Americas and having the most divers in Southeast Asia (Sirirugsa, Larsen, & Maknoi, 2007).

Bentong Ginger, Black Ginger and Black Turmeric (also identified scientifically as *Zingiber officinale* var Bentong, *Kaempferia parviflora* and *Curcuma caesia* respectively) are members of Zingiberaceae tribe, subfamily to Zingineroideae. From sanctuaries these herbs are used as folk medicines. The fact supported by (Shaneen, Christopher, & Deborah, 2015)

anaesthetic effects of Zingiberaceae extracts have been discovered in preclinical research. Bentong Ginger, Black Ginger and Black Turmeric were registered for rich in antioxidant and varies nutrients that are good to human health. As affirmation by MARDI show that Black Turmeric have lots of antioxidants, stabilize hormone, as anti-depressant, inflammation preventor, and etc. (Zuraida, et al., 2016). Besides being used in traditional remedy, some of Zingiberaceae species are important in industries of food and nutraceuticals because the presence of significance value of nutrient and phytochemical characteristics (Dana, et al., 2016) In additional, Zingiberaceae also utilized as ornamental plants, and essential oil extraction for therapeutical activities (Berihu, 2018).

The demand for Bentong Ginger in both domestic and global markets is influenced by its uniqueness features; thick rhizomes, less fibrous pulp and more aromatic (Akerele, Zainab, & Ayoade, 2017). Meanwhile, increasing market trend for Black Ginger and Black Turmeric were supported by its role-play in cosmetic and nutritional supplement production and its market price for both Black Ginger and Black Turmeric could exceed RM200 per kg (Zuraida & Ayu, 2015) and FAMA (2021).

In conventional cultivating method, high amount of ginger's rhizomes (Bentong Ginger, Black Ginger and Black Turmeric) needed as the planting material, as a result of slow growing rate and less of shoots sets per rhizomes (Solanky, Patel, & Patel, 2013). Large proportion of ginger rhizomes used to replant for the next planting seasons will lead to detrimental impact on gingers market sustainability; because the rhizomes are one of the most commercially gingers part. Furthermore, soil-borne pathogens such as *Ralstonia (Pseudomonas) solanacearum* frequently infect ginger vegetative reproduction (Trujillo, 1964). As a result, the availability of ginger deteriorated. Besides, highly chance for diseases and bacterial such as rhizome rots spread to other plants.

Therefore, a strategic approach needs to devised to assure the market continuity of ginger rhizomes. Utilised micropropagation or *in-vitro* method is known to facilitate mass propagation of planting materials in diverse crops such as banana, orchid, herbs, etc. The ability to multiply planting materials had successfully overcome the shortage of available seedling in many crops' species. In addition, free-disease explants were generated though micropropagation methods (Sathyagowri & Thayamini, 2011). However, to control yet eliminate bacteria in the explant, proper pre-treatment needed to minimize the contamination as well as maximize the survival rate of the explant (Nisar, Hawa, & Mansor, 2021). Afterward, the implementation of Auxin and Cytokinin in micropropagation as Plant Growth Regulator required to promote the mass production of shoot and root of the gingers (Seied, 2019)

1.2 Problem Statement

Infection of soil borne diseases become major factor to utilize mass propagation of ginger seedling using t *in-vitro* culture of *Zingiberaceae* (Bentong Ginger, Black Ginger and Black Turmeric). Proper pre-treatment should be conducted to prevent infection from bacteria and fungi that cause the disease. Moreover, to maximize the shoot and root production of *in-vitro* Bentong Ginger, Black Ginger and Black Turmeric, the enhance the protocol in the *in* vitro ginger production such as the combination of plant growth regulator needed for their optimum growth and development.

1.3 Objective of Study

The objectives of this study are;

a. To evaluate the effect of pre-treatment for the initiation of Bentong Ginger, Black Ginger and Black Turmeric.
b. To study the effect of the different combination of Plant Growth Regulator used on the growth and development of Zingiberaceae's (Bentong Ginger, Black Ginger and Black Turmeric).

1.4 Hypothesis

H_0: Difference pre-treatment and different combination of plant growth regulator give similar effect on the growth of Bentong Ginger, Black Ginger and Black Turmeric.

H_A: Difference concentration of pre-treatment and different combination of plant growth regulator give different effect on the growth of Bentong Ginger, Black Ginger and Black Turmeric.

1.5 Scope of Study

In this research, three different species of Zingiberaceae (*Zingiber officinale* Var. Bentong, *Kaempferia parviflora* and *Curcuma caesia*) were cultured using *in-vitro* methods using MS (Murashige & Skoog, 1962) media. The use of antibiotics (Ampicillin)

as pre-treatment and PGR to induce the plant growth and development in different concentration on the percentage of contamination, survival rate of the explant and the percentage of the shoots and roots induction for Bentong Ginger, Black Ginger and Black Turmeric were investigated. This had been conducted for about 4-5 months.

1.6 Limitation of Study

This study only manages to cover up to two-time multiplication stage and after initiation culture. Further study is needed to investigate the effect of different combination of plant growth regulator to the shoot's induction ratio, shoots elongation and roots formation. Moreover, further study is also needed to evaluate the best acclimatation protocol for commercial production of Bentong Ginger, Black Ginger and Black Turmeric seedlings.

1.7 Significance of Study

Micropropagation could facilitate the mass propagation of various crops species. The outcome of this study could overcome the shortage of Bentong Ginger, Black Ginger and Black Turmeric planting materials (rhizomes) in the market. Therefore, these could reduce the price for planting material (rhizome) and thus increase the Bentong Ginger, Black Ginger and Black Turmeric production and will subsequently increasing the farmers income and accelerating the production of pharmaceutical products based on ginger.

CHAPTER 2

LITERATURE REVIEW

2.0 ZINGIBERACEAES

The Zingiberaceae family is well-known for its pharmacological properties, and it is found throughout the tropics, especially in Southeast Asia. Zingiberaceae is one of the family under Zingiberales order, and it is the largest family among other families located within it. The fragrance feature presence of the Zingiberaceae family distinguishes it from other families, moreover the most extensive regarding to it uses (Ujang, Nordin, & Subramaniam, 2015). The most commonly produced species of Zingiberaceae are *Zingiber offcinale, Curcuma* sp., *Alpina galanga,* and *Kaempferia parviflora* as it widely uses in daily utilization. According to diagram below (Figure 2.0(a)), *Zingiber officinale* (Bentong Ginger), *Kaempferia parviflora* (Black Ginger) and *Curcuma caesia* (Black Turmeric) are located under Zingiberaceae Tribe, which is one of the tribe or sub family under Zingiberaceae family.

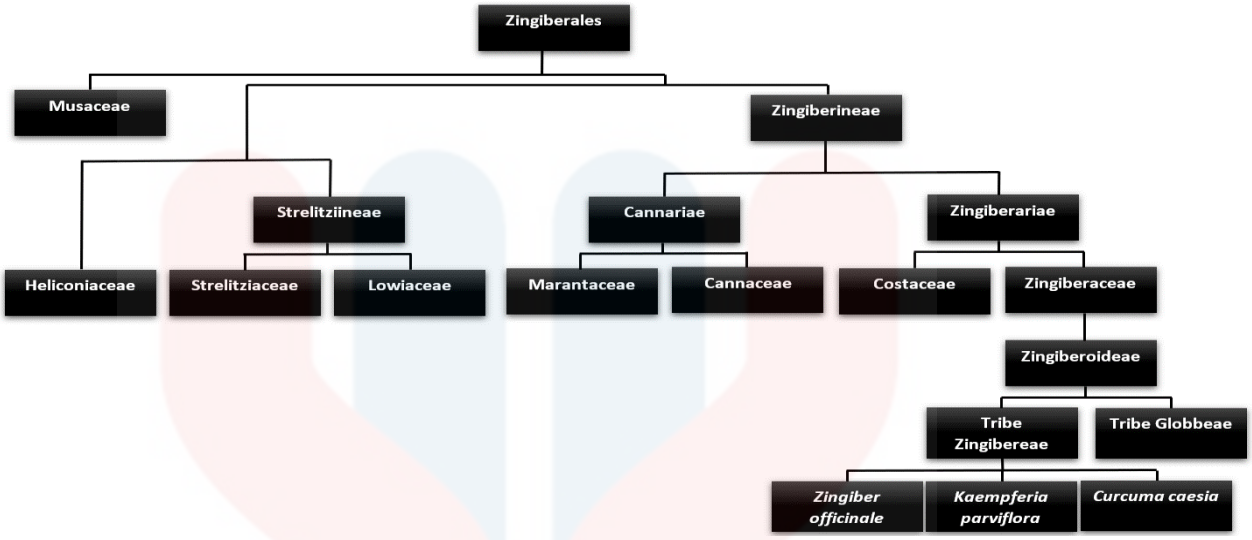


Figure 2.0 (a): Phylogeny of Order of Zingiberales (Location of Bentong Ginger, Black Ginger and Black Turmeric in Hierarchy) (Sass et.al, 2016)

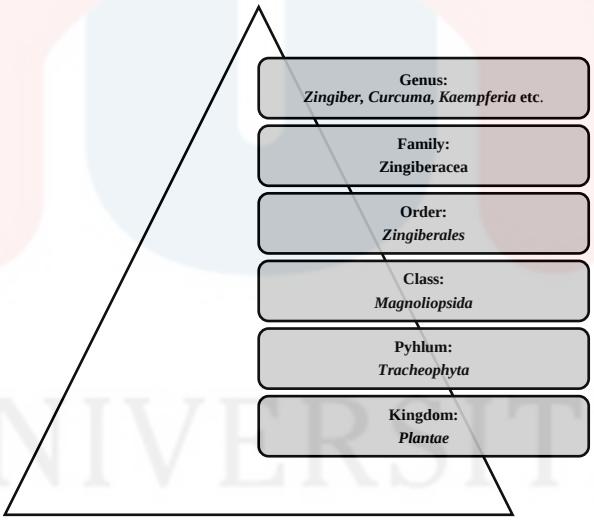


Figure 2.0 (b): Hierarchy of Scientific Classification of Ginger Family (ITIS, 2021)

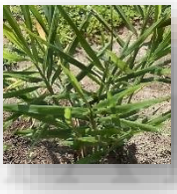
2.1 MORPHOLOGY

Zingiberaceae or ginger family's members are rhizomatous annual and perennial herbs. The rhizome has distinguishable segments and has sympodial branches. Rhizomes colours are differed for each species, might be pale yellow, deep yellow, greenish blue, pink, dark blue or even the mixture of these colours. Zingiberaceae plant can grew up to 6 meters.

