UNIVERSITI PUTRA MALAYSIA

IN VITRO CLONAL PROPAGATION OF GINGER (Zingiber officinale Roscoe) var. Bentong THROUGH DIRECT SHOOT AND MICRORHIZOME ORGANOGENESIS

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By

ZAHID NISAR AHMAD

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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DEDICATION

I dedicate this thesis to my loving parents, grandmother and wife, cute son (Sohail), and my siblings, who have always supported and prayed for my success and improvement in life. Your invaluable love and support were my main motivations for my hard-working during this academic journey. I am amazed by your patience in bearing my absence for more than two years. My heartfelt thoughts also go to my relatives and friends, who are always genuinely happy with my progress.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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June 2021

Chairman : Mohd Hakiman bin Awang @ Mansor, PhD
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Bentong ginger is an important variety of *Zingiber officinale* Roscoe in Malaysia. Due to the poor flowering and seed set, ginger is vegetatively propagated through its rhizome. Using rhizome as planting material is bulky and has often caused yield loss due to the soil-borne disease's transmittance in ginger cultivation. Hence, micropropagation could be the best solution for these problems associated with ginger's conventional propagation. Therefore, the present study aims to optimize different stages of two different techniques (direct shoot regeneration and microrhizome induction) of Bentong ginger micropropagation. Bentong ginger rhizome sprouted bud explants were surface sterilized with 70% (v/v) ethanol for 1 minute and then followed by surface sterilizing with Clorox® (5.25% NaOCl) at 30, 40, 50, 60 and 70% (v/v) for 30 minutes. Using 70% Clorox® resulted in the highest percentage of aseptic cultures (75%), with 83.60% survivability in the culture medium. In the first experiment of shoot multiplication, the addition of different types of cytokinins (zeatin, 6-benzylaminopurine (BAP) and kinetin at 10 µM and thidiazuron (TDZ) at 5 µM) in Murashige and Skoog (MS) medium were assessed. Zeatin was found more effective than BAP, kinetin and TDZ for shoot multiplication of Bentong ginger. In the second experiment of shoot multiplication, zeatin at 0, 5, 10, 15 and 20 µM in combination with three different types of basal media vis MS, Linsmaier and Skoog (LS) and Gamborg et al. (B5) media was studied. MS medium supplemented with 10 µM of zeatin resulted in the highest number of shoots per explant (4.28) after six weeks of culture. In the last experiment of shoot multiplication, the addition of different types of auxins (indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) at 0, 2.5, 5 and 7.5 µM) in MS medium supplemented with 10 µM zeatin was evaluated. The addition of 2.5 µM NAA in MS medium supplemented with 10 µM zeatin resulted in the highest number of shoots per explant (6.7) after eight weeks of inoculation. After shoot multiplication, the micropropagated plantlets were subjected to different

growing media mixed of (1) soil + peat moss + vermiculite (1:1:1(v/v/v)), (2) soil + peat moss + perlite (1:1:1(v/v/v)), (3) soil + coco peat + vermiculite (1:1:1(v/v/v)), (4) soil + coco peat + perlite (1:1:1(v/v/v)) and (5) soil+ sand (1:1(v/v)). A growing media mixed of soil + coco peat + vermiculite resulted in the highest survival (94.8%) of the plantlets in the *ex vitro* conditions. The acclimatized plantlets were successfully established with a 100% survival in a shade house under a 50% black shade net. In the second part of the study, Bentong ginger's microrhizome induction was studied. In the first experiment, zeatin and BAP at 0, 5, 10, 15 and 20 µM in MS medium supplemented with 80 g L^{-1} sucrose and 2.5 µM NAA were examined for microrhizome induction of Bentong ginger. Zeatin was found more effective than BAP for microrhizome induction and 10 µM zeatin resulted in the highest number (4.50) and fresh weight of microrhizomes per explant (3.61 g) and the maximum diameter of microrhizome (7.82 mm). In the second experiment, different concentrations of sucrose (30, 45, 60, 75 and 90 g L^{-1}) combined with NAA at 0, 2.5, 5 and 7.5 µM in MS medium supplemented with 10 µM zeatin were assessed. The addition of 60 g L^{-1} sucrose and 7.5 µM NAA in MS medium supplemented with 10 µM zeatin was the best combination for microrhizome induction. Finally, 93% of the microrhizomes were sprouted in the moist sand inside a room and 100% of the sprouted microrhizomes were successfully established in the open field conditions. In conclusion, both micropropagated plantlets and microrhizomes can be used as disease-free planting materials for the commercial cultivation of Bentong ginger.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PROPAGASI KLONAL *IN VITRO* HALIA (*Zingiber officinale* Roscoe) var. Bentong MELALUI ORGANOGENESIS SECARA LANGSUNG PUCUK DAN MIKRORIZOM

