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MICROPROPAGATION OF Artocarpus heterophyllus LAM. AND ASSESSMENT OF GENETIC STABILITY USING ISSR MARKERS

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MICROPROPAGATION OF A*rtocarpus heterophyllus* LAM. AND ASSESSMENT OF GENETIC STABILITY USING ISSR MARKERS

By

NURUL HUSNA BINTI MUSTAFA KAMAL

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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Specially Dedicated

To

My parents,

Mustafa Kamal Abdul Satar & Saniyah Ismail

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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Chairperson: Associate Professor Maheran Abdul Aziz, PhD
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Jackfruit (*Artocarpus heterophyllus*) is a multi-purpose species that provides the source of food, timber, fuel, fodder, medicinal and industrial products. The plant, however, is mostly known for its edible fibrous medium-sized fruit which is crunchy, juicy and sweet. Jackfruit can be propagated through seeds and vegetative means. However, the seeds are known for its recalcitrant nature, thus it cannot be stored outside for a long period of time. Thus, this study was carried out in order to produce uniform planting materials in large quantity using *in vitro* technique. Micropropagation has been proven to successfully mass produce plantlets in a short time as well as producing disease-free plantlets. In the experiment on optimization of seed sterilization procedure, the whole seeds were sterilized with 70% ethanol for 1 min followed by different concentrations (30, 40 and 50%) of Clorox® (5.25% sodium hypochlorate) for 20 min and rinsed once with sterile distilled water. This was followed by immersion in 10, 20 and 30% Clorox® for 15 min then rinsed five times with sterile distilled water. Tween 20 was added into the Clorox® solution as surfactant. The seeds were successfully sterilized using 40% Clorox for 20 min + 20% Clorox for 15 min and 50% Clorox for 20 min followed by 20% Clorox for 15 min. For the effect of different concentrations of hormones on shoot regeneration from seeds, aseptic seeds were cultured on half-strength MS medium supplemented with different concentrations (0, 1.0, 2.5, 5.0, 7.5 and 10.0 mg/L) of BAP and KIN separately. BAP at 2.5 mg/L was chosen as the most suitable concentration producing a mean number of 7.33 shoots. Shoot induction using shoot tip and different node positions (node 1 and node 2) of seed derived shoots showed that there were no significant differences on mean number of shoots produced per explant between the node positions. Nevertheless, node 2 gave the highest mean number of shoots (4.47). For the effect of decapitation on shoot regeneration of *in vitro* shoots there was significant difference between the decapitated and non-decapitated shoots on mean number of shoots produced per explant. The mean number of shoots for the decapitated shoots reached 12.73. In the shoot multiplication experiment, node 1 and node

2 were cultured on half-strength MS medium supplemented with 3 different cytokinin types at 5 different concentrations each; BAP (1.0, 2.5, 5.0, 7.5 and 10.0 mg/L), KIN (1.0, 2.5, 5.0, 7.5 and 10.0 mg/L) and TDZ (0.05, 0.1, 0.5, 1.0 and 2.0 mg/L). BAP at 1.0 mg/L gave the highest mean number of shoots (17.13). BAP at 5.0 mg/L, on the other hand, gave the highest mean shoot length (2.95 cm). BAP at 1.0 mg/L however was chosen as the most suitable treatment in producing multiple shoots. For rooting of shoots, shoots which were 5 – 6 cm in length were separated individually and placed on half-strength MS medium containing different concentrations (0, 1.0, 2.5 and 5.0 mg/L) of IBA and NAA separately. Medium containing 2.5 mg/L IBA gave the highest mean number of roots (18.73). The control however had the highest mean root length (3.37 cm). Nevertheless, 2.5 mg/L IBA was chosen as the best treatment for rooting. The completely rooted plantlets were tested on 4 different potting mixtures which were organic soil and topsoil (1:1), perlite and sand (1:1), peat moss and sand (1:1) and organic matter, topsoil and sand (1:1:1). The acclimatized plantlets were observed to perform best in potting medium containing organic soil and topsoil (1:1). The percentage of plantlet survival was 88.89%. ISSR markers were used to assess the genetic stability of the regenerants at the fifth subculture and it was observed that some of the regenerants evaluated showed variability compared to the mother plant. Out of 19 regenerants assesed, 15 had Jacaard's similarity coefficient of 1.0000, signifying that nearly 80% of the regenerants were identical to the mother plant. From this study, it can be concluded that *in vitro* technique can be used as a suitable means of propagating clonal *A. heterophyllus* in large quantity. Moreover, the propagules produced exhibited high genetic stability even at the fifth subculture.