The presence of scale leaves provide protection for immature rhizomes and axillary buds. True aerial stems are present only in certain genera and leafy shoots are often avascular. In some genera such as *Kaempferia* sp. and *Zingiber* sp., true short stem might presence while pseudo-stems might presence in *Curcuma* with clasping leaf sheaths. The leaves arrangement of Zingiberaceae are distichous, and the structure, shape, size, texture and venation are varied morphologically accordingly to each genus. Phenolic oils cells are present and leaves are distichously oriented transverse or aligned to the rhizome. Labellum are varying in size and showy. In some genera, lateral staminodes are present, filament is long, thin and exserted. Epigynous glands are normally two, linear or non-exist ovary are either unilocular or trilocular.

Generally, *Gingiberaceaes* rhizomes and fruits are pungent, nutritive and impetuses. The presence of volatile oils and oleoresins are used to distinguished the *Zingiberaceae* members. Some are consumed because they are high in starch and produced constrictive and perspiring juice. *Zingiberaceae* includes the genera *Alpinia, Amomum, Curcuma, Elettaria, Hedychium, Kaempferia* and *Zingiber* are important genera for having medicinal properties.

Table 2.1: Morphology, Origin and Genera of Bentong Ginger, Black Ginger and Black Turmeric

Criteria	Bentong Ginger	Black Ginger	Black Turmeric
Scientific Name	*Zingiber officinale* Roscoe	*Kaempferia parviflora*	*Curcuma caesia*
Genera	*Zingiber*	*Kaempferia*	*Curcuma*
Origin (Native)	Southeast Asia (Thailand, China, India Subcontinent and New Guinea) (Ravindran, Nirmala, & Kandaswamy, 2007)	Southeast Asia (Bangladesh, Cambodia, Myanmar and Thailand) (Ahmed, 2008)	Southeast Asia (Southern China, Indian Subcontinent, New Guinea and Northern Australia) (Techaprasan, Klinbunga, Ngamriabsakul, & Jenjittikul, 2010)
Rhizomes			
Leaves			

Stems



Flower

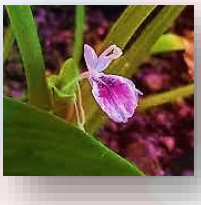
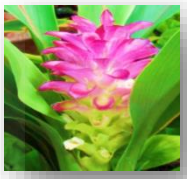




Photo Sources: https://myagri.com.my/2019/01/khasiat-halia-bentong/ , https://myagri.com.my/2019/12/cara-cara-menanam-kunyit-hitam/ , and https://mysihat.com/khasiat-kunyit-hitam/

2.2 History and Economic Important of Zingiberaceaes (Bentong Ginger, Black Ginger and Black Turmeric)

The Zingiberaceae family was being used as a cuisine, folk medicines, spices, seasoning, dye and flavouring from sanctuaries ago. In Malaysia, over 150 native and cultured Zingiberaceae species were identified, with up to 50 species being extensively used for it diverse uses including in culinary (Larsen, Ibrahim, & Khaw, 1999). Traditionally, ginger rhizomes are used to flavouring cuisine and are consumed raw or cooked.

One of the most popular ginger varieties in Malaysia is *Zingiber officinale* Roscoe Var. Bentong. Bentong Ginger named after the place it cultivated, which is mountain range in Pahang, Malaysia, also popularly known as Genting Highlands. Due to fertile soil and geographical factors, Bentong Ginger is well developed and cultivated, therefore

rhizome produced in Bentong is extra pungent, peppery and more expensive apart from standard commercial ginger (Ghasemzadeh, Jaafar, & Rahmat, 2016). In 2015, the Department of Agriculture, Pahang has recognized Bentong Ginger as a Geographical Indication (Tabbassum, Zeeshan, & Low, 2021). The terms “Indication” is referring to a symbol that is assigned onto the products with specified geographic origins.

On the hands, *Kaempferia parviflora* or botanically known as Black Ginger. This Thailand’s native pharmaceutical herbs can be discovered within 75 to 500 meters in elevation (Sirirugsa, 1991). In Thailand, it is locally known as *Krachai-dam* (Kitwetcharoen, Thanonkeo, Klanrit, & Thanonkeo, 2020) while, in Malaysia known as *Cekur Hitam*. It also can be discovered in other Asia countries include Laos, Myanmar, India, and China.

Other species of Zingiberaceae is *Curcuma caesia* which is Black Turmeric natively to Northeast, Central of India and Bangladesh. It knowingly as wild turmeric species and widely known as *Kali Haldi* or Black Turmeric (Shahinozzaman, Ferdous, Faruq, Azad, & Amin, 2013). Black Turmeric also broadly planted in Southeast Asia such as China, Taiwan, Sri Lanka, Indonesia, Peru, Australia and West Indies (Ghosh, Saha, Chatterjee, & Ghosh, 2013). Due to its industrial importance, Black Turmeric extensively spread in pantropical countries. This plant is currently classified as an endangered species because its natural habitat is being destroyed due to human activities such as overharvesting for medicine, industrial activities and urban development (Shahinozzaman, Ferdous, Faruq, Azad, & Amin, 2013).

2.2.1 Zingiberaceae Species (*Zingiber officinale* Roscoe, *Kaempferia parviflora* and *Curcuma caesia)* in Folk Medicine

Zingiberaceae family has long been valued for its nutritional and therapeutical properties. For centuries, Zingiberaceae rhizomes were being used as spices and sauces (Joy, Thomas, Matthew , & Skaria, 1998). Besides, women in confinement used to consume ginger rhizome and maternity midwife belief that ginger juice has a calming effect on breastfeeding women who frequently experience nausea and vomiting (Stanisiere, Mousset, & Lafay, 2018). Ginger also promotes appetites, metabolism, gastric production, and intestinal wall peristalsis. On the others hand, gingers also could remediate colds symptoms by promoting "good perspiration" and aids in the removal of excess "heat" away from the body, as well as removing and encounter infections and toxic in the body. Anti-inflammatory presence in ginger can alleviate with muscle cramp and tendinopathy caused by swelling and redness (Mashhadi, et al., 2013). Women are much prone to have cold hands and feet after giving birth, correspondingly gingerol compound content in the ginger potentially wider blood capillaries, improve circulation and warm up their body (Bode & Dong, 2011).

For generations, the *Kaempferia parviflora* rhizomes has already been utilised as natural remedy, health supplement herbs and indigenous medicine to treat ulcers, colic syndromes, allergies, gout, osteoarthritis, and inflammation (Saokew, et al., 2017). Despite being used as medicine, Black Ginger rhizomes also used as food and beverages. The raw rhizomes are used to make wine, while dried rhizome was crushed and powdered as sachet teas (Toda, Hitoe, Takeda, & Shimoda, 2016). It has been turned into a variety of nutritional supplements, including in form of medical honey-liquor, pills (powder form rhizome mixed with honey), caps, and tabs.

Curcuma caesia on the other hands, has become a great derivation of natural stock for human wellness prolonging. In ethnomedicine, *Curcuma caesia* Roxb rhizomes, both raw and processed (dried) have been used in the treatment of wounds, asthma, tumours, bronchitis, piles, and leukoderma (Das, Mondal, & Zaman, 2013). Not only that, Black Turmeric rhizomes are grated and used as massage remedy to relieved cramp muscle (Arulmozhi, Sridhar, Veeranjaneyulu, & Arora, 2006). *Curcuma caesia* plant is considered particularly fortunate in Madhya Pradesh, India and it is said that anyone who has it will never go hungry. While in Arunachal Pradesh, India, Adi natives consume a rhizome stock during diarrhoea (Kagyung, Gajurel, Rethy, & Singh, 2010) and Khamti tribes used rhizomes paste to treat snake and scorpion bites (Tag, Das, & Loyi, 2007). Nevertheless, Black Turmeric crushed rhizome paste is coats onto cuts or injuries to prevent haemorrhage and fasten recovery (Trivedi, 2003), prescribed in tonsil swelling (Mia, Kadir, Hossan, & Rahmatullah, 2009), and its roots powder are brewed to cure gastrointestinal issue (Badola, Idrisi, & Singh, 2010).

2.2.2 Zingiberaceae Species (*Zingiber officinale* Roscoe, *Kaempferia parviflora* and *Curcuma caesia)*: Pharmacological and Clinical Evidence

Enthusiastic phytochemicals found in Zingiberaceae species have been shown to have a variety of biological and therapeutic effects, including antitumor, antioxidants, anti-inflammatory, and significantly more. The therapeutic characteristics of Zingiberaceae must be exploited for modern application such as pharmaceuticals, nutrition products and beauty products, in order to meet customer needs for ethically, organic, maintainable, efficient, and cost-effective products. Several factors of operations

cost of Zingiberaceae should indeed be addressed in order to generate a high-value product, including sustainable supply chain management, standardisations and quality inspection, effective extraction procedures, continuity, and verification of its effectiveness.

Zingiber officinale Roscoe was being used as herbal remedy to cure a variety of ailments and conditions, including bowel inflammation diseases, arthritis, colds, dyspepsia, colon cancer (Zhang, et al., 2021) and Alzheimer's diseases. Extraction of ginger has anti-diabetic, anti-allergic, anti-emetic, anti-inflammation, anti-nauseant, antioxidants, anti-parasitic, anti-platelet, anti-pyretic, anti-tussive, cardiovascular, digestive and hypoglycaemic characteristics. Study by Choi, Kim and Oh, 2017 confirm that ginger's active compounds have therapeutic efficacy in age-related neurological disorders (ANDs). Ginger also aided COVID-19 patients in minimizing their recovery time and alleviating their severe symptoms (Safa, et al., 2020). The abundance of significant phenolics and flavonoids in the leaves, stems, and rhizomes has indeed been recognized as a major role in its pharmacological properties. Flavonoid compound such as rutin, kaempferol, quercetin, catechin, naringenin, and epicatechin can be extracted from Bentong Ginger leaves and its rhizomes (Ghasemzadeh, Jaafar, & Rahmat, 2010).