Oleh

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Halia Bentong merupakan satu varieti halia yang penting dari *Zingiber officinale* Roscoe di Malaysia. Oleh kerana proses pembungaan dan biji benih yang lemah, halia dibiakkan secara vegetatif menggunakan rizom. Penggunaan rizom sebagai bahan penanaman adalah bersaiz besar dan sering menyebabkan kehilangan hasil kerana penularan penyakit bawaan tanah. Oleh itu, pembiakan mikro boleh menjadi satu jalan penyelesaian terbaik untuk masalah berkaitan propagasi halia secara konvensional. Justeru itu, kajian ini dijalankan bertujuan untuk mengoptimumkan pelbagai tahap dari dua teknik yang berbeza (regenerasi secara langsung pucuk dan induksi mikrorizom) dalam pembiakan mikro halia Bentong. Eksplan tunas yang terhasil daripada rizom halia Bentong disterilkan permukaan mengunakan 70% (v/v) etanol selama satu minit dan kemudiannya diikuti dengan pensterilan permukaan menggunakan Clorox® (5.25% NaOCl) pada kepekatan 30, 40, 50, 60 dan 70% (v/v) selama 30 minit. Penggunaan 70% (v/v) Clorox® menghasilkan peratusan kultur aseptik tertinggi (75%) dengan 83.60% kelangsungan hidup di dalam media pengkulturan. Di dalam eksperimen pertama untuk penggandaan tunas, pelbagai jenis sitokinin (zeatin, 6-benzilaminopurina (BAP), dan kinetin pada kepekatan 10 µM dan thidiazuron (TDZ) pada kepekatan 5 µM) di dalam media kultur Murashige dan Skoog (MS) telah dinilai. Zeatin didapati lebih efektif berbanding BAP, kinetin dan TDZ dalam penggandaan tunas halia Bentong. Di dalam eksperimen ke dua untuk penggandaan pucuk, zeatin pada kepekatan 0, 5, 10, 15, 20 µM dengan kombinasi tiga jenis media kultur berbeza iaitu MS, Linsmaier dan Skoog (LS) dan Gamborg et al. (B5) telah dikaji. Media MS yang ditambah dengan zeatin pada kepekatan 10 µM menghasilkan jumlah pucuk per eksplan tertinggi (4.28) selepas enam minggu pengkulturan. Pada eksperimen terakhir untuk penggandaan pucuk, penambahan pelbagai jenis auksin (indol-3-asid asetik (IAA), indol-3-asid butirik (IBA) dan 1-naftalena asid asetik (NAA) pada kepekatan 0, 2.5, 5 dan 7.5 µM) ke dalam media MS yang ditambah dengan 10 µM zeatin telah dinilai. Penambahan 2.5

