UNIVERSITI PUTRA MALAYSIA

IN VITRO PROPAGATION AND MUTATION INDUCTION OF TORCH GINGER (Etlingera elatior J.)

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By

MUHAMAD FAHMI YUNUS

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

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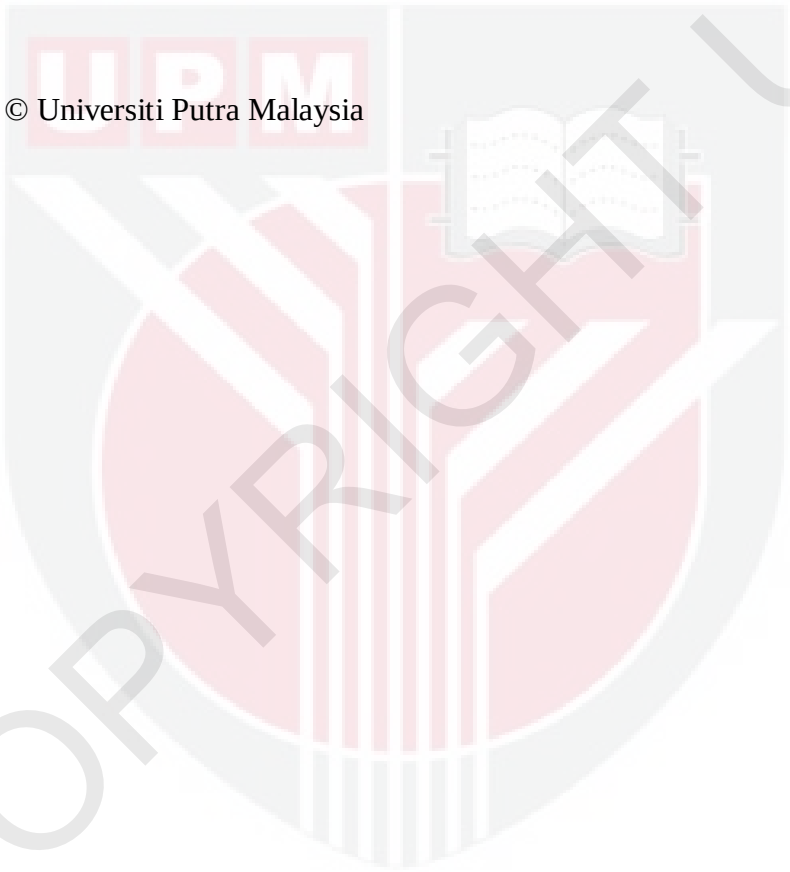

Dedicated to:

My dearest parents

Yunus bin Jamaludin

Zawiah binti Basnun

and

My Siblings

Muhamad Aqqat bin Yunus

Muhammad Ehsan Sabri bin Yunus

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

IN VITRO PROPAGATION AND MUTATION INDUCTION OF TORCH GINGER (_Etlingera elatior_ J.)

By

MUHAMAD FAHMI YUNUS

October 2013

Chairman : Associate Professor Maheran Abd Aziz, PhD

Faculty : Faculty of Agriculture

The aim of this study was to develop a protocol for *in vitro* propagation and mutation induction of *Etlingera elatior* by using gamma ray irradiation. The study included establishment an efficient *in vitro* plant propagation system in *E. elatior*, investigation of the optimum dose for radio sensitivity test, to determine the effects of various doses of gamma irradiation on multiple bud induction and also to determine the variation in genomic DNA of regenerated shoots by using random amplification of polymorphic DNA (RAPD) technique.

In this study, an efficient and systematic protocol for complete plant regeneration from suckers of *Etlingera elatior* (J.) has been developed. The addition of N6-benzyl amino-purine (BAP) (0, 3, 5, 7 and 10 mg L^{-1}) to the culture medium comprising of Murashige and Skoog (MS) basal salts, 3% sucrose, 0.4% gelrite did not show any significant effects on percentage of shoot induction and mean number of shoots produced. However, BAP at 3 mg L^{-1} was chosen as the best medium for shoot induction due to economic feasibility and it gave the highest result in all four

parameters recorded. Various concentrations of BAP, 6-furfurylaminopurine (kinetin) and N6-(2-isopentenyl) adenine (2-iP) alone at 0, 3, 5, 7 and 10 mg L^{-1} were tested for shoot multiplication. BAP at all levels were found suitable for the multiplication of shoot. However, the low level of 3 mg L^{-1} BAP was chosen as the best concentration of BAP due to economic feasibility. The best root proliferation was observed on MS medium without plant growth regulator (PGR). Assessment of various potting media for acclimatization showed medium containing soil: sand: peat moss (1:1:1) produced high survival of plantlets, number of leaves produced per plant and the plant height.