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

PEMBIAKAN MIKRO A*rtocarpus heterophyllus* LAM. DAN PENILAIAN STABILITI GENETIK MELALUI PENANDA ISSR

Oleh

NURUL HUSNA BINTI MUSTAFA KAMAL

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Nangka (*Artocarpus heterophyllus*) merupakan spesies pelbagai guna yang membekalkan makanan, kayu, bahan api, makanan, perubatan dan produk industri. Tumbuhan ini kebanyakannya dikenali untuk buah berserabut bersaiz sederhana yang rangup, berair dan manis. Nangka boleh dibiakkan melalui biji benih dan tampang. Walau bagaimanapun, biji benihnya terkenal dengan bersifat rekalsitran, lantas ia tidak boleh disimpan di luar untuk tempoh masa yang panjang. Oleh itu, kajian ini dijalankan untuk menghasilkan bahan penanaman seragam dalam kuantiti yang besar menggunakan teknik *in vitro*. Mikropropagasi telah terbukti berjaya menghasilkan anak pokok dalam jumlah yang besar dalam masa yang singkat serta menghasilkan anak pokok yang bebas penyakit. Dalam eksperimen untuk mengoptimumkan prosedur pensterilan biji benih, keseluruhan biji benih telah disterilkan dengan 70% etanol untuk 1 minit diikuti dengan kepekatan Clorox® (5.25% natrium hypochlorate) (30 , 40 dan 50%) yang berbeza selama 20 min dan dibilas sekali dengan air suling steril. Ini diikuti dengan rendaman dalam 10 , 20 dan 30% Clorox® selama 15 min kemudian dibilas lima kali dengan air suling steril. Tween 20 telah ditambah ke dalam larutan Clorox® sebagai surfaktan. Biji benih telah berjaya disterilkan menggunakan 40% Clorox selama 20 min diikuti dengan 20% Clorox selama 15 min dan 50% Clorox selama 20 min diikuti dengan 20% Clorox selama 15 min. Bagi kesan kepekatan hormon pada percambahan pucuk dari biji benih, biji benih aseptik telah dikulturkan dalam medium MS separuh kekuatan ditambah dengan kepekatan BAP dan KIN yang berbeza (0, 1.0, 2.5, 5.0, 7.5 dan 10.0 mg/L) secara berasingan. BAP pada 2.5 mg/L dipilih sebagai kepekatan yang paling sesuai dengan menghasilkan purata min bilangan pucuk 7.33. Induksi pucuk menggunakan hujung pucuk dan kedudukan nod yang berbeza (nod 1 dan nod 2) pada pucuk yang dicambah daripada biji benih menunjukkan bahawa tiada perbezaan yang signifikan bagi purata min pucuk dihasilkan per eksplan. Walau bagaimanapun, nod 2 memberikan purata min pucuk tertinggi (4.47). Untuk kesan dekapitasi pada pucuk *in vitro*, terdapat perbezaan yang signifikan di antara pucuk

