IN VITRO REGENERATION AND ACCLIMATIZATION OF *Carica papaya* L.

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ABSTRACT

The purpose of this reseach is to study *in vitro* organogenesis of *Carica papaya* L. through tissue culture system. *In vitro* plant regeneration, production of synthetic seeds and acclimatization of plantlets were investigated. The experimental design that was used in this research is Completely Randomized Design (CRD). For *in vitro* plant regeneration study, Benzylaminopurine (BAP) and Naphthalene Acetic Acid (NAA) concentrations and combinations were used ranging from 0.0 mg/L - 2.0 mg/L. The finding of this research shows that optimum callus induction was obtained when root explants were cultured on Murashige and Skoog (MS) medium fortified with 1.5 mg/L BAP and 0.5 mg/L NAA. Friable cream callus was produced. Complete plant regeneration was successfully achieved when root, stem, leaf and petiol aseptic explants were cultured on MS medium supplemented with various combinations of plant growth regulators. MS medium supplemented with 1.0 mg/L BAP was found to be the optimum medium and stem explant was the most responsive explant producing 28.73 ± 6.03 shoots per explant. MS medium (with 1.5 mg/L NAA) was most optimum for root induction. Meanwhile, synthetic seeds of *Carica papaya* L. were produced when micro shoots were encapsulated with 4.0% sodium alginate solution added with 1.5 mg/L BAP and 1.0 mg/L NAA. Synthetic seeds germination rate was 2.56 ± 0.43 shoots per explant. Finally, acclimatization of *Carica papaya* L. was accomplished when plantlets were transferred to the combination of black and red soil (2:1) with 86.67% survival rate. In conclusion, the research showed that *in vitro* propagation of *Carica papaya* L. through tissue culture system was successfully achieved. It proves that plant tissue culture technology could be an alternative solution to achieve high quality of papaya plants, therefore could increase the crop production.

REGENERASI *IN VITRO* DAN AKLIMATISASI BAGI *Carica papaya* L.

ABSTRAK

Tujuan kajian ini adalah untuk mengkaji organogenesis secara *in vitro* bagi *Carica papaya* L. melalui sistem kultur tisu. Regenerasi tumbuhan secara *in vitro*, penghasilan biji benih tiruan dan juga aklimatisasi plantlet telah dikaji. Reka bentuk kajian yang digunakan dalam penyelidikan ini adalah Reka Bentuk Rawak Secara Penuh. Bagi kajian regenerasi tumbuhan secara *in vitro*, Benzylaminopurine (BAP) dan Napthalene Acetic Acid (NAA) pada kombinasi dan kepekatan yang berbeza telah digunakan dalam julat antara 0.0 mg/L – 2.0 mg/L. Hasil kajian menunjukkan induksi kalus yang optimum diperolehi apabila eksplan akar dikultur pada medium Murashige dan Skoog (MS) yang diperkaya dengan 1.5 mg /L BAP dan 0.5 mg /L NAA. Kalus berstruktur rapuh dan bewarna krim dihasilkan. Regenerasi tumbuhan yang lengkap berjaya dicapai apabila akar, batang, daun dan petiol aseptik dikultur pada medium MS ditambah dengan pelbagai kombinasi penggalak pertumbuhan tanaman. Medium MS yang telah ditambah dengan 1.0 mg /L BAP didapati sebagai medium optimum dan eksplan batang adalah explan paling responsif yang menghasilkan 28.73 ± 6.03 pucuk bagi setiap explan. Medium MS (dengan 1.5 mg /L NAA) adalah paling optimum untuk induksi akar. Sementara itu, biji benih tiruan *Carica papaya* L. berjaya dihasilkan apabila pucuk mikro dikapsulkan menggunakan larutan natrium alginat 4.0% ditambah dengan 1.5 mg /L BAP dan 1.0 mg /L NAA. Kadar percambahan biji benih tiruan ini adalah 2.56 ± 0.43 pucuk bagi setiap eksplan. Akhir sekali, aklimatisasi lengkap *Carica papaya* L. ke persekitaran luar telah berjaya dicapai apabila plantlet dipindahkan kepada campuran tanah hitam dan merah (2:1) dengan kadar kemandirian sebanyak 86.67%. Secara kesimpulannya, kajian yang telah dilakukan ini menunjukkan bahawa propagasi pesat tanaman *Carica papaya* L. melalui sistem kultur tisu telah berjaya dicapai. Ini membuktikan bahawa teknologi kultur tisu tumbuhan berupaya menjadi satu penyelesaian alternatif untuk mendapatkan tanaman betik yang berkualiti dan seterusnya penghasilan tanaman ini dapat dipertingkatkan.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BA	Benzyl Adenine
BAP	Benzylaminopurine
C	Celcius
Ca	Calcium
$CaCl_2.2H_2O$	Calcium Chloride Dehydrate
Clorox	Sodium Hypochlorite
CO_2	Carbon Dioxide
CRD	Completely Randomized Design
DMRT	Duncan's Multiple Range Test
FAMA	Federal Agricultural Marketing Authority
g	Gram
GDP	Gross Domestic Product
IAA	Indole-3-Acetic Acid
IBA	Indolebutyric Acid
M	Mol
MARDI	Malaysian Agricultural Research and Development Institute
mg/L	Milligram per liter
mL	Milliliter
mM	milliMol