The extraction of *Kaempferia parviflora* (Black Ginger) rhizomes has been proven in pharmaceutical researches to have anti-gastric ulcer, anti-microbacterial, anti-viral properties, anti-inflammation (Tewtrakul, Subhadhirasakul, Karalai, Ponglimanont, & Cheenpracha, 2009), anti-depression (Wattanathorn, Pangpookiew, Sripanidkulchai, Muchimapura, & Sripanidkuchai, 2007), anti-cholinesterase (Sawasdee, Sabphon, Sitthiwongwanit, & Kokpol, 2009), anti-cancer, and aphrodisiac (Wattanapitayakul, et al., 2007). Preliminary study by Jeong, et al., 2016 verified that Black Ginger ethanol extraction have the ability to suppress the establishment of *Cronobacter* spp. and

Enterohemorrhagic Escherichia coli (EHEC). Despite that, Black Ginger also commonly utilised as complementary medicine to treat variety of ailments, includes fungal infection, gastrointestinal disorders, diminished vigour, and allergies (Tewtrakul, Subhadhirasakul, & Kummee, 2007). Furthermore, recent study found that Black Ginger intake enhanced blood flow due to induce of vasorelaxation across cyclooxygenase and nitric-dependent processes, resulting in a synergistic impact of increasing cardiac output and anti-inflammation actions which might aid in muscle strengthen (Promthep, Eungpinichpong, Sripanidkulchai, & Chatchawan, 2015). Besides, Matsushita, et al., (2015) found that *Kaempferia parviflora* extract can significantly improve entire body energy intake by activating brown adipose tissue, which beneficially act as anti-obesity regimen.

Curcuma caesia was been reported for exhibited anti-fungal activity, anti-asthmatic, anti-oxidant, anti-bacterial activity, anti-convulsant and anti-ulcer (Bordoloi, Phukan, & Singh, 2012). On the other hands, extraction of *Curcuma caesia* also significantly aids in locomotor depressant, muscle relaxant effects (Arulmozhi, Sridhar, Veeranjaneyulu, & Arora, 2006) and Central Nervous Systems (CNS) depressant effects (Karmakar, et al., 2011). Correspondingly, studies established that Black Turmeric ethanol extraction show significant neuropharmacological action (Karmakar, Saha, Sarkar, S, & Haldar, 2011), anti-ulcer efficacy and reduce volume and production of gastric acid in the stomach. The presence of phenolic compound in extraction of Black Turmeric could inhibits *Bacillus subtilis* bacteria activity (Nambisan, Angel, & Vimala, 2012). It is also found that, extraction of volatile oil from Black Turmeric leaves has the ability to germinate rice seeds and help in secondary root formation activity (Lalitha, Raveendran, & Salim, 1995). Black Turmeric has been shown to be a potent anxiolytic and antidepressant herb. Study Karmakar, et al., (2011) by found that methanol extract of

Black Turmeric could inhibit the pentobarbitone in which induce sleep duration significantly.

2.3 Crop Improvement Through Micropropagation

Ginger's demand has skyrocketed in the global marketplaces as a result of all of these distinctive attributes. In additional, ginger is sterile plant that can only be cultivated through vegetative reproduction. For few agricultural productions, micropropagation has become a significant means of economic reproduction (Miri & Roughani, 2018). Due to the fact, conventional ginger breeding operations are limited caused by deficiency of fertility and pure seed (rhizome) sets (Teferra, et al., 2013). However, because *Pythium* spp. and *Ralstonia solanacearum* are significantly induce ginger rhizome rots, *in-vitro* propagation can be employed to establish diseases-free explant (Hasanloo, Kermani, Dalvand, & Rezazadeh, 2019).

Micropropagation defined that plant is propagated by cultivating explants (tissues/cells/organs/seeds/embryos or other plant parts) in controlled environment (temperature, photoperiod, pH, and nutrients) on an artificial media (nutrient medium) by *in-vitro* method (Saurabh, Kiran, Randhir & Tanmoy, 2015). Moreover, *in-vitro* propagation methods is a useful approach to investigate and conserving rare, endangered, and vulnerable medicinal and native herbs (Kapai, Kapoor, & Rao, 2010), including significantly facilitate clonal multiplication of genetically stable and pure type plant progeny (Bhojwani, 1996). The ability of a plant to undergo micropropagation determined by its totipotency which is defined as the ability to develop into whole plants

or plant organs *in-vitro*. Its ability is also influenced by the presence of plant hormones which are auxin and cytokinin.

Generally, auxin is hormones that trigger the cells in plants to elongate, while cytokinin hormones are responsible for stimulating cell multiplication of plant roots. Both hormones are important for plant growth and development. There are few factors impact the success of micropropagation of plants; growth media, environment factors, explant source and explant genetics. Minerals, carbons sources and plant growth regulators (PGR) are the basis of the artificial planting media. Each of the plants required a different amount of mineral, carbons and PGR for its growth and development. Meanwhile, growth and development of explant is also controlled by environment such as photoperiod, light and temperature. Besides that, age of explant, organ type and genetics of plant species also will determine the resourcefulness of the micropropagation. In accordance with that, plant micropropagation proves that the propagation process can be fastened, besides speeding up the selection process for crop improvement without changing the genetics of the plants. Therefore, time saving and time management could be controlled. Moreover, micropropagation also allows for the mass production of a large number of identical individuals from a small amount of starting material. Seasonal limitations for germination or cultivation also can be overcome, while diseases such as soil borne disease could be prevented successfully.

Gingers are vegetative reproductive plants. It reproduces through rhizomes. New shoots will be formed from the rhizome which later develop into new plants naturally. In ginger micropropagation, 0.5-2.0 cm of new shoots will be cut off from mature rhizomes (Ghosh, Saha, & Chatterjee, 2013). Kitwetcharoen, Thanonkeo, Klanrit and Thanonkeo (2020) emphasize that rhizomes must be at least 10 months before they undergo micropropagation. It will undergo surface sterilization with detergent and running tap

water to wash out the dirt's, and next subjected to 5 min immersion of 70% ethanol treatment and 0.1% mercuric chloride solution (Ghosh, Saha, & Chatterjee, 2013). While, Khairudin, Haida and Hakiman (2020) suggested that use of 5.25% of sodium hypochlorite with 50% concentration added with 2-3 drops of Tween-20 followed with 3 times distilled water rinsed gave positive effects toward contamination prevention protocol. Contamination indicates the presence of fungal and bacteria that will infect the explants. To eliminate and handle the contamination culture, it needs to be autoclaved first to kill all possible bacteria and fungus. Autoclaved contaminated media are thrown into a hazardous plastic bag and jars are washed and dried before being used again (Carey, Payton & McDaniel, 2015). Labrooy, Abdullah and Johnson (2020) stated that sterilized explant cultured into jar that contain Murashige and Skoog (1962) media supplemented with 6% sugar (sucrose) and PGR (4.44 μM BA + 1 ml NAA) have significantly show most functional for generation of micro-rhizomes.

2.4 Antibiotic Application in Contamination Controlling

Plants from nature are frequently infected with diseases including bacteria and fungi, surface disinfection is required to ensure explants are free contamination. Surface sterilisation has also been accomplished using a variety of methods, including physical and chemicals treatments, which is ultrasonic and heat treatments, and sodium hypochlorite, ethanol, anti-fungal and also anti-biotics (Benzie & Strain, 1997). According to Reed, Bucley and DeWilde, (1995), antibiotics application was efficient towards bacterial isolation, however they might not be utilised to contamination treatment of explant tissues because of phytotoxicity or defective penetration into plant cells.

Nevertheless, the harmful impacts on plant culture and the probability of producing antibiotics resistant microorganisms, continuing usage of overdosage antibiotics in growth medium is prohibited (Shehata, Wannarat, Skirvin, & Norton, 2010).

Rhizome borne pathogens, play significant role in diseases transmission throughout infected plants, soils and water, resulting in accelerated interest for healthy planting materials (sprouted rhizomes) for large and sustainable farming (Dohroo, 2005). A study by Tewelde, Patharajan, Teka and Shatu (2020) reported that culture colonized by bacterial and fungal contamination has become a major problem in most plant tissue culture companies. In order to cope with these contamination problems, Wojtania, Pulawska and Gabryszewska (2005) had suggested the use of antibiotics in the culture media. From previous research, antibiotics mixture (kanamycin, gentamicin, streptomycin and cefotaxime) added to MS culture media show less effectiveness toward bacteria and fungus infection. Moreover, explant die for antibiotics more than 50 mg/L. On the other case, Jain, Aravindraram & Pal, (2016), suggested that, bacterial growth was inhibited effectively using 100 mg/L Cephotaxime in ginger establishment stage of micropropagation with no presence of toxicity side effects.

2.5 PLANT GROWTH REGULATOR TO ENHANCE GROWTH PERFORMANCE

The aseptic culture establishment, shoot regeneration capability, roots, and acclimation are all important factors in the *in vitro* technique's effectiveness. Rhizome buds, a common source of explants in the Zingiberaceae family, have been shown to be more active. Shoot regenerate capability, on the other hand, is influenced by the growth medium composition and plant growth regulators (PGR), primarily cytokinin. For ginger shoot multiplication, Murashige and Skoog (MS) medium enriched with 6-benzylaminopurine (BAP) is more usually utilised (Thakur, Sharma, & Kumari, 2018).

Furthermore, a well-developed root system is an important phase in plant vegetative propagation, ensuring a high survival rate of micro-propagated plantlets during the acclimation period (Miri, 2020). Study done by Mehaboob, et al., 2019, show that Auxins supplement in the growth media improves *in-vitro* root induction of ginger. Meanwhile, *in-vitro* root induction in distinct Zingiberaceae species was impacted differently by different types and amounts of auxin (Jualan, Nurul Humairah, Devina, & Hartinie, 2015). Therefore, Identifying the best type and concentration of auxin can improve ginger root induction *in vitro* and, as a result, make acclimation and successful establishment of in vitro-raised plantlets easier in the field.