Mutation breeding techniques in combination with tissue culture and molecular marker methods provide a powerful tool for improvement of vegetatively propagated plants. The results of irradiation on *in vitro* buds of *E. elatior* showed that LD_{50} to be 10 Gy with the survival of explants being sharply reduced after this dosage. The gamma irradiated shoots were subcultured for three cycles (M_1V_1 to M_1V_3) to obtain potential mutant lines. This study showed that RAPD marker was efficient in differentiating the induced mutants from the untreated control of *E. elatior*. All eight selected gamma irradiated regenerants were differentiated from the untreated control based on the banding patterns obtained using 9 primers which generated 59 reproducible bands, whereby 35 (55.31%) were found to be polymorphic. The Jaccard's coefficient of similarity values ranging from 0.537 to 0.860 were indicative of the level of genetic variation among the mutants studied. For comparison between the potential lines (PL) and the control, a maximum similarity value (0.814) was observed in PL1 mutant while the minimum value (0.537) was observed in PL7. The

presence of polymorphic bands in 8 potential lines suggested that genetic variation occurred in all the treatments as compared to the control.

In summary, the combination of techniques of *in vitro* propagation, multiplication, gamma irradiation, and RAPD analysis for early screening of mutants can facilitate breeding programme of *E. elatior*.

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

PROPAGASI *IN VITRO* DAN ARUHAN MUTASI POKOK KANTAN (*Etlingera elatior* J.)

Oleh

MUHAMAD FAHMI YUNUS

Oktober 2013

Pengerusi : Professor Madya Maheran Abd Aziz, PhD

Fakulti : Fakulti Pertanian

Tujuan penyelidikan yang dijalankan ialah untuk membangunkan teknik propagasi *in vitro* dan aruhan mutasi *Etlingera elatior* dengan menggunakan penyinaran sinar gamma. Kajian ini merangkumi penghasilan satu sistem propagasi tumbuhan secara *in vitro* bagi *E. elatior*, kajian mengenai dos optimum bagi ujian sensitiviti radio, penentuan kesan-kesan pelbagai dos sinaran gamma dan juga penentuan variasi pada genom DNA dari pucuk yang dihasilkan dengan menggunakan teknik penanda molekul amplikasi rawak DNA polimorfik (RAPD).

Di dalam penyelidikan ini, protokol yang effisien dan sistematik untuk regenerasi tumbuhan dari sulur *E. elatior* (J.) telah dibangunkan. Penambahan N6-benzil amino-purin (BAP) pada kepekatan 0, 3, 5, 7 dan 10 mg L^{-1} pada kultur medium yang mengandungi nutrien asas Murashige dan Skoog (MS), 3% sukrosa, 0.4% Gelrite tidak menunjukkan sebarang kesan yang signifikan terhadap peratusan penginduksian pucuk dan juga jumlah min penghasilan bilangan pucuk. Walaubagaimanapun, BAP pada kepekatan 3 mg L^{-1} telah dipilih sebagai paras kepekatan terbaik disebabkan faktor ekonomi dan ia memberikan keputusan terbaik

di dalam semua empat parameter yang direkodkan. Pelbagai kepekatan tunggal pengawalatur pertumbuhan BAP, 6-furfurilaminopurin (Kinetin) dan juga N6-(2-isopentenil) adenin (2-iP) pada kepekatan 0, 3, 5, 7 dan 10 mg L^{-1} telah diuji untuk penggandaan pucuk. BAP pada setiap kepekatan telah diuji berkesan untuk penggandaan pucuk. Sungguhpun demikian, BAP pada kepekatan 3 mg L^{-1} telah dipilih sebagai paras kepekatan terbaik berdasarkan sifat ekonomi yang dimiliki. Medium penggandaan akar yang terbaik ialah medium MS tanpa sebarang penambahan pengawalatur pertumbuhan. Penilaian pelbagai media berpasu bagi tujuan aklimitasi menunjukkan bahawa medium yang mengandungi tanah: pasir: tanah gambut berlumut (1:1:1) memberikan kadar kemandirian yang tinggi kepada anak pokok, penghasilan daun per anak pokok dan juga tinggi anak pokok.

