IN VITRO MORPHOGENESIS AND ACCLIMATIZATION OF *Asparagus officinalis* L.

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SULTAN IDRIS EDUCATION UNIVERSITY

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ABSTRACT

The purpose of this research was to examine morphogenesis of *Asparagus officinalis* L. through tissue culture system. The experimental design used in this study was Completely Randomized Design (CRD) and 30 samples were used in each treatment. Complete plant regeneration was successfully achieved when stem and root aseptic explants was cultured on MS medium supplemented with various combinations of plant growth regulators. MS medium supplemented with 2.0 mg/L BAP was found to be the optimum medium and stem explant was the most responsive explant producing 6.233 ± 0.810 shoots per explant. MS medium supplemented with 1.5 mg/L NAA was most optimum for root induction with 10.567 ± 0.841 roots per explant. Optimum callus induction was obtained when root explants were culture on MS medium fortified with 1.0 mg/L BAP and 1.0 mg/L NAA. Friable cream callus was produced. Meanwhile, the study with other auxin and cytokinin also showed the complete plant regeneration. MS medium supplemented with 0.5 mg/L BAP + 1.5 mg/L IAA was the best treatment and root explant was the most responsive for shoot regeneration with 10.200 ± 1.011. MS medium supplemented with 0.5 mg/L Kinetin + 1.5 mg/L NAA was found to be the optimum treatment for root induction with 12.033 ± 1.054. The best callus induction was obtained when stem explants were cultured on MS medium supplemented with 0.5 mg/L Kinetin + 1.5 mg/L NAA (2.319 ± 0.035). For the production of synthetic seeds, *Asparagus officinalis* L. were produced when microshoots 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 5.672 ± 0.430 shoots per explant. Finally, acclimatization of *Asparagus officinalis* L. was accomplished when plantlets were transferred to the combination of black and red soil (2:1) with 93.33% survival rate. In conclusion, the main result showed that morphogenesis of *Asparagus officinalis* L. through tissue culture system was successfully achieved. The implication of this research is higher crop production could be obtained in agriculture industry especially in Malaysia.

MORFOGENESIS *IN VITRO* DAN AKLIMATISASI *Asparagus officinalis* L.

ABSTRAK

Kajian ini bertujuan untuk mengkaji morfogenesis dari *Asparagus officinalis* L. melalui sistem kultur tisu. Rekabentuk ekperimen yang digunakan adalah Rekabentuk Rawak Penuh (CRD) dan 30 sampel digunakan untuk setiap eksperimen ini. Penjanaan semula tumbuhan yang lengkap berjaya dicapai apabila batang dan akar aseptik dikultur dalam medium MS yang ditambah dengan pelbagai kombinasi penggalak pertumbuhan tanaman. Medium MS yang ditambah dengan 2.0 mg/L BAP didapati sebagai medium optimum dan eksplan batang adalah eksplan yang paling responsif yang menghasilkan 6.233 ± 0.810 pucuk bagi setiap eksplan. Medium MS dengan 1.5 mg/L NAA merupakan medium yang paling optimum untuk induksi akar dengan 10.567 ± 0.841 akar bagi setiap eksplan. Induksi kalus yang paling optimum diperolehi apabila eksplan akar dikultur di dalam medium MS yang diperkaya dengan 1.0 mg/L BAP dan 1.0 mg/L NAA. Kalus berstruktur rapuh dan berwarna krim dihasilkan. Sementara itu, kajian yang menggunakan auksin dan sitokinin yang lain juga menunjukkan penjanaan semula tumbuhan yang lengkap. Medium MS yang ditambah dengan 0.5 mg/L BAP + 1.5 mg/L IAA merupakan rawatan yang paling baik dan eksplan akar adalah eksplan yang paling responsif untuk penjanaan semula pucuk dengan 10.200 ± 1.011. Medium MS ditambah dengan 0.5 mg/L Kinetin + 1.5 mg/L NAA didapati adalah rawatan yang paling optimum untuk induksi akar dengan 12.033 ± 1.054. Induksi kalus yang terbaik diperolehi apabila eksplan batang dikultur dalam medium MS yang diperkaya dengan 0.5 mg/L Kinetin + 1.5 mg/L NAA (2.319 ± 0.035). Untuk penghasilan benih sintetik, benih *Asparagus officinalis* 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 benih sintetik ini adalah 5.672 ± 0.430 pucuk bagi setiap eksplan. Akhir sekali, aklimatisasi lengkap *Asparagus officinalis* L. ke persekitaran luar telah berjaya dicapai apabila planlet dipindahkan kepada campuran tanah hitam dan merah (2:1) dengan kadar keterushidupan sebanyak 93.33%. Kesimpulan, dapatan untuk kajian ini menunjukkan bahawa morfogenesis *Asparagus officinalis* L. melalui sistem kultur tisu telah berjaya dicapai. Implikasi kajian adalah lebih banyak tanaman dapat dihasilkan dalam industry pertanian terutamanya di Malaysia.

