MICROPROPAGATION OF GINGER (*Zingiber officinale* Rosc. cv Tambunan)

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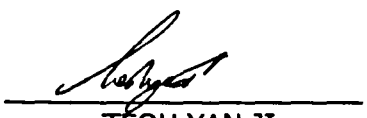

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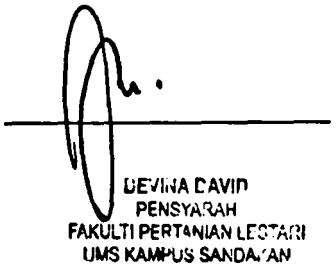

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ABSTRACT

This study was conducted at the Tissue Culture Laboratory Faculty of Sustainable Agriculture in Universiti Malaysia Sabah, Sandakan, Sabah to investigate the effect of NAA and BAP hormones at different concentrations on *in vitro* growth of ginger (*Zingiber officinale* Rosc cv Tambunan). The experimental design was Complete Randomized Design (CRD) using 0 mg/L BAP and NAA as a control, BAP (1, 2 and 3 mg/L) and NAA (1, 2 and 3 mg/L) alone or combination of both for shoot regeneration, rooting and multiplication. Each of the treatment was replicated ten times and the observation was done every two weeks. Parameters such as number of shoots, number of leaves, number of roots, shoot and root length (cm), days to shoot initiation and survival rate (%) was calculated. The collected data was analyzed using SAS (Statistical Analysis Software) version 9.4 software. ANOVA with mean separation by Duncan's multiple range test ($P < 0.05$) was used to compare means of different treatments. Based on the result, 3 mg/L BAP and 1 mg/L NAA had significantly induced the highest number of shoots (6.14 ± 0.91) after 10 weeks of culture and observed that the day to shoot initiation were shorter compared to other treatments. MS media supplemented with single hormone 2mg/L NAA achieved the highest number of root production (34.40 ± 1.81) with the highest root length at 4.52 ± 0.20 cm for root multiplication and elongation of axillary bud culture of *Zingiber officinale* Rosc. cv. Tambunan after 10 weeks of culture. Therefore, MS media supplemented with combination of 3 mg/L BAP and 1 mg/L NAA and single hormone treatment 2 mg/L NAA was recommended for better growth and development of *Zingiber officinale* Rosc. cv. Tambunan.

MIKROPROPAGASI KE ATAS HALIA (*Zingiber officinale* Rosc. cv Tambunan)

ABSTRAK

Kajian ini telah dijalankan di makmal kultur tisu, Fakulti Pertanian Lestari, Universiti Malaysia Sabah, Sandakan, Sabah untuk mengkaji kesan pengawalatur pertumbuhan NAA dan BAP ke atas pembentukan halia (*Zingiber officinale* Rosc cv Tambunan) dengan Rekabentuk Eksperimen Rawak Lengkap. Tujuan kajian ini adalah untuk membuat perbandingan kesan pengawalatur pertumbuhan NAA dan BAP dalam kepekatan yang berbeza ke atas pembentukan halia. Dalam kajian ini, 0 mg/L BAP dan NAA akan dijadikan sebagai kawalan media, kepekatan BAP (1, 2 dan 3 mg/L) dan NAA (1, 2 dan 3 mg/L) telah diguna sebagai rawatan. Setiap rawatan mempunyai sepuluh replikasi dan permerhatian dijalankan setiap dua minggu. Data parameter adalah seperti bilangan pucuk, bilangan akar, bilangan daun kepanjangan pucuk dan akar (cm), hari permulaan pucuk dan kadar survival (%) akan dikirakan. Data yang diperolehi dinalisis menggunakan perisia SAS (Statistical Analysis System) version 9.4 dengan analisis varians (Analysis of Varian, ANOVA). Melalui hasil kajian didapati bahawa media mengandungi 3 mg/L BAP and 1mg/L NAA telah menunjukkan bilangan pucuk yang paling tinggi (6.14 ± 0.91) selepas sepuluh minggu pengkulturan dan hari permulaan pucuk juga didapati lebih awal berbanding dengan rawatan yang lain. MS media mengandungi 2 mg/L NAA menunjukkan bilangan akar yang paling banyak (34.40 ± 1.81) dan ukuran akar yang paling panjang (4.52 ± 0.20 cm) selepas sepuluh minggu pengkulturan ke atas pembentukan halia (*Zingiber officinale* Rosc cv Tambunan). Secara keseluruhan, MS media mengandungi combinasi 3 mg/L BAP and 1 mg/L NAA dan 2 mg/L adalah media yang sesuai untuk pembentukan halia (*Zingiber officinale* Rosc cv Tambunan).