CHAPTER 3

METHODOLOGY

3.1 Preparation of Planting Materials

Zingiber officinale Rosc. "Bentong" was obtained from herbal shop in Bentong Pahang. Meanwhile *Kaempferia parviflora* and *Curcuma caesia* were purchased online from Bandar Tun Abdul Razak Pahang and Parit Raja Johor respectively. Rhizomes of the gingers and turmeric were cultivated in moist peatmoss for at least 3 weeks at Plant Tissue Culture nursery. Buds' rhizomes about 0.5-1.5 cm were cut and used as explant (Figure 3.1) for initiation culture at Plant Tissue Culture Laboratory, University Malaysia of Kelantan Jeli Campus.

Figure 3.1: Explant size (Left 0-1 cm, Right 1-2 cm)

3.2 Experimental Design

The experiment was conducted using Complete Randomized Block Experimental Design (CRBD) with the factorial 3 X 3 X 2 x 6 consist of 3 type of plant species,3 treatments, 2 different rhizome size and 6 replications (Table 3.1). The total number involve for initiation culture was 108 samples. Rhizomes are survived for the second experiment (refer Table 3.2 b), shoots that produced from first experiment (initiation stage) was subjected to multiplication stage against 5 Plant Growth Regulator (PGR) treatments based on its concentration with factorial 3 X 5 X 6, which consist of 3 type of plant species, 5 PGR concentration and 6 replications. Thus, the total culture involved in this experiment was 144 samples.

Table 3.2 (a): Treatments for initiation culture of Bentong Ginger, Black Ginger and Black Turmeric

Type of Explant	Size of explant (cm)	Code of treatment	Type of Treatment	Number of samples
Bentong Ginger	0.1-1.0	Control	MS media + 0 mg/L ampicillin	6
		T1HBI	MS media + 20 mg/L ampicillin	6
		T2HBI	MS media + 50 mg/L ampicillin	6
	1.1-2.0	Control	MS media + 0 mg/L ampicillin	6
		T1HBII	MS media + 20 mg/L ampicillin	6
		T2HBII	MS media + 50 mg/L ampicillin	6
Black Ginger	0.1-1.0	Control	MS media + 0 mg/L ampicillin	6
		T1BGI	MS media + 20 mg/L ampicillin	6
		T2BGI	MS media + 50 mg/L ampicillin	6
	1.1-2.0	Control	MS media + 0 mg/L ampicillin	6
		T1BGII	MS media + 20 mg/L ampicillin	6
		T2BGII	MS media + 50 mg/L ampicillin	6
Black Turmeric	0.1-1.0	Control	MS media + 0 mg/L ampicillin	6
		T1BTI	MS media + 20 mg/L ampicillin	6
		T2BTI	MS media + 50 mg/L ampicillin	6
	1.1-2.0	Control	MS media + 0 mg/L ampicillin	6
		T1BTII	MS media + 20 mg/L ampicillin	6
		T2BTII	MS media + 50 mg/L ampicillin	6
			TOTAL SAMPLE	**108**

Table 3.2 (b): Treatments for shoot and root initiation of Bentong Ginger, Black Ginger and Black Turmeric

Type of Explant	Type of Treatment	Code of Treatment	Number of Sample
Bentong Ginger	MS Media + 0 PGR	Control HB	6
	MS Media + 1.0 mg/L BAP + 0.5 mg/L NAA	HBT1	6
	MS Media + 2.0 mg/L BAP + 1.0 mg/L NAA	HBT2	6
Black Ginger	MS Media + 0 PGR	Control BG	6
	MS Media + 1.0 mg/L BAP + 0.5 mg/L NAA	BGT1	6
	MS Media + 2.0 mg/L BAP + 1.0 mg/L NAA	BGT2	6
Black Turmeric	MS Media + 0 PGR	Control BT	6
	MS Media + 1.0 mg/L BAP + 0.5 mg/L NAA	BTT1	6
	MS Media + 2.0 mg/L BAP + 1.0 mg/L NAA	BTT2	6
		TOTAL	**54**

3.3 Culture Media Stock Preparation

First and foremost, micronutrients, macronutrients, vitamins and ferum sources were prepared as a stock. These stocks were required for later used in MS media preparation. In this research, 20X, 200X, 500X and 200X concentration for macronutrients, micronutrients, vitamins and ferum sources respectively was prepared. Table 3.3 (a) shows the amount of compound needed for macronutrients, micronutrients and vitamins stock preparation.

Stock was prepared by weigh the required amount of chemicals (according to the table 3.3 (a)) and was dissolved one by one to avoid chemical reaction among chemicals. Note that each stock requires a different conical flask. Once all the chemicals completely dissolve in the solution, the solution was filled up to the final volume of the conical flask used. The stock then needs to wrap with aluminium foil, labelled and keep in the refrigerator at 4 degrees Celsius.

On the other hand, PGR stock was also prepared for the MS media which is used for initiating the shoots and roots formation of the Bentong Ginger, Black Ginger and Black Turmeric. Table 3.3 (b) shows the PGR stock preparation.

Table 3.3 (a): Stock Media Solution Preparation Table

Stock (Component)	Concentration (Amount)
Macronutrient Stock	**20X/500 ml**
Ammonium Nitrate (NH_4NO_3)	16.56 g
Potassium Nitrate (KNO_3)	19 g
Calcium Chloride ($CaCl_2.2H_2O$)	4.4 g
Magnesium Sulphate ($MgSO_4.7H_2O$)	3.7 g
Micronutrient Stock	**200X/500 ml**
Manganese Sulphate ($MnSO_4.4H_2O$)	2.23 g
Zinc Sulphate ($ZnSO_4.H_2O$)	0.86 g
Boric Acid (H_3BO_3)	0.62 g
Potassium Iodide (KI)	0.083 g
Sodium Molybdate ($Na_2.MoO_4.2H_2O$)	0.025 g
Cupric Sulphate ($CuSO_4.5H_2O$)	0.025 g
Cobaltous Chloride ($CoCl_2.6H_2O$)	0.025 g
Vitamin Stock	**500X/125 ml**
Myo-inositol	6.25 g
Glycine ($C_2H_5NO_2$)	0.125 g
Thiamine-HCl (Vitamin B1)	0.0625 g
Nicotinic Acid (Vitamin B3)	0.03125 g
Pyridoxine-HCl (Vitamin B6)	0.03125 g
Ferum Source	**200X/ml**
Ferrous Sulphate ($FeSO_4.7H_2O$)	2.78 g
Sodium Ethylenediaminetetraacetic Acid ($Na_2EDTA.2H_2O$)	3.73 g

Table 3.3 (b): PGR Stock Preparation Table

PGR Stock (100 X)	
Component	**Amount (g/100 ml)**
BAP	0.1
NAA	0.2

3.4 Culture Media Preparation

Media that used in this micropropagation of Bentong Ginger, Black Ginger and Black Turmeric is MS (Murashige & Skoog, 1962) media with plant growth regulator stocks. The basal medium was used to provide all the compounds needed for the plant (Bentong Ginger, Black Ginger and Black Turmeric) growth and development. MS media were consisted of 95% water, macronutrients, micronutrients, vitamins, amino acids, sugars and plant growth regulators. Each stock was required in different amounts in the media. The volume of micronutrients added was much less than macronutrients due to its function contributing to the plant growth development. Organic substances such as sugar (sucrose, fructose, maltose or glucose) and vitamins provide essential and basic needs for plant life. Vitamins help in metabolic function while sugar acts as carbon sources required by the plant to generate photosynthesis effectively. Meanwhile, plant growth regulators (PGR) were required to initiate the formation and growth of plant shoots and roots. The PGR were proposed in this research are auxin and cytokinin. Morphogenesis of BG and BT were determined by the ratio of PGR used.

Appropriate nutrient medium and media ingredients were giving optimal results to the mass propagation of Bentong Ginger, Black Ginger and Black Turmeric. Supplement mediums in plant tissue culture give water, mineral dietary requirements, nutrients, development controllers, admittance to air for gas trade and mechanism for evacuation of plant metabolite squander.

3.5 Surface Sterilization of Explant

Bentong Ginger, Black Ginger and Black Turmeric rhizomes were washed under running tap water before undergoing surface sterilization. After 2 hours soaking under running tap water, the rhizomes were soaked in 75% ethanol to kill the bacteria on the surface of the rhizomes. After that, the rhizomes were rinsed with distilled water. In the same beaker, 50% of Clorox and 1-3 drops of Tween-20 was added and left for 20 minutes. Rhizomes were fully immersed in the solution for surface sterilization. Then, the rhizomes were rinsed with distilled water 5-6 times before trimming the buds. All the aseptic steps done in laminar flow by done surface sterilized with 75% ethanol and UV light. Then, the rhizomes were trimmed into 1.5 to 2 cm and cultured into the MS media. Jars were sealed and kept in the growth room.

3.6 Initiation Culture

Subsequently, the sterilised explants were then placed in test tube containing basal media (Murashige and Skoog, MS media) treated with 0 mg/L, 20 mg/L, and 50 mg/L ampicillin antibiotics respectively. The test tube containing explants then incubated at standard culture growth room at 23-27 ºC with an intensity of light of 2000 Lux and a photoperiod of 16 h. explants condition were observed after 15-17 days. Any presence of contamination and death of explants were recorded as in the Table 3.2 (a). The *in vitro* plantlets were used for the multiplication stage.

3.7 Subculturing

Successfully established explants were subcultures in test tube containing basal media (MS Media) with 0 mg/L BAP and NAA, 1 mg/L BAP + 0.5 mg/L NAA, and 2 mg/L BAP + 1 mg/L NAA respectively. The test tubes were incubated at standard culture growth room at 23-27 ºC with an intensity of light of 2000 Lux and a photoperiod of 16 h. Number of shoots, shoots length and number of roots were recorded as in Table 3.2 (b) in one month's incubation period intervals.

3.8 Data Collection and Analysis

Change or presence of contamination were observed after a week of initiation culture and after 4 weeks of subculturing of Bentong Ginger, Black Ginger, and Black Turmeric on its roots and shoots induction. The effect of pre-treatment and concentration of PGR were analysed by observing the number of survive explants, number of contamination explants, and survival rate of the explant, effects of PGR on shoots, roots induction, and length of explants shoots of Bentong Ginger, Black Ginger, and Black Turmeric. The entire data were analysed using Statistical Package for Social Science (SPSS 27.0) and the mean between all treatments were calculated using Analysis of Variance (ANOVA). The process of experiments can be described as in Figure 3.0.