CONTENT

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LIST OF ABBREVIATIONS

Al	Aluminium
ANNOVA	Analysis of variance
B	Boron
BA	Benzyl Adenine
BAP	Benzylaminopurine
C	Celcius
$C_{10}H_{13}N_5O$	Zeatin
$C_{15}H_{15}NO_8$	8-Hydroxyquinoline citrate
Ca	Calcium
$CaCl_2.2H_2O$	Calcium Chloride Dehydrate
CHO	Carbohydrate
Cl	Chlorine
Co	Cobalt
CO_2	Carbon dioxide
Cu	Cuprum
DMRT	Duncan's Multiple Range Test
EDTA	Ethylenediamine tetraacetic acid
Fe	Ferum
g	gram
HCl	Hydrochloric acid

$HgCl_2$	Mercuric Chloride
I	Iodine
IAA	Indole-3-Acetic Acid
IBA	Indole-3-butyric Acid
K	Kalium
Kinetin	6-furfurylaminopurine
M	Mol
Mg	Magnesium
mg/L	Milligram per liter
ml	milliliter
mM	milliMol
mm	millimeter
Mn	Manganese
Mo	Molybdenum
MS	Murashige and Skoog
N	Nitrogen
Na	Natrium
NAA	Naphthalene Acetic Acid
$NaC_6H_7O_6$	Sodium alginate
NaClO	Sodium hypochlorite
NaOH	Sodium hydroxide
Ni	Nickel
NOA	Naphthoxyacetic acid
O_2	Oxygen

P	Phosphorus
pCPA	p-Chlorophenoxyacetic acid
PGRs	Plant Growth Regulators
pH	potential Hydrogen
Pro	Protein
PRSV	Papaya Ringspot Virus
rpm	Rotation per minute
S	Sulphur
SE	Standart Error
SEM	Scanning Electron Microscope
SPSS	Statistical Package for the Social Sciences
TDZ	Thiadiazuron
2ip	2-isopentenylaminopurine
2,4,5-T	Trichlorophenoxyacetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
4-CPA	4-Chlorophenoxyacetic acid
µM/L	Micro Molar per Liter
µM	Micro Molar

CHAPTER 1

INTRODUCTION

1.1 Introduction

Asparagus is a large genus comprising more than 150 different species of high economic value perennials crop with 2n = 20 as its chromosome number. It is grown worldwide and basically originated from Asia, Africa, and Europe (Prohens *et al*., 2008). Asparagus is the main genus of the *liliaceae* family (Kanno and Yokoyama, 2011), and it is the most economically important which is a highly priced vegetable (Stajner *et al*., 2002). Asparagus is a popular vegetable because it is one of the first field crops to be harvested in spring and it can provide both growers and consumers with an early season fresh commodity. As a dioecious crop, asparagus is inevitably cross - pollinating. Male and female flowers are born on different plants. Generally, male plants have more commercial advantages over the female plants. They have

higher productivity (Falloon and Nikoloff, 1986) and produce more stalks (Gonzalez Castanon, 1990).