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LIST OF SYMBOLS, UNITS AND ABBREVIATIONS

%	Percentage
°C	Degree Celsius
µM	Micromolar
ADS	Adenine sulphate
ANOVA	Analysis of variance
ARS	Agricultural Research Station
BAP	6-Benzylaminopurine
cm	Centimeter
CRD	Complete Randomize of Design
cv	Cultivar
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
mg/L	Milligram / liter
min.	Minutes
mm	Millimeter
mM	Millimolar
MS	Murashige and Skoog
NAA	1-Naphthaleneacetic acid
NN	Nitsch and Nitsch
Rosc.	Roscoe
SAS	Statistical Analysis System
SE	Standard Error
T	Treatment
v/v	Volume/volume percent
var	Variety

LIST OF FORMULAE

CHAPTER 1

INTRODUCTION

1.1 Introduction

The ginger plant (*Zingiber officinale* Rosco) is belong to the family of *Zingiberaceae*. *Zingiber officinale* Rosc. is an important tropical horticultural plant, valued throughout the countries as an important spices for its medicinal properties. The ginger plant is an erect perennial growing from one to three feet in height with stems surrounded by the sheathing bases of the two-ranked leaves. A club-like spike of yellowish, purple-lipped flowers have showy greenish yellow bracts beneath. The ginger have a thick scaly rhizomes, they able to grow with the length of 7-15 cm, 1-1.5 cm broad and laterally compressed (Ghosh *et al.*, 2011). The branches of ginger arise obliquely from the rhizome are about 1-3 cm long and terminate in depress scars or in undeveloped buds (Ghosh *et al.*, 2011). Ginger is vegetatively propagated through underground rhizomes with a low multiplication rate and mainly cultivated in many countries including India, China, Japan, Indonesia, Australia, Nigeria and West Indies. Besides that, *Zingiber officinale* Rosc. is a native plant in tropical South East Asia and it was introduced into the West Indies, Africa and other warmer regions of the world. In the past, Marco Polo was the first European to see ginger growing in this natural habitat in the late 13th century, and Chinese records show that ginger was cultivated in the Malacca region (Malaysia) in 1416 (Weiss, 2002).

In Malaysia, about 30-40 species of *Zingiberaceae* have long been used and cultivated as food, traditional medicine or spices and ornamentals (Ibrahim, 1992). For example, *Etlingera coccinea* or Tuhau is consumed widely in Sabah and the rhizome of *Alpinia galangal,* also known as lengkuas are used for digestive, stomach problems and skin disease (Kulip, 2007). This plant has a natural dietary component and has been

known to have antioxidant and anti-carcinogenic properties. It also possesses anti-inflammatory effect, antiplatelet effect, anti-ulcer principles, cardiovascular effect and anticonvulsive and analgesic effects (Zachariah, 2008). The important factor that makes ginger so popular is the ginger oil, oleoresin. The benefits of this ginger oil not only possesses aroma and flavour, but also the lacking of pungency that makes it to be the main ingredient in flavouring of beverages, confectionery and perfumery (Zachariah, 2008). On the other hand, ginger plant can also be planted as ornamental in gardens. The Kaempferia species that are commonly planted as ornamental ginger due to its attractive coloured or patterned leaves and Curcuma species are cherished because of the colourful and long-lasting inflorescences (Kuehny *et al.*, 2002).

Zingiber officinale Rosc. cv Tambunan is a highly valued medicinal spice plant and also one of the registered intellectual property in Malaysia (Berita Wilayah, 2007). In Sabah, Tambunan is among the main producer of ginger production in Malaysia. However the main problem in ginger cultivation is high infestation by pest and disease (Dohroo and Edison, 1989) such as bacterial wilt, yellows, *Phyllosticta* leaf spot, storage rots and rhizome rot (Nada *et al.*, 1996). Rhizome rot is one of the major disease that cause by *Pythium* spp. in ginger *Zingiber officinale* Rosc. (Nirmal Babu *et al.*, 2005). This problem has caused the degradation of ginger supply in Sabah.