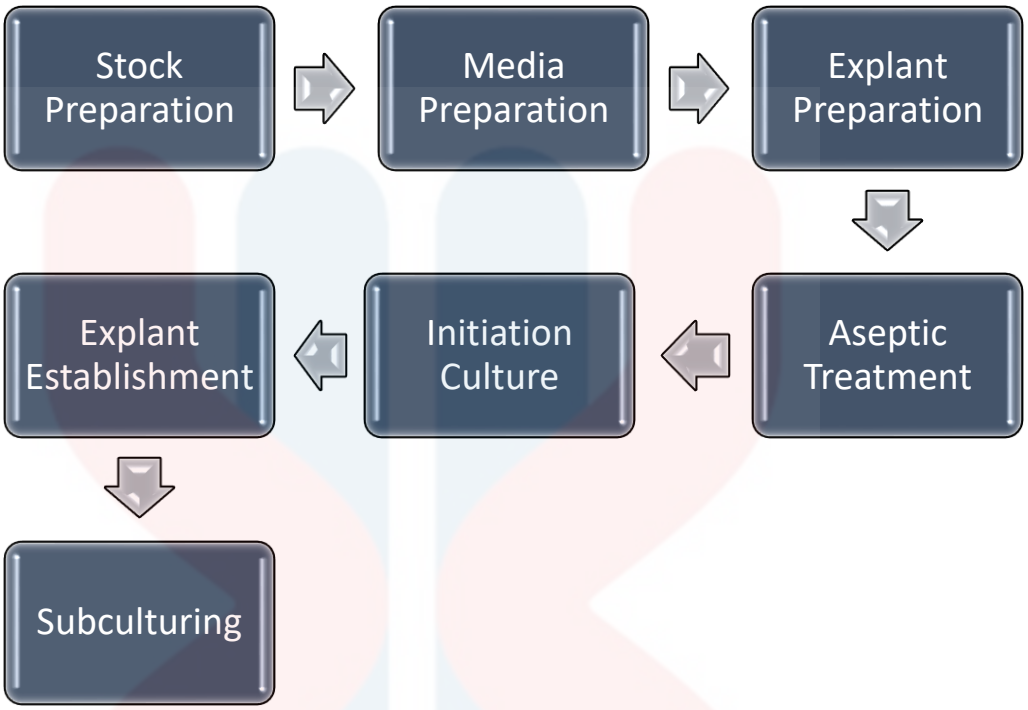


Figure 3.0: Experiment process of Bentong Ginger, Black Ginger and Black Turmeric

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Initial Culture Establishment

As indicated in the literature review, the aim of this analysis was to construct an *in vitro* propagation procedure and analyse the effect of combining PGR (BAP and NAA). Although the rhizome produced buds were a fast-responding explant, contamination-free culture establishment proven to be difficult. The contamination of ginger rhizome produced bud explants was significantly reduced after surface sterilisation with Clorox (Sodium hypochlorite). Regardless, the survival rate of explants in the culture was reduced due to the harmful effects of a high Clorox concentration and long-term exposure. Antibiotics should be used as a pre-treatment to ensure contamination-free explants. As a result, the first section of the research looked into the influence of antibiotics (Ampicillin) on explant survival rates explants, accordingly to the size of explants.

4.1.1 Explant Survival Rate Across the Size of *Zingiber officinale* Var. Rocs cv. Bentong Explant Against Ampicillin Concentration

According to the Table 4.2.1 and Figure 4.1.1 below, the survival rate was significantly high for rhizomes' size between 1.1-2.0 cm which is 55.56% survival explant. For the percentage survival explant between treatment of antibiotics, there is no difference for both size of explant of the same 50 mg/L ampicillin treatment which is 66.66% respectively. Meanwhile, the lowest survival rate explant was recognised in 0.1-1.0 cm explant size with 0 mg/L treatment of ampicillin concentration. This due to contamination rate higher compared to treated explant with ampicillin antibiotics. Contradictorily, the least number of contaminated explants successfully maintaining a greater percentage of survival.

Table 4.1.1: The Effect of Various Bentong Ginger's Rhizome Size Explants Against the Concentration of Antibiotics

Size of Explant (cm)	Ampicillin (mg/L)	Number of survival Explant	No. of Contaminate Explant	Percentage Survival Explant (%)
0.1-1.0	0	1	5	**16.67**
	20	2	4	**33.33**
	50	4	2	**66.66**
	Total	**7**	**11**	**38.89**
1.1-2.0	0	2	4	**33.33**
	20	4	2	**66.66**
	50	4	2	**66.66**
	Total	**10**	**8**	**55.56**
TOTAL		**17**	**19**	**47.22**

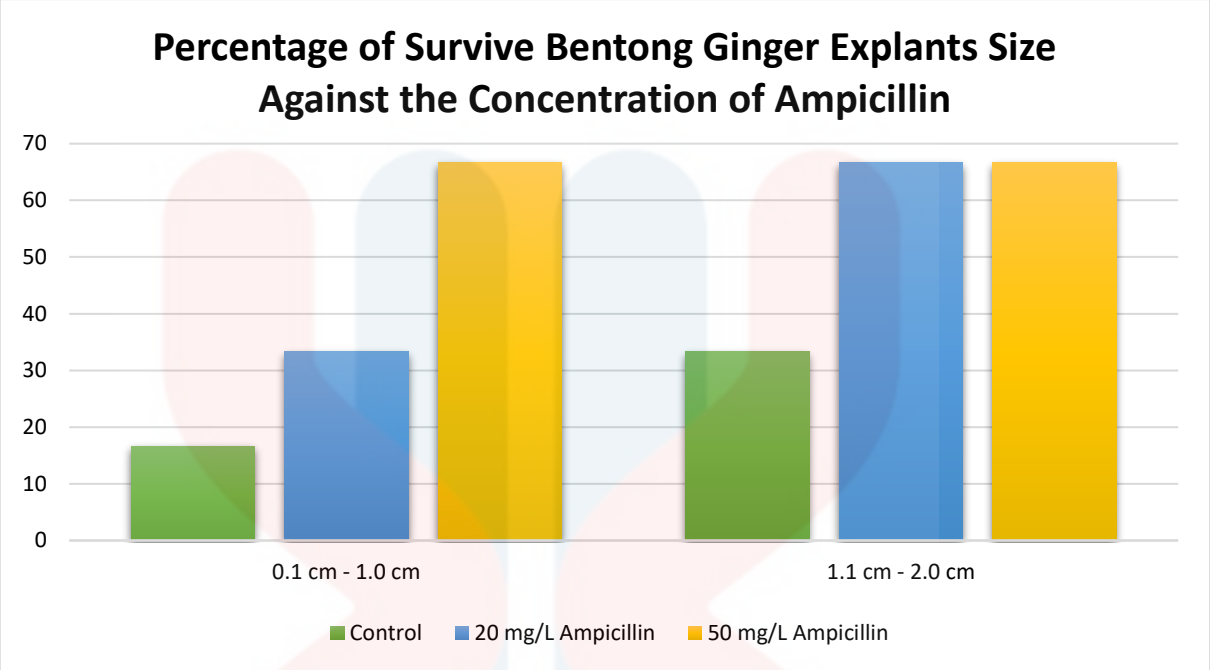


Figure 4.1.1: Percentage of Survive Bentong Ginger Explants Size Against the Concentration of Ampicillin

4.1.2 Explant Survival Rate Across the Size of *Kaempferia parviflora* (Black Ginger) Explant Against Ampicillin Concentration

On the other hand, after one week of initiation culture, the percentage of survive explant is higher for the explant size 1.0-2.0 cm as compared to 0.1-1.0 cm, with 66.67% and 38.89% respectively (Table 4.1.2 & Figure 4.1.2). Within the explant size between 0.1 cm to 1.0 cm, explant treated with 50 mg/L ampicillin shows the highest rate of survival at 66.66% while the control (non-ampicillin treated) the survival rate is only 20%. By contrast, explant treated with 50 mg/L ampicillin for explant size between 1.0 cm to 2.0 cm, show the highest survival rate with 83.33% with 5 explants survive from 6 explants. From the tabulated data, contamination cause by fungus and bacteria had affect the survival rate of both explants size.

Table 4.1.2: The Effect of Various Black Ginger's Rhizome Size Explants Against the Concentration of Antibiotics

Size of Explant (cm)	Ampicillin (mg/L)	Number of survive Explant	No. of Contaminate Explant	Percentage Survive Explant (%)
	0	1	5	**20**
0.1-1.0	20	2	4	**33.33**
	50	4	2	**66.66**
	Total	**7**	**11**	**38.89**
	0	3	3	**50**
1.0-2.0	20	4	2	**33.33**
	50	5	1	**83.33**
	Total	**12**	**6**	**66.67**
TOTAL		**19**	**17**	**52.78**

