PLANT TISSUE CULTURE OF POMEGRANATE, *Punica granatum* L.

MUHAMMAD AL - AMIN BIN MAZLAN

THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE (AGRICULTURE SCIENCE)
(RESEARCH MODE)

FACULTY OF TECHNICAL AND VOCATIONAL
UNIVERSITI PENDIDIKAN SULTAN IDRIS

2018

ABSTRACT

The purpose of this study was to examine the propagation ability of *Punica granatum* L. through tissue culture system. In addition, callus induction was also carried out in the study to observe the rate of cell differentiation on explants. The experimental design that was used in this research is Completely Randomized Design (CRD). The complete regeneration of the *Punica granatum* L. plant and callus production was successfully produced through the tissue culture technique. Explants used in this study were leaves, stem and roots from 8-week-old aseptic seedlings. All cultures were stored at a temperature of 25 ± 1ºC and light 16 hours of light, 8 hours dark. Plant acclimatization was done on three different medium, garden soil, coco peat and vermiculite. All data was recorded and analyzed using ANOVA. Leaf explants were found to be very responsive and Murashige and Skoog (MS) medium added with 2.0 mg/l Benzylaminoupurine (BAP) + 1.5 mg/l Napthalene Acetic Acid (NAA) has been identified as the optimum medium for shoot regeneration. Leaf explant managed to produce the number of shoots *in vitro* with 1.3333±0.3228 per explant at week 8. Whereas from stem explant, MS media added with 0.5 mg/l BAP and 2.0 mg/l NAA gave the highest shoot development with 1.0000 ± 0.2837 shoots per explant. Combination of NAA and BAP, at 1.5 mg/l respectively was used for complete regeneration of *Punica granatum* L. *in vitro*. In addition, artificial seeds of *Punica granatum* were also produced. As a conclusion, this method was proven to be successful in producing new generation of *Punica granatum* L. and its features are preserved. This proves that plant tissue culture technology could be an alternative solution to achieve high quality of *Punica granatum* L, therefore could increase the crop production.

KULTUR TISU TUMBUHAN DELIMA, *Punica granatum* L.

ABSTRAK

Tujuan kajian ini dijalankan untuk menguji kemampuan propagasi *Punica granatum* melalui sistem tisu kultur. Selain itu juga penginduksian kalus juga dilaksanakan dalam kajian untuk melihat kadar pembentukkan sel pada bahagian eksplan. Reka bentuk kajian yang digunakan dalam penyelidikan ini adalah Reka Bentuk Rawak Secara Penuh. Regenerasi lengkap bagi tumbuhan *Punica granatum* dan penghasilan kalus telah berjaya dihasilkan melalui teknik kultur tisu Eksplan yang digunakan dalam kajian ini ialah bahagian daun, batang dan akar daripada anak benih aseptik yang berumur 8 minggu. Semua kultur disimpan pada suhu 25 ± 1ºC dan kala cahaya 16 jam cahaya, 8 jam gelap. Aklimatisasi tumbuhan dilakukan pada tiga jenis medium yang berbeza iaitu tanah kebun, *coco peat* dan *vermiculite*. Semua data direkodkan dan dianalisis menggunakan ANOVA. Medium Murashige dan Skoog (MS) dengan kombinasi hormon 2.0 mg/l Benzylaminopurine (BAP) + 1.5 mg/l Napthalene Acetic Acid (NAA) telah dikenalpasti sebagai medium optima bagi regenerasi pucuk manakala eksplan daun pula telah dikenalpasti sebagai eskplan yang terbaik dalam kajian ini. Eksplan daun telah berjaya menghasilkan pucuk *in vitro* dengan peratus perhasilan pucuk sebanyak 1.3333± 0.3228 pucuk dihasilkan bagi setiap eksplan. Manakala bagi eksplan daripada batang kombinasi terbaik ialah 0.5 mg/l BAP + 2.0 mg/l NAA dengan bilangan pucuk terhasil sebanyak 1.0000 ± 0.2837 setiap eksplan. Kombinasi auksin seperti NAA dan sitokinin seperti BAP pada 1.5 mg/l telah digunakan untuk tujuan regenerasi lengkap tumbuhan *Punica granatum* secara *in vitro.* Biji benih tiruan *Punica granatum* juga telah berjaya dihasilkan Kesimpulannya, kaedah tisu kultur ini merupakan kaedah yang terbaik dalam penghasilan generasi baru *Punica granatum* dan ciri-cirinya adalah dikekalkan. Ini membuktikan bahawa teknologi kultur tisu tumbuhan berupaya menjadi satu penyelesaian alternatif untuk mendapatkan tanaman *Punica granatum* yang berkualiti dan seterusnya penghasilan tanaman ini dapat dipertingkatkan.