Mm	Millimeter
MS	Murashige and Skoog
NAA	Naphthalene Acetic Acid
$NaC_6H_7O_6$	Sodium alginate
NaOH	Sodium hydroxide
NIEs	Newly Industrializing Economics
O_2	Oxygen
PCPA	4-Chlorophenoxyacetic acid/ Parachlorophenoxyacetate
PGRs	Plant Growth Regulators
pH	potential Hydrogen
PRSV	Papaya Ringspot Virus
Rpm	Rotation per minute
SE	Standard Error
SEM	Scanning Electron Microscope
SPSS	Statistical Package for the Social Sciences
Tween 20	Polyoxyethylene sorbitan monolaurate
USDA	United States Department of Agriculture
2ip	2-isopentenyl aminopurine
2,4-D	2,4-Dichlorophenoxyacetic acid

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CHAPTER 1

INTRODUCTION

1.1 Introduction

The constant improvements in technology and crop productivity through selection and breeding of plants have contributed to the growth of human civilization (Taji, Kumar, & Lakshmanan, 2002). Agricultural development had continued to increase since the agricultural revolution (Rahardja & Wahyu, 2003). Agriculture has been identified as the economy's third engine of growth (Austin & Amir, 2012). Agriculture is one of the fundamental sector of develop economic. This sector was not only important in providing basic necessities of life, but also provided food and employment opportunities (Raza, Zulkipli & Mohd, 2013).

The increasing of various crops, especially the cereal, during the past 50 years through traditional and improvised agricultural techniques leading to the "green revolution" of the 1960s and 1970s is perhaps the most commendable achievement of plant breeding (Taji et al., 2002). Agriculture plays the important role in contributing approximately 10% of Malaysia's GDP, and at least one-third of the country's population depends on this sector with 14% of them are farmers (Austin & Amir, 2012). However, the world population increase has been exponential in the past century and it is projected to reach 8.3 billion by 2020, before stabilizing at around 11 toward the end of the twenty-first century (Taji et al., 2002).

Malaysia has become a developing country, emerging a multi-sector factor in terms to increase the country's economic capita. Malaysia focus on the primary natural resources development and agricultural products and stand as a rapidly industrializing country (Austin & Amir, 2012). In the context of the second tier newly industrializing economies (NIEs) in Southeast Asia (Indonesia, Malaysia and Thailand) showed a successful selective intervention in agriculture, and contributed directly to the growth and development of the industrial economy (Rock, 2002).

Agriculture is the important key role in developing the rise of human civilization, whereby farming of domesticated species created food surpluses that nurtured the development of civilization. Taji et al. (2002) stated that, as the human population increases, the demand for more land area for housing and industrial activities also increases, forcing the conventional agriculture into marginally productive land. Furthermore Austin and Amir (2012) claimed that, in addition, the perspective of Malaysian land use, approximately 39.2% of the total area was planted with tree crops, such as rubber, oil palm, cocoa, coconut, fruits and vegetables.

There are natural limits in increasing the agriculture productivity by conducting an environmental manipulations. Thus, biotechnology was created in order to improve and maintain a sustainable agriculture (Taji et al., 2002). Biotechnology defined as the use of living organisms in terms of production of goods and services (www.cbd.int). Biotechnology is perceived as scientific research idea in resulting a new variant of plants or animal. Agricultural biotechnology was one of the biotechnology implementation in agriculture area. Agricultural biotechnology has been practiced for a long time, as people have sought to improve agricultural organism by selection and breeding (Wieczorek, 2003).

Biotechnology technique has been farmer's and researcher's consideration in solving a problem in agriculture. However, a research by Buckwell and Moxey (1990) perceived that biotechnology was not well defined and divisible on the farm level. Several researchers believed that biotechnology did not offer a significance effect on agriculture. However, biotechnology has being an alternative technique in developing the agriculture production.