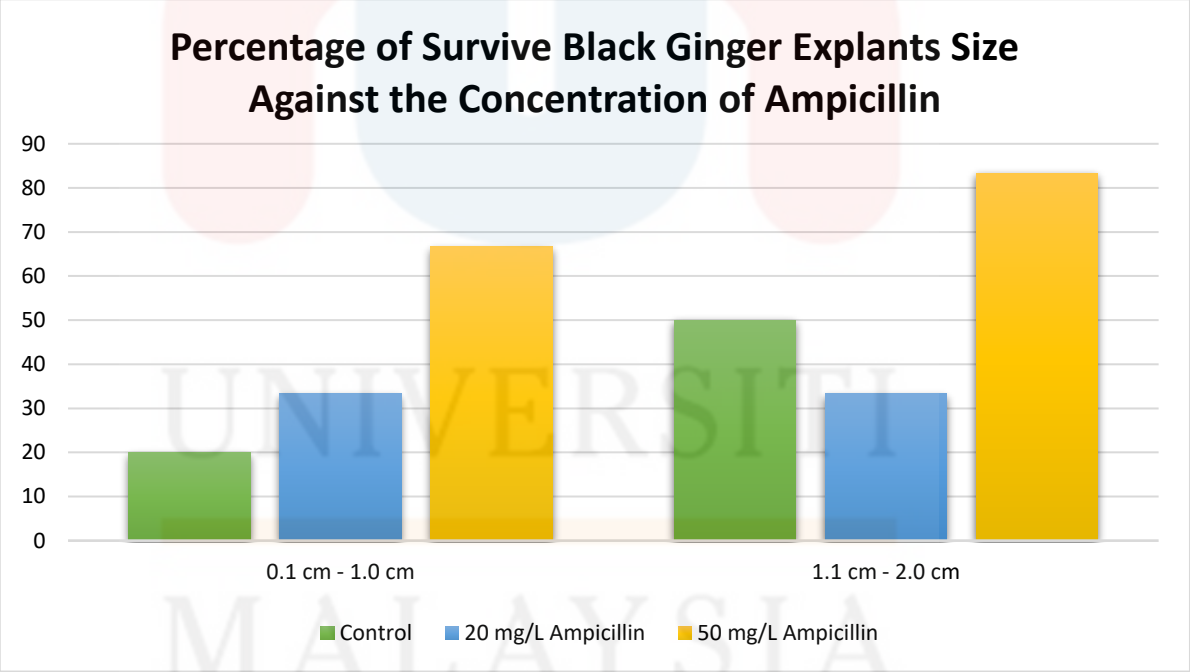


Figure 4.1.2: Percentage of Survive Black Ginger Explants Size Against the Concentration of Ampicillin

4.1.3 Explant Survival Rate Across the Size of *Curcuma caesia* (Black Turmeric) Explant Against Ampicillin Concentration

As stated in Table 4.1.3 and Figure 4.1.3 below, black turmeric explant shows higher percentage of survival rate for the size of explant used (between 1.1cm to 2.0 cm) as compared to the size explants of 0.1 cm to 1.0 cm, at 66.67% and 33.33% respectively. The highest number of contaminated explants observed was for non-ampicillin treat of explants size between 0.1-1.0 cm at 0% set as compared to the 1.2-2.0 cm explant size. This indicate that, the size explant influences the rate contamination in the establishment of sterile explants. Furthermore, the highest survival rate had detected in explants treated with 50 mg/L ampicillin with the explants size of 1.1-2.0 cm at 83.33%.

Table 4.1.3: The Effect of Various Black Turmeric's Rhizome Size Explants Against the Concentration of Antibiotics

Size of Explant (cm)	Ampicillin (mg/L)	Number of survival Explant	No. of Contaminate Explant	Percentage Survival Explant (%)
	0	0	6	**0**
0.1-1.0	20	2	4	**33.33**
	50	4	2	**66.67**
	Total	**6**	**12**	**33.33**
	0	3	3	**50**
1.1-2.0	20	4	2	**66.67**
	50	5	1	**83.33**
	Total	**12**	**6**	**66.67**
TOTAL		**18**	**18**	**50**

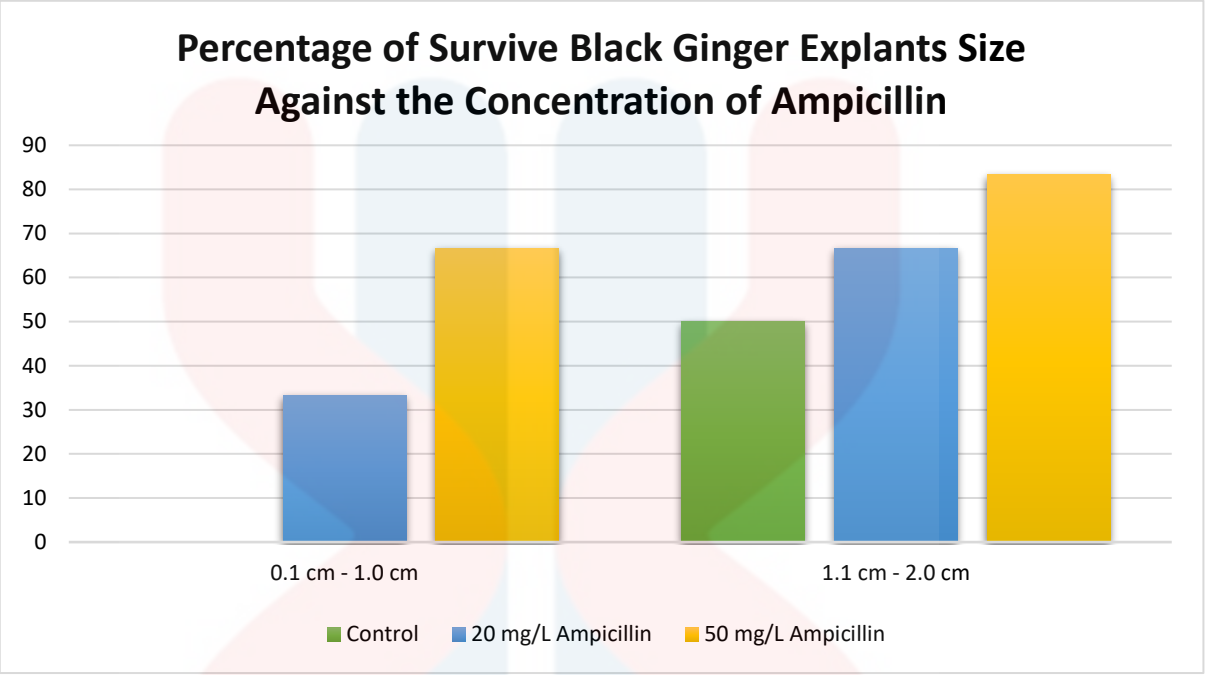


Figure 4.1.3: Percentage of Survive Black Turmeric Explants Size Against the Concentration of Ampicillin

The findings revealed that the thinner the explant, the more difficult the culture, hence the culture media normally includes additional components. To keep the culture sustained, larger explants potentially have higher nutritional reserves and endogenous plant growth regulators. This finding contradicts with the widely held belief that the tinier the explants, the lesser the chance of contamination (Seran, 2011). The source of contamination was observed and identified. Most of the contaminants are caused by bacteria and fungi (Figure 4.3) which are might found on the surface or trapped in scratch, scale, utensils and environment. According to (Islam, Kloppstech, & Jacobsen, 2004), some researchers claimed that contamination of underground rhizomes was extremely high, making it difficult to generate contamination-free cultures.

Decline in survival rate for the explants size between 0.1 cm to 1.0 cm is possibly caused by endogenous bacteria presence in the cultures, fungus spore contains in the air,

water and utensils or nutrient reduction in the media. Large number of contaminations observed for explants size between 0.1-1.0 cm might due to death of explant caused. These lead to slow recovery and affecting the nutrient intake form the planting media to establish the shoots. Meanwhile the bigger explants recover quicker utilising obtainable nutrient in the explants. In addition, high concentration of Ampicillin could lead to elimination and death of explant tissues. Moreover, Tewelde, Patharajan, Teka and Sbhatu (2020) suggest that, single antibiotics might be ineffective in preventing bacterial contamination of explants and media. As a results, a combination of antibiotics is recommended. However, limited study to investigating the effect of ampicillin towards small rhizome explant was reported.

Nevertheless, significant amount of available nutritional in explants (1.1-2.0 cm) might be the factors of high mitogenic reaction that influencing the survival rate of the explants. In additional, Ei Boullani et al., (2017), stated that the larger explants size, the faster recovery and higher survival rate of the explants. Antibiotics were frequently utilised to control the bacteria and fungi contamination in *in vitro* explants, and over dosage or concentration of antibiotics potentially kill explants critical tissues. However, larger surface area of explant size (1.1-2.0 cm) and its thickness could prevent the antibiotics damaging the tissues cell. Therefore, explants of 1.1-2.0 cm, treated with 50 mg/L Ampicillin successfully prove the highest survival rate among those treatments. In this finding, we can conclude that, different concentration of ampicillin, present independent effect on survival of explant, nevertheless dependent toward explant size.

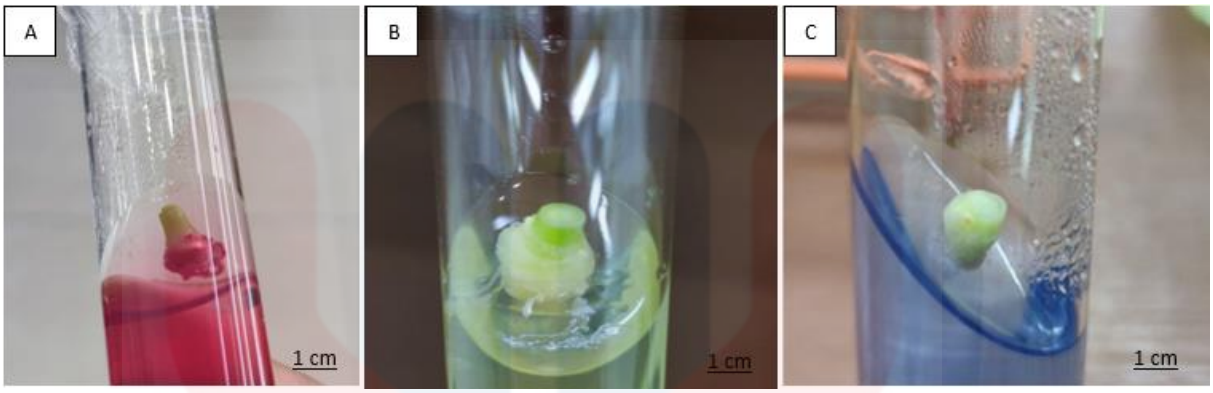


Figure 4.1 (a): Survive Explant After 1 Week of Initiation A; Bentong Ginger, B; Black Ginger and C; Black Turmeric in MS Media Treated with 50 mg/L Ampicillin respectively.