In order to overcome these disease problem, the tissue culture protocol for this ginger were introduced as well as to improve the quality of ginger food shortages. Previous studies had reported the application of tissue culture techniques on propagating ginger (Abdelmageed *et al.*, 2011; Kambaska and Santilata, 2009; Mohamed *et al.*, 2011).Tissue culture techniques have been applied typically when traditional methods of propagation have either failed or proved inadequate. *In vitro* propagating technique is considered the best alternative that can supply enough planting materials for commercial planting. Philips and Hubstenberger (1995) proved that axillary bud proliferation typically can results in an average tenfold increase in shoot number per monthly culture passage.

1.2 Justification of Study

The general cultivation practices of ginger is based on the use of rhizomes as planting materials due to the habit of rare flowering and non-viable seed production (Malamung *et al.*, 1991). Ginger cultivation is threatened by disease that are spread through infected rhizome. While farmers usually used part of one season's yield as planting stock for the next season, thereby increasing the possibility of perpetuating endogenous diseases such as rhizome rot diseases that is currently the most severe problem for ginger cultivation in Sabah and competes with the use of ginger rhizomes for human consumption (Villamor, 2010). Poor seed setting has been a major constrain in crop improvement of ginger. It is therefore necessary to find the alternative source of disease free planting materials to establish large scale production (Thayamini, 2013). One of the method of producing disease-free planting stock in a large amount is by tissue culture. This technique has been used as an implement for propagation of many crop plant species, not only ease the commercial propagation and also prevent fatal disease to be introduced (Villamor, 2010).

Unfortunately, disease might have the chance to invade the plantlets through the ginger cultivation site although disease-free planting stock has been introduced. Therefore this study could be as reference in optimizing the production of high quality ginger plant to provide enough planting materials when new resistant ginger variety has been developed and thus fulfill the high demand of ginger in Sabah.

1.3 Objective

To compare the effect of NAA and BAP hormones at different concentrations on *in vitro* axillary bud culture of *Zingiber officinale* Rosc. cv Tambunan.

1.4 Hypothesis

Ho: There is no significant different between the different concentration of NAA and BAP and the *in vitro* respond of the explant.

Ha: There is significant different between the different concentration of NAA and BAP and the *in vitro* respond of the explant.

CHAPTER 2

LITERATURE REVIEW

2.1 Ginger

Ginger, botanically known as *Zingiber officinale* Rosc, belongs to the family *Zingiberaceae* in the order of Zingiberales (Ravindran and Nirmal Babu, 2005; Zachariah, 2008). *Zingiberaceae* family includes some 40 genera, containing hundreds species of perennial tropical and subtropical herbs. The genus *Z. Boehmer,* the most important of which is represented by *Z. officinale* Rosc. *Zingiber officinale* is a creeping perennial, usually cultivated as an annual, with purple flowers and a robust branched rhizome, which spreads underground horizontally near the soil surface. Moreover, there are two types of ginger roots such as fleshy and fibrous. For fibrous roots, many roots having indefinite growth grow out of the base of the sprouts after planting and the number of such roots will keep on increasing with the growth of tillers. These fibrous roots are thin, having root hair and their main function is to absorb water and nutrients (Ravindran *et al.*, 2005). As a ginger plant grows further, several fleshy roots of the indefinite growth are produced from the lower nodes of the primary fingers and mother ginger. These roots are thicker, with a few root hairs, no lateral roots, milky white in colour and take the function to support as well as absorption (Figure 2.1).

Ginger is a modified stem for the vegetative propagation and food materials storage. The stem comprises nodes with scale leaves and internodes. All nodes have axillary buds, except for the first few nodes. When the rhizome bit is used for planting purpose, there may have one or more apical buds on the rhizome; however, only one bud will become active. If large pieces are planted, there will have more than one bud may develop simultaneously (Shah and Raju, 1975).