Agricultural biotechnology would also concern about horticulture. Horticulture is defined as the science and art of growing fruits, vegetables, flowers or ornamental plants (Relf, 1992; Malaysian Agricultural Research and Development Institute [MARDI], 2008). Through this definition, several scholars confused about the purpose of horticulture in the implementation among the farmers. Based on Parrot, Pedelahore, De Bon and Kahane (2010), horticulture is considered as an innovation in improving the level of capabilities that could increases the chances of adopting horticulture.

Adoption and utilization of modern technology in the horticulture sector is an important factor to enhance productivity and sustain competitiveness in an increasing liberalized global market that demands good agricultural practices to ensure food quality, safety and sustainability (MARDI, 2008). Furthermore, MARDI (2008) also stated that generally horticulture plant are grouped into vegetables, fruits, medicinal plants, and also ornamental vegetables. These kind of crops have good prospective, particularly tropical fruits and flowers, which has high demand value and able to compete with others; pineapple, mango, papaya, and orchids.

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Plants cultivation is also described as *in vitro* plantation technique, in pots, green house, or the field. The cultivation technique is grouped into several research focus, agriculture food crops, horticulture, agriculture farms, forestry, plant breeding, and so on (Zulkarnain, 2014). In the early 1980s, the *in vitro* tissue and cell culture technique have been a possible in regenerating of whole plants from a single cell or small piece of tissue (Ruttan, 1999). The evidence of cell totipotency (total genetic potential) was the beginning of tissue culture practice. This theory was proposed by Schwann and Schleiden in 1838 (Yusnita, 2004). This technology is appropriate to be implemented into agricultural system and perceived could enhance agriculture productivity.

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1.2 Background of Study

Since the 1980s until now, plant tissue culture techniques have grown very rapidly. This situation has caused the culture activities difficult be monitored. Moreover, many breakthroughs have high commercial value achieved by research institutions from unpublished companies. In addition to plant propagation, the field of genetic engineering was aimed to improve the genetic quality of agricultural crops. There have been many varieties, even new species created through protoplast fusion techniques. Similarly, the application of the tissue culture techniques to the elimination of disease, especially viral diseases and secondary metabolite production with the help of *Agrobacterium* has become a routine technique performed by experts around the world (Zulkarnain, 2014).

To date, there are many plants that have been successfully propagated through tissue culture system. Malaysian local fruit crops are among it, such as banana, star fruit, papaya and many others. The papaya is popular as a backyard tree in many developing countries but increasingly becoming more important in commercial plantings for domestic markets and in countries like Mexico and Malaysia, for export. Advantages in papaya cultivation include a rapid return to investment and intensive cultivation, and also high crop yield. Most papayas in the tropics can be harvested eight or nine months after sowing and yields can range from 60-100 t/ha/year for improved varieties. The ripe fruits have a delicate aroma and sweetness, and high contents of vitamins A and C. There is a great diversity in the size, shape and quality of the fruit. In unselected germplasm or backyard trees, fruits are usually very large and are not very palatable, but for varieties such as ‘Solo’ and ‘Eksotika’ specifically selected for export or up-market, they are usually small for convenient packaging and have much better taste and storage attributes (MARDI, 2008). *Carica papaya* L. is a very good example of a crop that is extremely vulnerable, but economically very important (Saha, Phatak, & Chandra, 2004).

Papaya is one of the important food crops in Malaysia. The demand for the fruit is so high for consumption both by people within the country and abroad. According to Nogeh Gumbek (2017), Malaysia is able to become a major exporter in the world in next few years.

1.3 Problem Statement

A plant tissue culture in the vessel can be assumed as a miniature of greenhouse in transplant production (Aitken-Christie et al., 1994 in Kozai, Fujiwara, & Kitaya, 1995). However, there should be similarity between the two cultures from the environment factors, such as temperature effects, light, CO_2 concentration, and medium nutrient composition. However, a mistake in actual compositing of adding substances could causes various culture result (Nicomrat & Anantasaran, 2015).

Along with the development of genetic science and plant maintenance, the researchers conducted a research review of the papaya. Basically, the general purpose of papaya review was to find a high quality culture than the existing. The preferred types of superior papaya plants, that has low features tree, fast flowering periods, high productivity and resistance ability to degenerative and disease creatures (Sriani &

Ketty, 2010). Furthermore, Sriani and Ketty (2010) stated that papaya plant could grow in low and also highland. However, there were several issues and problems if papaya grows at the highland. At the condition with an altitude of more than 500 m above sea level, growth is slow and papaya fruit will have less sweet flavor. This has led to the cultivation of papaya in the highlands is not recommended. In addition to affecting the taste, papaya grown in the highlands are also vulnerable to disease because of the relatively high air humidity.