µM NAA ke dalam media MS yang ditambah dengan 10 µM zeatin menghasilkan jumlah pucuk per eksplan tertinggi (6.7) selepas lapan minggu inokulasi. Selepas proses penggandaan tunas, anak pokok *in vitro* ditanam di dalam campuran media pertumbuhan yang berbeza iaitu (1) tanah + lumut gambut + vermikulit (1:1:1 (v/v/v)), (2) tanah + lumut gambut + perlite (1:1:1 (v/v/v)), (3) tanah + coco peat + vermikulit (1:1:1 (v/v/v)), (4) tanah + coco peat + perlite (1:1:1 (v/v/v)) dan (5) tanah + pasir (1:1 (v/v)). Media pertumbuhan campuran tanah + coco peat + vermikulit menghasilkan kelangsungan hidup anak pokok tertinggi (94.8%) pada keadaan *ex vitro*. Anak pokok yang diaklimatisasi berjaya dihasilkan dengan kadar kelangsungan hidup 100% di rumah teduhan yang mempunyai jaring teduhan hitam pada 50% kadar peneduhan. Pada bahagian ke dua kajian, induksi mikrorizom halia Bentong telah dikaji. Dalam eksperimen pertama, zeatin dan BAP pada kepekatan 0, 5, 10, 15 dan 20 µM dalam media MS yang ditambah dengan 80 g L^{-1} sukrosa dan 2.5 µM NAA telah dikaji untuk induksi mikrorizom halia Bentong. Zeatin didapati lebih efektif berbanding BAP untuk induksi mikrorizom dan zeatin pada kepekatan 10 µM menghasilkan bilangan terbanyak (4.50) dan berat segar mikrorizom per eksplan (3.61 g) serta diameter maksimum mikrorizom (7.82 mm). Dalam eksperimen ke dua, kepekatan sukrosa yang berbeza (30, 45, 60, 75 dan 90 g L^{-1}) dengan kombinasi NAA pada kepekatan 0, 2.5, 5 dan 7.5 µM di dalam media MS yang ditambah dengan 10 µM zeatin telah dinilai. Penambahan 60 g L^{-1} sukrosa dan 7.5 µM NAA dalam media MS yang ditambah dengan 10 µM zeatin merupakan kombinasi terbaik bagi induksi mikrorizom. Akhirnya, 93% mikrorizom tumbuh dalam pasir lembap pada keadaan bilik dan semua mikrorizom berjaya bercambah di keadaan kawasan terbuka. Secara kesimpulannya, kedua-dua anak pokok pembiakan mikro dan mikrorizom dapat digunakan sebagai bahan tanaman yang bebas penyakit untuk penanaman komersial halia Bentong.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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2,4-D	2,4-Dichlorophenoxyacetic acid
2-iP	2-Isopentenyl adenine
AA	Auto-analyzer
AAS	Atomic Absorption Spectrophotometer
AG	Gibberellic acid
ANOVA	Analysis of Variance
ARR	*Arabidopsis* Response Regulator
B5	Gamborg et al. (1968) medium
BAP	6-Benzylaminopurine
CEC	Cation Exchange Capacity
Ci	Intercellular carbon dioxide concentration
CRD	Completely Randomized Design
DMRT	Duncan Multiple Range Test
DMSO	Dimethyl sulfoxide
EC	Electrical Conductivity
Gs	Stomatal conductance
HCl	Hydrochloric acid
$HgCl_2$	Mercuric chloride
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
K_2SO_4	Potassium sulphate
LS	Linsmaier & Skoog (1965) medium
MS	Murashige and Skoog (1962) medium
NAA	1-naphthaleneacetic acid
NaOH	Sodium hydroxide

NH_4OAc	Ammonium acetate
NN	Nitsch & Nitsch (1969) medium
OM	Organic Matter
PGR	Plant Growth Regulator
Pn	Photosynthesis rates
SAS	Statistical Analysis Software
SE	Standard Error
SO_4	Sulphate
SPAD	Soil Plant Analysis Development
TDZ	Thidiazuron
tms1	Agrobacterium auxin biosynthesis gene
TOC	Total Organic Carbon
UV	Ultraviolet
WPM	Woody Plant Medium

CHAPTER 1

INTRODUCTION

Medicinal plants have long been used in the treatment of several diseases throughout the world. Ginger (*Zingiber officinale* Roscoe), an herbal monocotyledon plant from the Zingiberaceae family, is one of these plants. It is an important spice and medicinal crop that originated in South East Asia and has been widely cultivated in many tropical and subtropical countries (Ravindran & Babu, 2005). Ginger underground rhizome has been used as spice and medicine for treating cancer (Zhang et al., 2021), cardiovascular disease (Ghafoor et al., 2020), diabetes (Said et al., 2020), and several other illnesses such as cold, nausea, asthma and cough (Choi et al., 2018). Due to the global pandemic of COVID-19, ginger consumption gained more interest. It alleviates COVID-19 positive patients' severe symptoms and reduces the recovery time in those patients (Rangnekar et al., 2020; Safa et al., 2020). Ginger rhizome extract is a rich source of antioxidants, and due to its antimicrobial activity, it is also demonstrated as a natural candidate in food preservation (Beristain-Bauza et al., 2019; Si et al., 2018). Due to all these unique characteristics of ginger, its demand is highly increased in the world markets.