Figure 2.1 Sketch of the ginger plant showing the origin of shoots, inflorescence, and flower. AS: Aerial shoot, R: Rhizome, Fl: Fower, P: Peduncle (scape), S: Spike.

Source: Ravindran and Nirmal Babu, 2005

2.2 Ginger cultivation

The plant is believed to have originated in Southeast Asia but it was under cultivation from ancient times in India as well as in China. Now, there are many main ginger growing countries such as India as the largest producer, China, Jamaica, Taiwan and including Malaysia (Ravindran and Nirmal Babu, 2005). Ginger is grown from smallholder level to large-scale fully mechanized operations, to service a range of markets and as a major cash crop in the Caribbean, China, India, Sri Lanka and West Africa (Weiss, 2002). In fact, ginger is commercially propagated vegetatively through pieces of rhizomes from the previous year harvest, which have a limitation in reproduction and productivity of

the crop, but can also micropropagated using meristem, rhizome sections or tissue culture (Arimura *et al.*, 2000). The breeders finds difficulties to breed because of sterility. So major ginger-growing area have cultivars only suited to that particular area (Ai *et al.*, 2005).

2.3 Ginger medicinal and pharmacological properties

In the past few decades, the world has witnessed a considerable enhancement of interest in the value of ginger as a spice in almost all system of medicine. Among the herbal drug, ginger is the one raw drug that has undergone considerable of study. In Ayurveda, ginger is known as *Sunthi.* Ayurvedic system of medicine that are widely used in the Indian system, both dry and fresh ginger are used. Since ancient times, ginger has been widely used as a common household remedy for various illness (Remadevi *et al.*, 2005) and the use of this drug is mentioned in form of *Trikatu*, a popular Ayurvedic remedy for the treatment of digestive disorders. Furthermore, the drug in Ayurveda has been described as appetizer pharmacologically and also indicated in the form of ointment for local pains applications (Samir and Amrit, 2003).

2.3.1 Anti-inflammatory effect

Recently, a number of studies have been conducted which show various pharmacological effects of the plants. Ginger is one of the plants that contains pungent phenolic substances with pronounced antioxidative and anti-inflammatory activites. It is suggested that ginger may have capability of treating arthritis (Remadevi *et al.,* 2005). The World Health Organization (WHO) document 1999 reported that five to ten percent of ginger extract administration may brought about partial or full pain relief, joint function recovery and a decrease of swelling in patients with chronic rheumatic pain and lower back pain. [6]- Gingerol, a pungent ingredient of ginger, has antibacterial, anti-inflammatory and antitumour-promoting activites (Zachariah, 2008). Kim *et al.* (2005) found its antiangiogenic activity *in vitro* and *in vivo.* Angiogenesis, the formation of new blood vessels from pre-existing endothelium, in fundamental in a variety of pathological and physiological process, including embryo development, wound healing, chronic inflammation and tumour progression and metastasis. [6]-Gingirol also blocked capillary tube-like formation by endothelial cells in responds to vascular endothelial growth factor

(VEGF) and strongly inhibited sprouting of endothelial cells in rat aorta and new blood vessels formation in the mouse cornea in response to VEGF (Kim *et al.,* 2005).

2.3.2 Antioxidant effect

Ginger also contains some constituents that have antioxidant effects. The non-volatile fraction of the dichloromethane extract of ginger rhizomes exhibited a strong antioxidative activity using linoleic acid as the substrate in ethanol-phosphate buffer solution. Aeschbach *et al.* (1994) also studied the antioxidant effect of zingerone from ginger. Besides that, demonstration on the chemopreventive efficacy of ginger in colon cancer found that the number of tumours, as well as the incidence of cancer, was significantly decreased when treated with ginger (Manju and Nalini, 2005).

2.3.3 Antiplatelet effect

Gingerol with 0.5 – 20 μM concentration inhibited the aggregation and release reaction of arachidonic acid and collagen-induced rabbit platelets. In human platelet-rich plasma, gingirol and indomethacin (indometacin) prevented the secondary aggregation and blocked release of ATP from platelets, but had no influence on primary aggregation (Zachariah, 2008).