The demand of Malaysian papaya is very good from Singapore, China, Hongkong and West Asia besides the new market in Europe and United State and therefore, the production of Malaysian papaya needs to be improved to accommodate the demand (Nogeh Gumbek, 2017).

Virus and disease attack are very harmful for papaya plants. Mohd Anim (2016) said that the number of papaya in Malaysia is now at a minimum production rate because of the logging and degradation of papaya trees disease. According to Malaysian Agriculture Department (2006), papaya ringspot virus (PRSV) was the most serious disease and can destroy the papaya industry. Ashriq Fahmi (2015) stated that papaya dieback disease has led to reduce the export of papaya fruit. Mohd Anim (2016) said that the problem occurred due to the ring spot virus attacks that damage crops and affect production. Sharif Haron (2017) claimed that in 2004 the value of papaya exports amounting to RM120 million was reduced to RM21 million in 2012 due to the spread of deaback disease. Nogeh Gumbek (2017) stated that Malaysian papaya exports fell to RM 21 million in 2012 and less than RM 3 million last year due to dieback disease.

The lack of papaya in the market has increased the price of the fruit from RM 4 to RM 5 per kilogram compared to about RM 2 to RM 3 per kilogram previously (Mohd Anim, 2016). The consequences of papaya dieback disease has caused, many entrepreneurs turn to other crops (Nogeh Gumbek, 2017).

The improvement of crop using conventional methods has several limitations (Gana, 2010). Crop cultural techniques by using conventional methods in soil or sand medium have often faced technical, environmental and time constraints problem. For example, plant propagation through seeds usually require a long time period and the results are different from its parent. Another obstacle that is faced is natural disturbance, either caused by living bodies, such as pests and diseases, and also environmental stresses that can interfere with the success of plant propagation in the field. The need of plant seeds in large quantities, quality, free of pests and diseases, availability in a short time, it often can not be met with conventional methods either generative or vegetative (Triwibowo, 2006). In horticultural crops, vegetative propagation in the maintenance of genetic uniformity and preservation of cloning identity is essential, so that an efficient vegetative propagation technique is required (Mumo, Rimberia, Mamati, & Kihurani, 2013).

Papaya plant is believed could enhance economic performance. Olubode, Odeyemi, and Aiyelaagbe (2016) stated on their research that optimizing the papaya management could improve the economic performance of the crop production and increasing the benefits from fruits growers. Furthermore, Dwi and Ida (2010) believed that to obtain papaya propagation technology through *in vitro* methods requires a better and consistent technique in various environments.

1.4 Objective of Study

The objectives of these study are:

1. To study callus induction of *Carica papaya* L. var Eksotika through tissue culture system.
2. To investigate complete regeneration process of *Carica papaya* L. var Eksotika through tissue culture system.
3. To study the production of synthetic seeds of *Carica papaya* L. var Eksotika from micro shoots obtained *in vitro.*
4. To investigate complete acclimatization process of *Carica papaya* L. var Eksotika

1.5 Significance of Study

Plant breeding in biotechnology and molecular biology has ability to overcome some of the problems that occur in conventional breeding (Muhammad, Sriani, & Rahmi, 2015). According to Yusnita (2004), tissue culture has advantages compared with conventional plant propagation, they are able to produce the number of seeds of plants in a relatively short period, does not require a large area, can be executed throughout the year without depending on the season, produce more healthy seeds and enabling genetic manipulation.

In the last 20 years, tissue culture techniques have assisted researchers, plant growers, and nursery industries in increasing a large quantity plants (Yusnita, 2004). The studies on plant propagation in tissue culture especially for *Carica papaya* L. var Eksotika have not been widely implemented. Finally, this study was expected to contribute to other researchers, as a knowledge for further research. In addition, this research can be applied in maximum in producing papaya plants that are resistant to disease, so it can help the farmers to get the best plant quality.

1.6 Scope and Limitation of Study

This study has been carried out using tissue culture system in *Carica papaya* L. The type of papaya selected is a kind of Eksotika papaya. Aseptic seedlings parts such as roots, stems, petioles and leaves were used as source of explants. In this study, only two types of plant hormones, Benzylaminopurine (BAP) and Naphthalene acetic acid (NAA) were used in various concentrations. Only the effects of these plant growth regulators on callus induction, plant regeneration and synthetic seeds poduction were investigated.The experiments were also limited by plant contaminations and to achieve the most effective sterilization protocol.