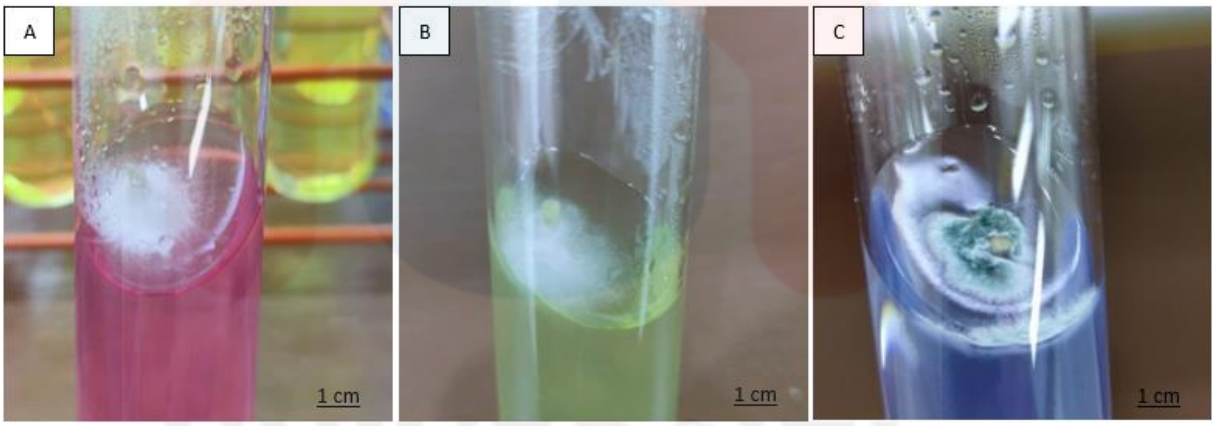


Figure 4.1 (b): Contaminated Explant After 1 Week of Initiation A; Bentong Ginger, B; Black Ginger and C; Black Turmeric in MS Media Treated with non-Ampicillin respectively.

4.2 Shoot Multiplication

Shoots from the initiation stage were propagated in MS media treated 0.0 mg/L BAP and 0.0 mg/L NAA, 1.0 mg/L BAP and 0.5 mg/L NAA, and 2.0 mg/L BAP and 1.0 mg/L NAA, respectively. After the fourth week of incubation, a clump of long and robust *in vitro* plantlets was developed (Figure 4.2).

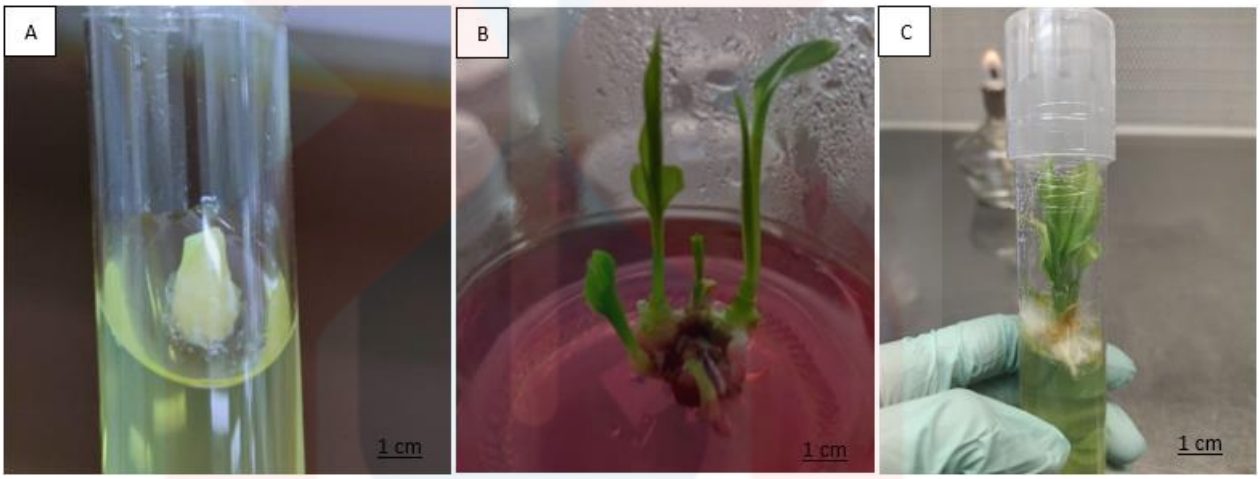


Figure 4.2 Micropropagation of Black Turmeric form first week to 4 weeks of subculturing.

Cytokinin and auxin stimulates cell growth and division, which prompt the shoots and roots initiation. The effect of cytokinin and auxin on the induction of shoots and the formation of roots are differed according to the type and concentration of the cytokinin and auxin. Zuraida et al., (2016) state that *in vitro* culture media, the higher concentration of BAP stimulates shoot growth more than the lowest concentration. In this study, we had successfully proved that higher number of shoots had been generated from plantlets cultured in 2 mg/L BAP and 1 mg/L NAA for the three types of explants (Bentong Ginger, Black Ginger, and Black Turmeric) as compared to their control treatments (Table 4.2). According to Table 4.2, we also found that response of explant elongation is differ for

each explant type. Bentong Ginger and Black Ginger explants show longest length of shoots in 1 mg/L BAP and 0.5 mg/L NAA at 2.81±0.63 cm and 2.07 ± 0.55 cm respectively, while Black Turmeric shoot elongation show greater response to 2 mg/L BAP and 1 mg/L NAA at 3.03 ± 1.41 cm. Roots formation for Bentong Ginger, Black Ginger and Black Turmeric explants show highest response toward 1.0 mg/L NAA and 2.0 mg/L BAP treatments at 6.17 ± 0.79 cm, 9.67 ± 1.26 cm, and 12.50 ± 1.41 cm respectively.

Table 4.2: The effects of BAP and NAA concentration towards number of shoots, number of roots induction, and length of shoots of Bentong Ginger, Black Ginger and Black Turmeric after 4 weeks of incubation.

Type of Explant	Treatment		Number of shoots per explant	Number of roots per explant	Length of shoot per explant (cm)
	BAP (mg/L)	NAA (mg/L)			
Bentong Ginger	0	0	7.17 ± 0.79[a]	4.33 ± 0.42[a]	1.98 ± 0.38[a]
	1.0	0.5	6.67 ± 1.12[a]	5.33 ± 1.63[a]	2.81 ± 0.63[a]
	2.0	1.0	7.83 ± 0.70[a]	6.17 ± 0.31[a]	2.12 ± 0.53[a]
Black Ginger	0	0	3.33 ± 0.56[a]	1.50 ± 0.62[a]	1.50 ± 0.57[a]
	1.0	0.5	6.50 ± 0.85[a]	6.33 ± 0.49[a]	2.07 ± 0.55[a]
	2.0	1.0	7.50 ± 0.67[a]	9.67 ± 1.26[a]	0.92 ± 1.56[a]
Black Turmeric	0	0	3.17 ± 0.60[a]	2.17 ± 0.60[a]	1.87 ± 0.93[a]
	1.0	0.5	6.50 ± 0.43[a]	7.67 ± 0.88[a]	1.60 ± 1.03[a]
	2.0	1.0	7.67 ± 1.05[a]	12.50 ± 1.41[a]	3.03 ± 1.41[a]

Values stated is means ± standard error (SE) of n = 6. BAP = 6-benzylaminopurine and NAA = 1-napthaleneacetic acid. [a] Not Significant at the ($p > 0.05$)

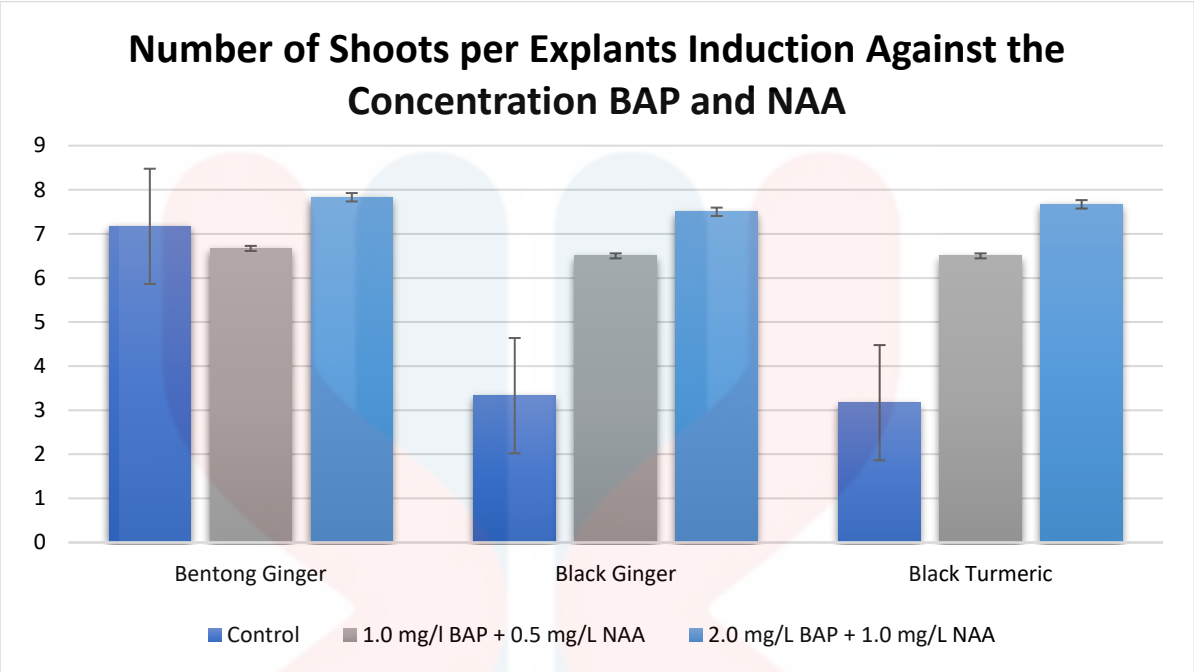


Figure 4.2 (a): Number of Shoots per Explants Induction Against the Concentration of BAP and NAA