dekapitasi dan pucuk tidak dekapitasi terhadap pengeluaran min bilangan pucuk per eksplan. Min bilangan pucuk bagi pucuk dekapitasi adalah 12.73. Dalam eksperimen penggandaan pucuk, nod 1 dan nod 2 telah dikulturkan dalam medium MS separuh kekuatan ditambah dengan 3 jenis sitokinin berbeza pada 5 kepekatan berbeza; BAP (1.0, 2.5, 5.0, 7.5 dan 10.0 mg/L) , KIN (1.0, 2.5, 5.0, 7.5 dan 10.0 mg/L) dan TDZ (0.05 , 0.1 , 0.5 , 1.0 dan 2.0 mg/L). BAP pada 1.0 mg/L memberikan jumlah purata pucuk tertinggi (17.13). Manakala BAP pada 5.0 mg/L memberikan purata min panjang pucuk tertinggi (2.95 cm). BAP pada 1.0 mg/L bagaimanapun dipilih sebagai rawatan yang sesuai dalam menghasilkan penggandaan pucuk. Untuk pengakaran pucuk, pucuk yang adalah 5 – 6 cm panjang telah dipisahkan secara individu dan diletakkan di atas medium MS separuh kekuatan yang mengandungi kepekatan IBA dan NAA yang berbeza (0 , 1.0 , 2.5 dan 5.0 mg/L) secara berasingan. Medium yang mengandungi 2.5 mg/L IBA memberikan jumlah purata tertinggi akar (18.73). Kawalan bagaimanapun mempunyai panjang min akar tertinggi (3.37 cm). Walau bagaimanapun 2.5 mg/L IBA dipilih sebagai rawatan yang terbaik untuk pengakaran. Anak pokok yang telah berakar sepenuhnyai telah diuji ke atas 4 campuran pot yang berbeza iaitu tanah organik dan topsoil (1:1), perlite dan pasir (1:1), tanah gambut dan pasir (1:1) dan bahan organik, topsoil dan pasir (1:1:1). Anak pokok aklimatisasi didapati menunjukkan prestasi terbaik pada medium pot mengandungi tanah organik dan topsoil (1:1). Peratusan hidup anak pokok adalah 88.89%. Penanda ISSR telah digunakan untuk menilai kestabilan genetik regeneran pada subkultur kelima dan diperhatikan bahawa regeneran yang dinilai menunjukkan kepelbagaian berbanding pokok induk. Daripada 19 regeneran yang dinilai, 15 mempunyai pekali persamaan Jacaard sebanyak 1.0000, menandakan bahawa hampir 80% daripada sampel itu serupa dengan pokok induknya. Daripada kajian ini, dapat disimpulkan bahawa kaedah *in vitro* boleh digunakan sebagai kaedah yang sesuai untuk propagasi klon *A. heterophyllus* dalam kuantiti yang besar. Di samping itu, propagul yang terhasil mempamerkan kestabilan genetik yang tinggi walaupun pada subkutur kelima.

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Declaration by Members of Supervisory committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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%	percentage
°C	degree centigrade
µl	microlitre
µM	micromolar
α	level of significance
ANOVA	analysis of variance
BAP	6-benzylaminopurine
bp	base pair
cm	centimeter
CTAB	cetyl trimethylammonium bromide
DMRT	Duncan multiple range test
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
e.g.	exempli gratia (for example)
et. al.	et alia
etc.	et cetera
g	gram
g/L	gram per litre
HCl	hydrochloric acid
IBA	indole-3-butyric acid
IAA	indole-3-acetic acid
ISSR	inter simple sequence repeat
Kin	kinetin
L	liter
M	molar
min	minute
mg	milligram
mg/L	milligram per liter
ml	milliliter
mm	millimeter
µ mol m^{-2} s^{-1}	micromole per meter square per second
µM	micromolar
MS	Murashige and Skoog
NAA	naphthalene acetic acid
NaOH	natrium hydroxide
ng	nanogram
NTSYS	numerical taxonomy and multivariate analysis system
PCR	polymerase chain reaction
PGRs	plant growth regulators
RCBD	randomized complete block design
RNase	ribonuclease
rpm	revolution per minute
SAS	statistical analysis system
TDZ	thidiazuron
TE	tris-EDTA
TRIS	Tris(hydroxymethyl)amino-methane
UPGMA	unweighted pair-group method using arithmetic averages
V	volt

v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

1.1 Background

Artocarpus heterophyllus Lam. is an evergreen tree that belongs to the mulberry family, *Moraceae.* It is known as jackfruit in English or nangka in Malay. Apart from jackfruit, other well known species in the genus *Artocarpus* include *Artocarpus altilis* (breadfruit), *Artocarpus altilis* 'Seminifera' (breadnut), *Artocarpus integer* (cempedak), *Artocarpus lakoocha* (lakoocha) as well as *Artocarpus odoratissimus* (marang) (Anonymous, 1996). The jackfruit, however, is the most well-known among all these species of *Atrocarpus,* including being well-known as Bangladesh national fruit (Hasan *et al.*, 2008). All parts of the plant can be used and have many economical values such as for food, timber, fuel, fodder, medicinal and industrial products (Hasan *et al.*, 2008).

In efforts to turn the agriculture sector into a major economic growth generator for the country, the Ministry of Agriculture and Agro-based Industry launched the Balance of Trade (BOT) Plan 2010 in the Ninth Malaysia Plan (2006-2010) which also included the jackfruit as one of the focused fruit species. In 2005, FAMA listed jackfruit as one of the focused fruits (Hasan, 2007). According to Haq and Hughes (2002), jackfruit production in Malaysia, Indonesia and Thailand had exceeded 7.5 million tonnes and will continue to grow due to the expanding market in processed products. In Asia, the main exporters of jackfruit are Thailand, China and Malaysia (Haq, 2006). It has been noted that Malaysia exports fresh jackfruits to Singapore and Hong Kong (more than 4633 tonnes) in 1995 and earned about US$ 740,000 (Azad, 2000). The demand will continue to expand over the years because the fruits are now being commercialized into value-added products such as chips, dried fruits, frozen and preserved in cans.