Teknik pembiakbaka mutasi dengan kombinasi teknik kultur tisu dan penanda molekul boleh menjadi teknik yang berkesan untuk penambahbaikan tumbuhan yang dibiakkan melalui kaedah tampang. Keputusan irradiasi tunas *in vitro E. elatior* menunjukkan bahawa LD_{50} ialah pada 10 Gy dengan kadar hidup berkurangan dengan drastik selepas dos ini. Pucuk yang telah disinari dengan sinar gamma telah disubkultur untuk tiga kitaran (M_1V_1 ke M_1V_3) untuk memperolehi titisan mutan yang berpotensi. Kajian ini menunjukkan RAPD adalah berkesan untuk membezakan antara mutan yang diaruh daripada kawalan *E. elatior* yang tidak diaruh. Kesemua lapan regenerasi yang diaruh dengan gamma telah dibezakan daripada kawalan yang tidak diaruh berdasarkan corak jalur yang diperolehi dengan menggunakan 9 primer yang menghasilkan 59 jalur reproduksi, di mana 35 (55.31%) adalah polimorfik. Nilai pekali pesamaan Jaccard berada di antara julat 0.537 hingga 0.860 menunjukkan paras kepelbagaian genetik antara mutan yang dikaji. Sebagai

perbandingan, antara titisan berpotensi (PL) dan juga kawalan, nilai kesamaan maksimum (0.814) telah diperolehi pada mutan PL1 manakala nilai minimum (0.537) diperolehi pada PL7. Penghasilan jalur polimorfik pada 8 titisan berpotensi menyarankan bahawa variasi genetik berlaku pada semua rawatan berbanding dengan kawalan.

Sebagai kesimpulan, kombinasi antara teknik propagasi *in vitro*, penggandaan, penyinaran gamma, dan juga analisis RAPD untuk penyaringan awal mutan boleh membantu program pembiakbakaan *E.elatior*.

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

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%	Percentage
µmol m^{-2} s^{-1}	Micromole per square meter per second
2, 4-D	2,4-Dichlorophenoxyacetic acid
^{60}Co	Cobalt-60
2-iP	N6-(2-isopentenyl) adenine
ANOVA	Analysis of variance
BAP	N6-benzyl amino-purine
cm	Centimetre
CTAB	Cetyl trimethylammonium bromide
DNA	Deoxyribonucleic acid
DMRT	Duncan's Multiple Range Test
EDTA	Ethylenediaminetetraacetic acid
g	Gram
Gy	Gray
IAA	indole-acetic acid
IBA	indole-3-butyric acid
KIN	6-furfurylaminopurine
LD	Lethal Dose
MARDI	Malaysian Agricultural Research and Development Institute
MS	Murashige and Skoog
NAA	1-naphthaleneacetic acid
PCR	Polymerase Chain Reaction
PPM	Plant Preservative Mixture
RAPD	Random Amplification of Polymorphic DNA

RCBD	Randomized Complete Block Design
SAS	Statistical Analysis System
SE	Standard Error
UKM	Universiti Kebangsaan Malaysia
UPM	Universiti Putra Malaysia

CHAPTER 1

INTRODUCTION

1.1 Background

Zingiberaceae is one of the largest families of the plant kingdom. It is an important family which provides many useful products for food, spices, medicines, perfume, dyes essentials oil and aesthetics to man (Jaafar et al., 2007; Poulsen, 2006). It provides plants of economic value mainly for its beautiful flowers, vegetable and also ingredient in a dish. These species are represented throughout the tropical and subtropical regions where the Indo Malayan (Indonesia, Malaysia, Brunei, Singapore, Papua New Guinea and the Southern Philippines) region is the centre of diversity for the Zingiberaceae. Of the 52 genera and 1500 species known in the world, at least 25 genera and 650 species can be found in Malaysia (Sirirugsa, 1999).