CONTENT

LIST OF TABLE

LIST OF FIGURE

ABBREVIATION

2,4,5-T	2,4,5-Tricholoro-phenoxyacetic acid
2,4-D	2,4-Dichlorophenoxy acetic acid
2iP	6-dimethylamino purine
4-CPA	4-chlorophenoxy acetic acid
B	Boron
BAP	Benzoacetic acid
C	Carbon
Ca	Calcium
$CaCl_2.2H_20$	calcium chloride dehydrate
Cl	Chlorine
cm	Centimeter
Co	Cobalt
Cu	Copper
dicamba	Dicholoro-2-methoxy-benzoic acid
DMRT	Duncan Multiple Range Test
DNA	Deoxyribonuclic acid
EDTA	Ethylene Diaminetethraacetic Acid
Fe	Ferum
FeEDTA	Iron chelate
H	Hydrogen
I	Iodine

IAA	Indole-3-acetic acid
IBA	Indole-3-butric acid
K	Potassium
Kinetin	N-2-furanilmethyl-1H-purine-6-amine
kPa	kilopascal
Mg	Magnesium
ml	Mililitre
mm	Milimetre
mM	Milimolar
Mn	Mangan
Mo	Molybdenum
MS	Murashige and Skoog
N	Nitrogen
Na	Sodium
NAA	Naphthelena acetic acid
$NaC_6H_7O_6$	Sodium Alginate
NaOH	Sodium hydroxide
O	Oxygen
P	Phosphorus
pCPA	p-chlorophenoxy acetic acid
pH	Percentage hydrogen
picloram	4-amino-3,5,6-tricholoro-picolinic acid
S	Sulphur
SE	Standard Error
SEM	Scanning Electron Microscopy

SPSS	Statistical Packages for The Social Science
SSOc	Synthetic seed *Ocimum basilicum*
TDZ	Thiazuron-N-phenyl-N-1,2,3 thiadiazol-5ylurea
Zeatin	6-4-hydroxy-3-methyl-trans-2-butenylaminoporine
Zn	Zinc
B5	Gamborg's medium
WPM	Woody Plant Medium

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Plant reproduction is needed to increase large number of plant on earth. It is important to maintain the variety and generation of plants. There are two types of plant reproduction which are propagation (sexual) and micropropagation (asexual). Seeds are ordinarily created from sexual proliferation; it is because the hereditary recombination has happened. A plant grow from seeds may have diverse attributes from its folks.

Seeds can be hard to get and few plants do not deliver seed. Plants have various systems for abiogenetic propagation. Some of these have been exploited by horticulturists and plant specialists to clone plants quickly. for example, tissue culture. The plants from a solitary parent are needed to keep up with the hereditary material. In

this manner, most vegetative proliferation techniques without fail deliver plants that are indistinguishable to the parent. Vegetative generation utilizes plants' organs, for example; roots, stems and clears out. Few plants seeds can be created without prepared treatment and the seeds contain the hereditary material of the parent plant.

Micropropagation is the art and science of plant multiplication *in vitro*. The procedure of micropropagation incorporates many strides, for example; stock plant care, explant selection and sanitization, media control to get expansion, establishment acclimation, and development of the liners. Other than that, micropropagation is the act of quickly increasing the stock plant material to deliver countless plants, utilizing present day plant tissue culture strategies. Micropropagation is utilized to multiply new plants, for example; those that have been hereditarily changed or reproduced through ordinary plant rearing strategies. It is additionally used to give an adequate number of plantlets for planting from a stock plant which does not deliver seeds, or does not react well to vegetative multiplication.

Micropropagation starts with the choice of plant material to be proliferated. Stock materials that are free of infections and growths are vital in the generation of the most advantageous plants. Once the plant material is decided for culture, the gathering of explants will commence and is reliant on the sort of tissue to be utilized. This includes stem tips, anthers, petals, dust and others plant tissues. The explant material is then surface cleaned, normally in numerous courses of blanch and liquor washes, and lastly flushed in disinfected water. This little segment of plant tissue, which is the solitary cell, is set on a development medium, commonly containing the sucrose as a

vital source and at least one plant development controllers will go about as plant hormones.