Jackfruit can be propagated through seeds and vegetative parts. However, there are still problems faced by growers. For propagation from seeds, the main problem is the recalcitrant nature of the seeds, thus the seeds cannot be stored outside for a long period of time. The seeds are also not true to type as it is not guaranteed that they will produce plants genetically or physically the same as the mother plant as jackfruit is naturally cross pollinated in nature which led to great varialibility (Aralikatti, 2005; Haq, 2006). Moreover, the time taken by seedling trees to reach fruit-bearing age is usually longer than for those trees propagated by vegetative methods. In addition, the trees grow taller than those propagated by vegetative methods, which is a constraint in management and harvesting (Hossain and Haq, 2006). On the other hand,

through vegetative propagation, the trees are often shallow rooted. The trees also tend to be dwarf and tend to produce branches at low levels, which results in lower quality timber with a shorter trunk (Hossain and Haq, 2006).

Tissue culture is the *in vitro* aseptic culture of cells, tissues, organs or whole plant grown under controlled environment and nutrition which is for their growth and multiplication and is generally applied to produce plant clones which are true-to type of the selected genotype (Thorpe, 2007; Hussain *et. al*., 2012). This technology apart from being used for large scale plant multiplication, it is also used to eliminate disease, plant improvement, production of secondary metabolites as well as conservation of endangered, threatened and rare species (Hussain *et. al*., 2012). Small pieces of tissue (named explants) can be used to produce hundreds and thousands of plants in a continuous process in relatively short time period and space under controlled conditions, irrespective of the season and weather on a year round basis (Akin-Idowu *et al.*, 2009). The micropropagation technology has a vast potential to produce plants of superior quality, isolation of useful variants in well-adapted high yielding genotypes with better disease resistance and stress tolerance capacities (Brown and Thorpe, 1995). Tissue culture technique has various benefits over the conventional propagation methods (seed, cutting, grafting, air-layering and so on). It is a very quick propagation processes which can lead to the production of virus-free plant. Other than that, by culturing pathogen free germplasm, higher yields have been acquired (García-Gonzáles *et al*., 2010).

However there is some problem regarding tissue culture technique that needs to be taken into consideration. It is important in tissue culture to decide the type of explants used as the initial explants as different part will give different respond. The explants age is also important juvenile explants respond better and quicker. Other than that, the hormone requirement also plays a major role. Some plant might multiply well in single hormone while some multiply well with combination homones. The concentration used should also be tested as different concentration exhibit different respond. The number of subculture used also affect the tissue culture growth as it is knows that frequent subculture could multiply the *in vitro* plant rapidly.

In addition, herbs are usually studied for tissue culture rather than woody species. Woody species are often labelled difficult to propagate species. This is because woody species contains less juvenile cell than herbs. Moreover, having higher phenolic compound could be toxic to the *in vitro* plant. Other than that, obtaining tap root is also a problem for woody species, thus making the tissue culture technique for woody species more adventurous.

In vitro culture techniques for *A. heterophyllus* have been researched for potential mass propagation in order to produce planting materials on a large scale basis. However, there is less report on using seeds as the initial explants

in order to produce aseptic culture for mass propagation of *A. heterophyllus*. Thus this study investigates the potential of micropropagating *A. heterophyllus* from seeds for mass production as the seeds are easily obtain all year round and can be found abundantly which makes it suitable for micropropagation. The effect of different plant growth regulators on shoot multiplication and rooting was also tested in order to find the optimum concentration for multiplication and rooting

As for the genetic stability of a culture, it should be tested as it is reported in many studies that the hormone alteration tested in tissue culture system could alter the cell cycle division within the plant thus exhibiting somaclonal variation. The number of subculture as well as the length of culture period could affect the stability of cultured plant as. Phenolic compound also was reported to damage the DNA within a plant due to the reactive oxygen species. Therefore in order to ensure a stable genetic make-up of regenerants produced over subcultures, a molecular-based investigation using ISSR markers was performed.

This study was done in order to develop an efficient system for mass production and assessing the genetic stability of *A. heterophyllus*.

1.2 Objectives

The objectives of this study were:

i. To develop an *in vitro* propagation system for mass production of *A. heterophyllus*.
ii. To asses the genetic stability of the regenerants produced using ISSR markers.

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