2.3.4 Anti-ulcer principles

Yoshikawa *et al.* (1994) detected an anti-ulcer principle, 6-gingesulphonic acid, and three monoacyl digalactosyl glycerols, ginger glycolipids A, B and C, from ginger rhizome from Taiwan when they investigated the stomachic principle of ginger. The dried rhizome of ginger is used in Chinese and Japanese traditional medicines to treat headaches, nausea, stomach ache and colds. Besides that, Yamahara *et al.* (1988) stated that zingiberene and 6-gingerol are the constituents which act as protectants against gastric lesion in medications containing ginger.

2.3.5 Cardiovascular effect

An atropine-resistant vasodilator activity was recorded from ginger phenolic constituents 6-, 8- and 10-gingerol, while a mild vasodilator had shown from 6-shogoal as well. According to Ghayur *et al.* (2005), the data shown aqueous ginger extract does lowers blood pressure through a dual inhibitory effect and this study gives a basis of sound mechanistic for the ginger utilization in hypertension and palpitations.

2.4 *Zingiber officinale* Rosc. cv Tambunan

Sabah is one of the 13 states within the Federation of Malaysia and is located in the northernmost part of Borneo Island. Sabah is the second largest state in Malaysia with a landmass of approximately 7.4 million hectares. In Sabah, the native communities commonly use the plants surrounding their house or in the forest for various purposes in their everyday life. Ginger are loosely called 'Halia' in Malay, and several species of economic importance. Ginger are usually characterized by their aromatic parts and are used as spices, made into conditions, essential oils and medicine, and grown as ornamentals. In Malaysia, about 30-40 species of *Zingiberaceae* have long been used in traditional medicine. A large number of species from the ginger family has been cultivated for use as food, medicine and ornamentals (Ibrahim 1992). However the usefulness of the native species has not been fully appreciated and exploited especially in Sabah. According to Kulip (2007), there are a total of 36 species in 12 genera of *Zingiberaceae* in Sabah was surveyed. Among all the genera, *Etlingera and Alpinia,* followed by *Zingiber* are the most common genera that utilized by the people in Sabah. *Zingiber officinale* Rosc. cv Tambunan which are cultivated in Tambunan is localized from Kg. Kaingaran, Tambunan and also known as Hayo/ Hazo by Kadazandusun or Halia by Malay. The rhizome of this ginger normally mixed with a little alcohol can use to massage sprained muscles or for rheumatism and also used to remove wind from body (Kulip, 2007).

Although *Zingiber officinale* Rosc. cv Tambunan is widely cultivated in Tambunan, it still cannot meet the high ginger demand of 24,000 kilograms a month in Sabah (The Borneo Post, 2012). Recently, a new ginger breeding technology using tissue culture techniques has been introduced to cope with this problem. Through this new breeding technology, introduced during the Agricultural Research Station (ARS) Golden Jubilee Open House at Ulu Dusun, 500 plantlets can be produced from only one ginger plant in

six months. Moreover, according to Sabah Agriculture director Datuk M.C Ismail Salam, this technology not only overcome the quality ginger food shortages but also produced disease-free plantlets especially from rhizome rot disease (The Borneo Post, 2012).

2.5 Micropropagation of Ginger

The reason for propagating ginger using tissue culture techniques is because the conventional breeding programs are hampered due to lack of fertility and natural seed set (Nirmal Babu *et al.*, 2005). Micropropagation of ginger have been previously reported by (Abdelmageed *et al.*, 2011; Kambaska and Santilata, 2009; Mohamed *et al.*, 2011; Mohanty *et al.*, 2008; Nirmal Babu *et al.*, 1996; Ricardo and Rolf, 1994) (Table 2.1).

2.6 Factors affecting *in vitro* propagation of ginger

2.6.1 Basal medium

The degree of success in any technology using plant cell, tissue or organ culture is related to few affecting factors. A significant factor is the choice of basal medium (Gamborg and Phillips, 1995). The nutritional component, the state of the medium and the concentration of the basal medium will have a direct effect on the regeneration and growth of explant. There are a relatively small number of mineral salts are used as components of plant tissue culture media. For most purposes, the medium should contain at least 30 mM each of inorganic potassium and nitrogen. Ammonium salts can be used at 2 – 20 mM that can have the effect vary from inhibitory to essential.