Usually the medium is thickened with agar to create a gel which supports the explant during its growth. Some plants are easily grown on simple media, but others require more complicated media for successful growth. The plant tissue develops and separates into new tissues relying upon the medium. For instance, media containing cytokinins are utilized to make spread shoots from plant buds. Multiplication is the taking of tissue samples produced during the first stage and increasing their number. Taking after the fruitful presentation and development of plant tissue, the foundation stage is trailed by augmentation. Through rehashed cycles of this procedure, a solitary explant test might be expanded from one to hundreds or a huge number of plants. Contingent upon the kind of tissue developed, duplication can include distinctive techniques and media. On the off chance that the plant material developed is callus tissue, it can be put in a blender and cut into little pieces and recultured on a similar sort of culture medium to develop more callus tissue. On the off chance that the tissue is developed as little plants called plantlets, hormones are frequently added that causes the plantlets to create numerous little branches that can be evacuated and recultured. This stage includes treating the plantlets or shoots that are created to support root development and "solidifying" process. It is performed *in vitro*, or in a sterile test tube environment.

Solidifying alludes to the readiness of the plants for a characteristic development environment. Until this stage, the plantlets have been developed in perfect conditions, intended to empower quick development. Because of the controlled way of their development, the plantlets regularly do not have completely useful dermal covers.

This causes them to be exceptionally helpless to malady and wasteful in their utilization of water and vitality. *In vitro* conditions are high in humidity, and plants under these conditions frequently do not shape a working fingernail skin and stomata that shield the plant from drying out.

At the point when removed from culture, the plantlets require time to change in accordance with more characteristic ecological conditions. Solidifying usually includes gradually weaning the plantlets from a high-humidity, low light, warm environment to what might be viewed as an ordinary development environment for the species. In the last phase of plant micropropagation, the plantlets are expelled from the plant media and exchanged to soil or preparing manure for proceeded with development by ordinary strategies. This stage is frequently consolidated with the pretransplant arrange.

1.2 Description *Punica granatum*

Pomegranate is a bush or little tree, around 15 feet or 30 feet high. The pomegranate is spread and pretty much barbed. To a great degree, few examples at Versailles are known to have survived for two centuries. It has a solid inclination to sucker from the base. The leaves are evergreen or deciduous, inverse or in whorls of 4 or 6, short-stemmed, oval lanceolate, 3/8 to 5 inches long, weathered. Pompous blossoms are home on the branch tips independently or upwards of 5 in a group. They are 1/4 inches wide and described by the thick, tubular, red calyx having 5 to 8 beefy, pointed sepals shaping a vase from which rise the 2 to 7 crinkled, red, white or variegated petals encasing the various stamens. About round, yet delegated at the base by the conspicuous calyx, the

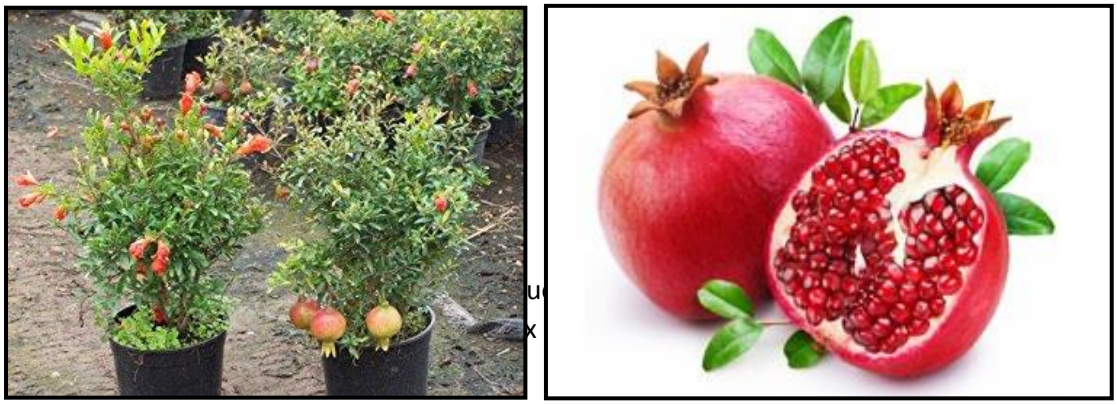
natural product, 2 1/2 to 5 inches wide, has an extreme, rugged skin or skin, essentially yellow pretty much overlaid with light or profound pink or rich red. The inside is isolated by membranous dividers and white light tissue into compartments stuffed with straightforward sacs loaded with tart, delightful, meaty, delicious, red, pink or whitish mash. In every sac, there is one white or red, rakish, delicate or hard seed. The seeds speak to around 52% of the heaviness of the entire natural product (Kew, 2010).

1.3 Importance of *Punica granatum*

Pomegranate is an economically important species of the tropical and subtropical locales of the world because of its scrumptious natural products, and pharmaceutical and fancy use. It is to a great extent utilized as a treat. The seeds alongside the meaty mash are dried and utilized as sauce (Jayesh 2004).