Bentong ginger is a well-known variety of ginger in Malaysia. It has bigger rhizomes with lower fibrous pulp than other Malaysian varieties of ginger (Suhaimi et al., 2012). Therefore, Bentong ginger is highly demanded in the domestic and international markets (Nafi et al., 2014). Due to the poor flowering and seed setting, ginger cannot be sexually propagated and it is vegetatively propagated by using its rhizome (Nair, 2019; Parthasarathy et al., 2012). The rhizome is an economically exploited part of the ginger plant. Using a high amount of rhizomes as starting material for ginger cultivation negatively affects its supply in the market (Ara et al., 2019). Besides, most of the diseases such as bacterial wilt (*Ralstonia pseudosolanacearum*), leaf spot or blast (*Pyricularia zingiberi*), soft rot (*Pythium* and *Fusarium* spp) and yellowing of leaf (*Fusarium oxysporum*) are easily transmitted through vegetative reproduction by fragmentation of the rhizomes (Abed-Ashtiani et al., 2016; Cosmas et al., 2016; Meenu & Jebasingh, 2020; Rai et al., 2018). The occurrence of these diseases can be caused for a high loss in the yield of ginger rhizomes. Furthermore, the bulkiness of ginger rhizomes as planting material makes it costly and laborious to handle. Therefore, micropropagation is a suitable alternative for the effective production of ginger (Kambaska & Santilata, 2009).

Micropropagation of ginger is vital to save the considerable amount of rhizomes used for planting and reducing ginger's planting cost. Micropropagation also prevents the transmittance of soil-borne disease in ginger cultivation. Therefore, it is more prolific to apply micropropagation techniques to ensure an extensive production of ginger plants with high phytosanitary quality (Das et al., 2013; Zuraida et al., 2016). The success of micropropagation largely depends on the aseptic culture establishment, shoot regeneration capacity, rooting, and acclimatization. Rhizome buds, which are

often used as the source of explants in Zingiberaceae, have been proven more responsive. However, the initial establishment of contamination-free culture is difficult due to the exposure of rhizomes to various soil-borne pathogens (Meenu & Kaushal, 2017; Thakur et al., 2018). These pathogens need to be eliminated by surface sterilization of the explants. For explant surface sterilization, the type and concentration of the disinfectants need to be carefully selected to prevent their toxic effect on plant tissues. In a previous study, mercuric chloride ($HgCl_2$) (0.1% for 15 minutes) by obtaining 86.66% aseptic cultures was found to be the most effective sterilant for surface sterilization of ginger sprouted rhizome explant. After $HgCl_2$, sodium hypochlorite (NaOCl) (3% for 10 minutes), by establishing 51.11% aseptic cultures, was found to be more effective than hydrogen peroxide (H_2O_2) and Bavistin® for surface sterilization of ginger rhizome sprouted buds explants (Khatun et al., 2016). $HgCl_2$, which significantly reduces the contamination level in the rhizome sprouted bud explant of ginger, is not recommended due to its high toxicity to either human or plant tissues (Ha et al., 2017; Thompson et al., 2009). Therefore, Clorox® bleach (5.25% NaOCl), which is relatively safe and easily available sterilant, could be the best alternative of $HgCl_2$ for effective surface sterilization of ginger rhizome sprouted bud explants. It needs to be studied by increasing its dose or exposure time for surface sterilization of the explants.