There are several researches previously done on the effect of basal medium in the micropropagation of ginger. For example in the report of Mohanty *et al.* (2008), *in vitro* growth of *Zingiber officinale* (cv. Suprava) is tested by using solid and liquid media. In the MS medium that supplemented with 1 mg/l of BA, solid media showed higher growth in shoot length (9.87 ± 0.17) cm than the one grown in liquid media. Though the multiplication rate remained the same in both media, plants grown in liquid media became weak and etiolated with stunted growth.

Table 2.1 *In vitro* responses of ginger.

Explant used	Media composition	*In vitro* responses	References
Vegetative buds and rhizome bits with axillary buds.	MS + 2 mg/L NAA (Liquid medium)	Multiple shoots and *in vitro* rooting	Nirmal Babu *et al.*, 1996
Axillary buds	MS + 22.2 µM BAP MS + 11.4 µM IAA	Increase in the number and length of shoots. Rooting regeneration.	Abdelmageed *et al.*, 2011
Axillary buds	MS + 4.0 mg/L BA + 1.0 mg/L IAA MS + 1.0 mg/L BA + 1.0 mg/L IAA + 100 Ads	Explant sprout. Shoot multiplication	Mohanty *et al.*, 2008
Sprouting buds	MS + 2.0 mg/L BAP + 0.5 mg/L NAA 0.5MS + 2.0 mg/L NAA	Shoot induction Root induction	Kambaska and Santilata, 2009
Axillary buds	MS + 4.5 mg/L BAP MS + 1.0 mg/L NAA	Shoot induction Root induction	Mohamed *et al.*, 2011
Axillary buds	MS + 10 µM BA + 5 µM NAA or IAA	Plant growth during bud establishment phase	Ricardo and Rolf, 1994
Callus derived from bud, ovary, leaf	MS + 10 mg/L BA, 0.2 mg/L 2,4-D	Callus induction and plant regeneration	Nirmal Babu, 1997

Besides that, the concentration of the basal medium also affect the growth and development of the ginger explant. According to Mohamed *et al.* (2011) in the study of *in vitro* propagation of *Zingiber officinale* Rosc., different types of nutrient media such as Heller's, Murashige and Skoog (MS), Gamborg B_5 and Nitsch and Nitsch (NN) media with different medium strength is experimented on the number of roots per shoot and the root length. Generally, half-strength of all different nutrient media improved the overall growth of roots compared to full strength salts of nutrient media for roots formation of *Z. officinale* as maximum number of roots formed per shoot is recorded with half strength of Gamborg B_5 (25.3), Heller's (23.7) and NN (19) media (Mohamed *et al.*, 2011).

2.6.2 Surface-sterilization process

All tissue culture studies which aim to obtain high-frequency of shoot regeneration, which is also a prerequisite for an efficient transformation system, should be performed under sterile conditions. Explant health is the main factor determining regeneration capacity, so viability of explant and the seedling from which the explant is excised, are very important for high frequency of shoot regeneration. The main purpose of surface-sterilization process is to eliminate all microorganisms such as bacteria that could easily grow under *in vitro* conditions. Since direct contact of explant with disinfectant during the sterilization process may have a severe effect on regeneration capacity of the tissue, the concentration of the disinfectant, application period and temperature should take into account to ensure explant's viability and regeneration capacity (Yildiz, 2012).

According to Mohamed *et al.* (2011), the excised bud sprouts of *Zingiber officinale* Rosc. was immersed in 70% of ethanol for 2 minutes for surface sterilization. After washed with sterile distilled water, the explant was immersed again in 50% (v/v) of Clorox solution with the addition of Tween 20 for 15 minutes. Besides that, in the research of *in vitro* induction for microrhizomes using silver nitrate in *Zingiber officinale* Rosc. var. Baishey and Nadia, they also practised sterilization method by drenching the excised buds with Labklin detergent around 10 to 15 minutes after washed with running tap water for 15 to 20 minutes. The explant was then treated overnight with gentamycin (8 mg/L) and later washed 5 to 10 times with distilled water to remove the antibiotic trace. After that, the explant was transferred into laminar flow and treated with 0.2% of mercuric chloride ($HgCl_2$) for 15 minutes. At last, explant was dipped into 70% ethanol

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