The organic product juice is a decent wellspring of sugars, vitamin C, vitamin B, pantothenic corrosive, potassium, cancer prevention agent polyphenols and a reasonable wellspring of iron. A few sections of the pomegranate tree, leaves, youthful natural products, organic product skin, blossom buds have been utilized customarily for their restorative properties and furthermore to tan of calfskin (Raj, 2010).

Other than that, the seeds of pomegranate take time to germinate by using traditional method. It happens because the seed coat of pomegranate is hard. The hard of seed coat is about 0.2mm thickness. So, by using the tissue culture it will help to reduce the time of pomegranate germinate (Kumar, 2010).



(a) (b)

Figure 1.1 (a) Tree of *Punica granatum* and (b) Fruit of *Punica granatum*

1.4 Plant Tissue Culture

Plants reproductives are needed to increase large number of plant in the earth. It is important to maintain their variety and generations of the plants. Plant reproductives have two types such as propagation and micropropagations. Propagations is referred to sexual propagation and asexual propagations whereas for the micropropagations is referred to plant tissues culture. The sexual propagation is referred to the seeds and spores it can be used for reproduction. Seeds are produced from sexual reproduction within a species, because genetic recombination has occurred.

A plant growth from seeds has different characteristics from its parents. Whereas asexual reproduction have many ways like by using stem cutting, leaf, root, onion and micropropagation, which is also known as plant tissue culture. A plant growth from asexual reproduction has the same characteristic from its parents because it does not exchange their genetically especially by using tissue culture.

Micropropagation process can increase the number of plants because it can use all part of plant to germinate such as by using stem, leaf and roots. From the stem, roots and leaf it can produce many plant from one explants.

Plant propagation through tissue culture may have been considered as early as 1902 when Haberlandt first attempt to regenerate plants from single cells. Although this pioneer work was failed, it inspired others to attempt in vitro culture of plants tissues (Conger, 1980). Whereas in the late 1930's, the first prolonged culture of unorganized plant tissues was reported independently in carrot by Gautheret and Nobecourt, and in tobacco by White (1963). Organization and developments of complete plants from cultured mass of carrot cells was demonstrated by Steward et al. (1958). In 1962, Murashige and Skoog formulated and published a define medium for tobacco culture which has probably been cited more than any other for culture of a wide range of blunt species, including both dicot and monocots.

As a conclusion, plant tissue culture is a gathering of procedures used to keep up or develop plant cells, tissues or organs under sterile conditions on a supplement culture medium of known creation. Plant tissue culture is broadly used to deliver clones of a plant in a technique known as micropropagation

1.5 Problem Statements

Punica granatum is an economically important species of the tropical and subtropical locales of the world because of its scrumptious natural products, and pharmaceutical and fancy use. It is to a great extent utilized as a treat.

The usual propagation method of *Punica granatum* was by seed. However, germination by seeds shows a high degree of variability because of cross-pollinated nature of the plant. Other than that, the seeds of *Punica granatum* take time to germinate by using traditional method. Its because the seeds have hard seed coat, so it will take time to break the seed coat. Thus, it is not attractive approach for producing a large number of elite plants within a short period of time. Besides, the percentage of seed germination through conventional method is lower than the culture tissue technique. It is because of the seed is surrounded by jelly, making the lifetime of the seed to survive is short in the germination period.

This problem gives effect to the wild stock of this important plant species that has been reduced slowly due to over exploitation and no efforts for its replenishment has been undertaken.

1.6 Objectives of the Research

1. To established organogenesis of *Punica granatum L*.
2. To establish callus induction and profeliration of *Punica granatum L.*
3. To identify the acclimatization optimal process for transplanting the *Punica granatum L.*
4. To achieve the potential of the synthetic seeds of *Punica granatum L.*

1.7 Scope and Limitation Study

1.7.1 Scope of Study

This experiment is conducted to establish organogenesis of *Punica granatum*. through tissue culture technique because it has high percentage of seed germination than conventional method. Thus, the problem of hardening the germination of the seed can be solved, and at the same time, the limitation number of this species of herbs can be solved too. The aspects looked into are the quality of seed and breeders. Through conventional method, the chances of having variety of characteristic to the plants are high because of the cross-pollinated nature of the plant.

1.7.2 Limitation of Study

There are few problems that need to solve and try other method to handle this experiment. One of the limitation studies is obtaining of quality seeds. Besides, the laboratory equipment's also are incomplete, thus it takes time to prepare and handling the experiment.