Besides, shoot regeneration capacity depends on the formulation of culture media and plant growth regulators (PGRs), mainly cytokinins and auxin. MS medium (Murashige & Skoog, 1962) supplemented with BAP is more commonly used for ginger shoot multiplication (Mehaboob, Faizal, Shamsudheen, et al., 2019; Thakur et al., 2018; Zuraida et al., 2016). Apart from BAP, kinetin (Ayenew et al., 2012) and TDZ (Lincy & Sasikumar, 2010) also differently influenced shoot multiplication of different varieties of ginger. The application of zeatin which is natural and biologically more active (Lomin et al., 2018), has not been reported in the micropropagation studies of ginger. In addition, the combination of auxins with cytokinins significantly enhanced shoot multiplication and *in vitro* root induction of ginger (Abbas et al., 2011; Mehaboob, Faizal, Raja, et al., 2019). A developed root system is crucial in plant vegetative propagation, ensuring a high survival and establishment of the micropropagated plantlets in field conditions (Miri, 2020). Determining the best type of basal medium, cytokinin, auxin and their optimum concentrations can significantly enhance shoot multiplication and rooting of ginger and subsequently facilitates acclimatization and successful establishment of the micropropagated plantlets in the *ex vitro* conditions.

Acclimatization of the micropropagated plantlets is commonly performed under controlled conditions of relative humidity, temperature and light intensity. A suitable growing medium is also crucial for their successful acclimatization of micropropagated plantlets. Different mixtures of growing media have varyingly affected the survival of ginger micropropagated plantlets during the acclimatization stage. A 100% survival of *Zingiber officinale* var. rubrum micropropagated plantlets was obtained when they were acclimatized in the mixture of organic soil + topsoil (1:1 (v/v)) (Zuraida et al., 2016). However, a low survival (64%) of ginger micropropagated plantlets was recorded when they were acclimatized in a mixture of sand and clay (1:4 (v/v)) (David et al. 2016). Kambaska & Santilata (2009)

obtained 95% survival of ginger micropropagated plantlets when ther were acclimatized in a mixture of soil + sand + farmyard manure (1:1:1(v/v/v)). The results of these studies indicate that growing media containing organic and nonorganic materials with characteristics of high water holding capacity and good aeration would be more suitable for the successful acclimatization of ginger micropropagated plantlets.

Besides the direct shoot regeneration, microrhizomes of ginger, which are modified stems or storage organs, can also be induced under *in vitro* conditions. They have the same anatomical features as the mature rhizomes of ginger and they can be sprouted and developed into a whole plant (David et al., 2018). *In vitro*-induced microrhizomes of ginger can be planted directly in the field without the process of acclimatization (David et al., 2018; Swarnathilaka et al., 2016). A high concentration of sucrose as a source of carbon and energy is the first metabolic signaling molecule involved in the *in vitro* storage organ formation (Yang et al., 2015; Zhou et al., 2020). Besides sucrose, cytokinins and auxins were also found the affecting factors of microrhizome induction of ginger (An et al., 2020). The optimum concentration of sucrose and PGRs for the best response of ginger microrhizome induction has been varyingly reported in different studies (Abbas et al., 2014; Mehaboob, Faizal, Shamsudheen, et al., 2019; Swarnathilaka et al., 2016). Hence, it is crucial to optimize sucrose and PGRs in the culture medium to obtain a better response of ginger for the *in vitro* microrhizome induction.

According to the two popular cultivars of Malaysian ginger, the *in vitro* regeneration of Halia Bara or red ginger cultivar was studied earlier (Zuraida et al., 2016). On the other hand, there is a lack of knowledge about Bentong ginger micropropagation. Therefore, this study aimed to establish a successful protocol for the direct shoot and microrhizome organogenesis of Bentong ginger. For this purpose, a fundamental understanding about explant surface sterilization, culture initiation, shoot multiplication, root development, *in vitro* microrhizome formation, acclimatization and survival of the micropropagated plantlets in the *ex vitro* conditions was required. Therefore, the specific objectives of the present study are as follow:

1. To determine the effective concentration of Clorox® for explant surface sterilization of Bentong ginger.
2. To determine the best basal media and the best type and optimum concentration of cytokinins and auxins for shoots multiplication of Bentong ginger.
3. To find out the suitable growing media mixture for acclimatization of the micropropagated plantlets of Bentong ginger.
4. To optimize sucrose, cytokinin and auxin for the *in vitro* microrhizome induction of Bentong ginger.

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LIST OF PUBLICATIONS

Zahid, N. A., Jaafar, H. Z. E., & Hakiman, M. (2021). Alterations in microrhizome induction, shoot multiplication and rooting of ginger (*Zingiber officinale* Roscoe) var. Bentong with regards to sucrose and plant growth regulators application. *Agronomy*, *11*(2), 320.

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