Etlingera elatior (Jack) R.M.Sm which belongs to the Zingiberaceae family is one of the most commonly known species of *Etlingera*. This species is also known as torch ginger or wax flower due to the striking resemblance of the inflorescence to a flaming torch. Torch ginger is widely cultivated in the tropical country and possibly native to Indonesia and Malaysia. It is known as *kantan* in Malaysia and *kecombrang* in Indonesia.

1.2 Problem Statement

E. elatior is one of the neglected plants in the Zingiberaceae family and scientific research on their propagation technique, production, biotechnology and ecology is limited. However, this ginger species offers great scope for the development of a large range of ornamental and cut flower types (Poulsen, 2006). Ismail (2009) reported that *E. elatior* is one of the 30 popular herbs or new industrial crops that have high demand in Malaysia. It is now cultivated on a commercial scale in places like Australia, Thailand and Costa-Rica for cut flower production (Ismail, 2009; Segalen, 2010). The plant itself makes a great garden landscape, their flowers having an immense ornamental value and also has a place in an eco-garden. To sustain in the ornamental and cut flower industry, torch ginger requires continuous improvement in certain characters like flower colour, morphology, longevity, size, odour and decreased time to flower formation. Unfortunately, conventional breeding of *E.elatior* is handicapped by the cross incompatibility and poor fruit set and also low seed production (Marcsik and Hoult, 2010). Due to these factors, alternative approaches for crop improvement of *E. elatior* such as through mutation induction could be explored.

In vitro culture techniques provide an alternative means of propagation and a tool for crop improvement (Zheng et al., 2008). Unfortunately, this medicinal plant has not received much attention from tissue culturists and to date, there is limited information on plant regeneration of this medicinal plant. Hence, with the increasing importance of torch ginger as an ornamental species, there is a need for an efficient

protocol for plant regeneration using tissue culture techniques. The protocol developed here will be helpful for regenerating plants at much higher rates than any other conventional breeding methods and also may serve as a potential source of new variants and further genetic improvement in this species (Xu et al., 2009). In addition, development of new torch ginger cultivars with improved characters is another approach that can be explored. Possible approaches to create genetic variability for the selection of useful plants are through conventional plant breeding, mutagenesis, somaclonal variation and genetic transformation.

1.3 Significance of the Study

In a modern and industrialized horticulture, there is always a demand and necessity for new cultivars. Modern day plant breeding is based on creating variations followed by selection, evaluation and multiplication of the desired genotypes. Induced mutations have played an important role in the improvement of plants and more than 2500 mutant cultivars have been developed through mutation breeding (Patade et al., 2008). Mutation breeding is an established method for plant improvement, thus encouraging the plant breeders to use induced mutagenesis. Induction of mutation can increase the possibility a thousandfold compared with spontaneous mutation under natural condition (Broertjes and Van Harten, 1988).

In a crop improvement programme, plant breeders often combine several techniques in order to increase efficiency and reduce the time needed for the development of a new cultivar. Such combination has been exploited for the creation of new and novel plant cultivars, particularly in vegetatively propagated species (Pinet-Leblay et al.,

1992; Broertjes and Van Harten, 1988). Successful outcome of a mutation depends on an efficient induction of mutation as well as an effective recognition and recovery of the desired mutant plants through repeated subculture (Puchooa, 2005).

The present study is divided into two parts: a) *In vitro* propagation and b) *In vitro* mutagenesis and RAPD analysis. A new technique is needed to standardize the management of chimeric tissues through multiple bud regeneration. Molecular techniques can provide an understanding of plant cell responses to mutation induction. It also facilitates a better understanding of the potential and limitations of mutation breeding, which can lead to early identification of useful variants. Major advantages of random amplification of polymorphic DNA (RAPD) are the low cost and effort required for its application (Dhakshanamoorthy, et al., 2011; Atienzar and Jha, 2006).

1.4 Objective

The objectives of this study are:

1) To establish an efficient *in vitro* plant regeneration system in torch ginger.

2) To investigate the optimum dose for radio sensitivity test and to determine the effects of various doses of gamma irradiation on multiple bud induction.

3) To determine the variation in genomic DNA of regenerated shoots by using RAPD technique.

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