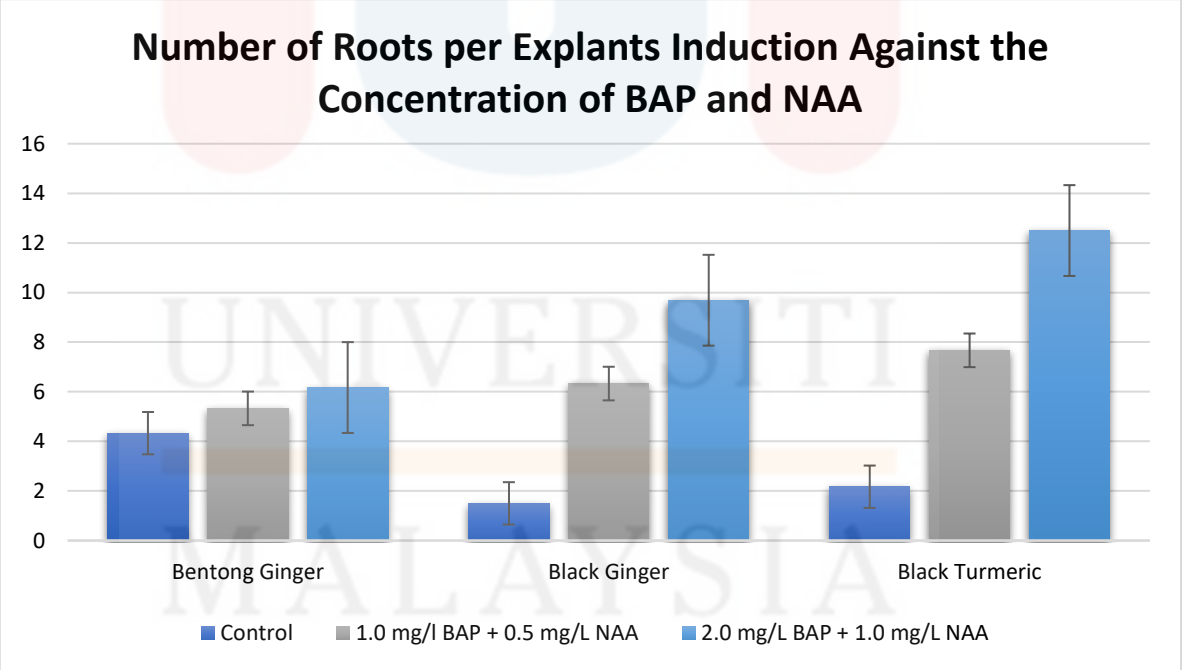


Figure 4.2 (b): Number of Roots per Explants Induction Against the Concentration of BAP and NAA

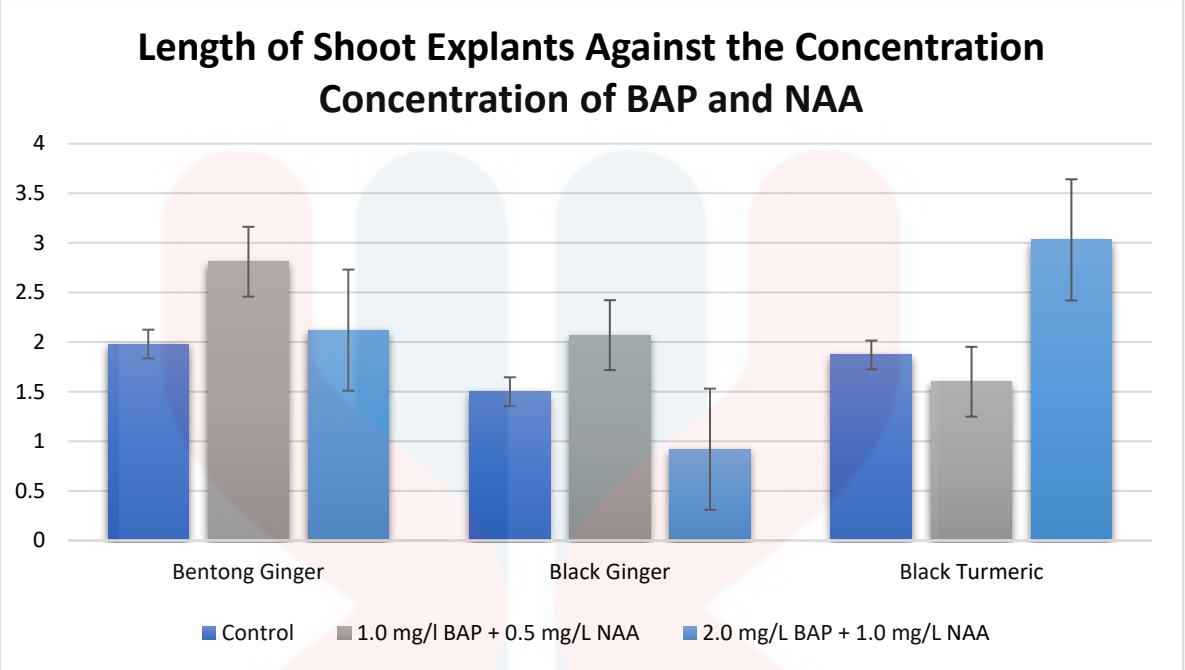


Figure 4.2 (c): Length of Shoots per Explants Induction Against the Concentration of BAP and NAA

The capability of high doses of BAP to produce many shoots was proven by in many crops' specie. Most of the herbaceous plants respond well to BAP treatments. Moreover, in this study explants treated with 2.0 mg/L BAP and 1.0 mg/L NAA produced extraneous shoots and roots. This supported by Hiremath (2006); the most numerous shoots forms in MS medium containing 2.0 mg/L BAP. However, it is contradictory with Seran (2011) with found that shoots multiplicate was observed in media treated with 5.0 mg/L BAP and 0.5 mg/L NAA. Furthermore, many researchers reported that combining PGRs had increased the number of root and shoot produced.

Increasing the combination of PGRs concentration had risen the shoots and roots formation. Overdose of PGRs will degraded the induction of explants shoots and roots (Zahid, Jaafar & Hakiman, 2021). Explant root was developed instantaneously during shoots formation. Previously, ginger *in vitro* roots explants were reported induce naturally as a result of shoots proliferation (Sathagowri & Thayamini, 2011). In the presence study,

it is shown that auxin (NAA) had effectively increases the roots formation although roots claimed to induce naturally during multiplication of shoots. This indicated that the use of auxin could help in nutrient absorption by the development of roots.

4.3 Contamination

Despite the fact that antibiotic is being used, contamination is still be observed during the subculturing (multiplication) stage. This may be due to the presence of fungal spore, microbes, and bacteria on the surface of utensil, surrounding environment and the media used during the culturing process. Aseptic handling is therefore critical during the culturing procedure. Temperature, light, and humidity, on the other hand, might act as factors that triggers and lead to contamination. Optimum yield could be possibly achieved if contamination were kept to a bare minimum. Figure 4.3 present the explants contaminate induced by mishandling procedures during culturing phase.

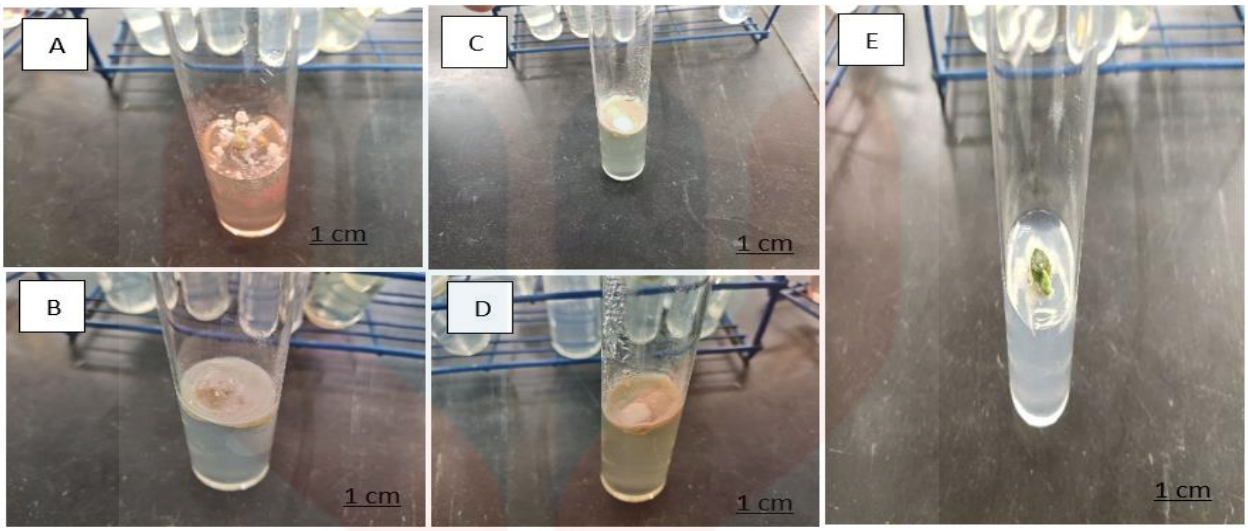


Figure 4.3 Explants Contamination. A & B During Multiplication Stage in 0 mg/L and 20 mg/L Ampicillin. C, D, & E Contamination During Initiation Stage in MS control media.

CHAPTER 5

CONCLUSION

Zingiberaceae becomes a valuable commodity crop that generates economic value in revenue for several countries. Infection of soil borne diseases become major factor that affect ginger cultivation in Malaysia. Moreover, lack of availability of seedling in market affect the price of the rhizome. As a result, in order to maximise the production of plantlets of Bentong Ginger, Black Ginger, and Black Turmeric, this paper presented the results of a research aimed at enhancing procedures for sterilising rhizome shoot explants, controlling bacterial contamination of *in vitro* ginger micropropagation through the use of antibiotics (ampicillin) application, and mass propagation using the optimize concentration of PGRs.

Result from this study reveal that, explant size between 1.0 cm to 2.0 cm is suitable for establishment of contamination-free explants with 50 mg/L of ampicillin. Shoots and roots of Zingiberaceae's (Bentong Ginger, Black Ginger and Black Turmeric) have shown significant response toward PGRs combination of 2 mg/L BAP and 1mg/L NAA Future research should focus on determining the best method for sterilising rhizome shoots explants and assessing contaminants in the explants, while minimising or eliminating phytotoxicity produced by explants during the *in vitro* propagation phase. In order to find less or nonphytotoxic approach, significantly different type of antibiotics

treatment could be further explored. Moreover, it is also essential to identify optimum concentration and combination of plant growth regulator to enhance shoot multiplication and development of the plantlets produced

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