The wild stock of this high value plant species has been rapidly diminished due to over exploitation and no efforts for its replenishment has been undertaken till date. The conventional method of propagation of this plant is through seeds. However, propagation of *Asparagus officinalis* L. through seeds is unreliable due to poor rate of seed germination and a slim chance to survive under natural conditions. The conventional method is not attractive approach for the production of abundant elite plants within a short period of time. In order to meet the extensive demand of this edible plant which also has medicine properties, the alternative method to be employed in conserving its diminishing population is *in vitro* culture. *In vitro* techniques are considered as easy and reliable methods for the rapid propagation of plants, especially medicinally important plants (Thomas and Philip, 2005; Thomas and Jacob, 2004). Large number of aseptic plants could be produced within short time using plant tissue culture protocols.

In fact, plant tissue culture is an alternative method for commercial propagation of abundant plant species that also include plants with medicine properties (Rout *et al.*, 2000). In recent years, *in vitro* culture techniques have been increasingly receiving interests because they not only offer a viable tool for mass multiplication, but they also offer germplasm conservation for rare medicinal plants that are endangered and threatened (Arora and Bhojwani, 1989; Vishwanath and Jayanthi, 1997; Anis and Faisal, 2005; Uppendra *et al.*, 2005). The *in vitro* technique

increases the availability of disease free stock plants and reduces culture space requirement as well as lowers the cost of production.

Due to its many uses, and very little work has been focused on this species, therefore the aim of the present research is to exploit this species. The studies will consist of the surveillance of propagation of this species cultured into media supplemented with various hormones and regeneration of new plants from explants. Therefore, this research is carried out consecutively to investigate and identify the advantages of this species. Two-month-old plantlets obtained from germination of aseptic seedlings were utilized as explants sources. Regenerations of *Asparagus officinalis* L. were carried out to investigate the effect of growth hormones and media components. Explant sources investigated included stems and roots which were cultured on Murashige and Skoog plant medium (MS) supplemented with different concentrations and combinations of auxins and cytokinins.

Regardless of adequate existing literature on the morphogenesis of *Asparagus officinalis* L., a study on callus induction is also of necessity. The production of callus depends mainly on sufficient balance of growth regulators. However, this balance has large variation pertaining to the type of explant and the species of plant investigated. It is important to manipulate the appropriate levels of auxins and cytokinins for defining growth regulators balance as to ensure that callus formation is induced in the various types of explants (Dezfuli *et al*., 2013).

Subsequently, the synthetic seed will produced from the explant to observe the regeneration of shoots. And then, the regenerated plants that had been acclimatized.

Plantlets obtained from *in vitro* were transferred to various sowing media or subtrates and transplanted to the green house.

1.2 Background of Study

Vegetables are the one type of food source for humans in the world. This is because vegetables have many benefits to the human body. Vegetables are rich sources of minerals, vitamins and dietary fibre essential for functioning of human body. They are considered as protective foods such as their use in preventing many diseases.

According to Gopalakrishnan (2001), vegetables are defined as an edible herbaceous plants or parts of plants used as raw or after cooking and rich in vitamins and minerals, low of calorific value and neutralize acid produced during the digestion of high energy foods. Vegetable crops includes a large number of plants, most annuals and some perennials, grown for edible stems, leaves, flowers, flower buds, roots and fruits. Vegetables are part of balanced diet and considered as 'protective foods' because of their use in preventing some diseases. They have an important role in the fight against malnutrition animals and humans.

Propagation is a way of multiplying the species. It was practiced for the production and maintenance of plant seedlings. The seeds are used for commercial production. Various method were used to disseminate useful plants. Different plants propagated by different methods with the ultimate goal of multiplication of certain plant species. To complete the requirements of a growing population, the

multiplication of useful plants is very important. Therefore, it is necessary to study the methods of breeding for different plants. There are two types of plant propagation which are sexual (seed) and asexual (vegetative).

Seed propagation is by far the simplest and oldest form of plant multiplication. Sometimes, it is the only means of propagating a specific crop. The production of seeds usually occurs from sexual reproduction between species as genetic recombination takes place. A plant has a tendency to possess different characteristics from its parents if it is grown from seeds. Further, some species produce seeds that might only germinate under special conditions such as cold treatment. Some plants species, such as many trees, do not have the ability to produce seeds until reaching maturity which might require many years to take place. While some plants are totally incapable of producing seeds, seeds can also in fact be difficult to obtain.

Plants possess a trait of some mechanism used for asexual or vegetative reproduction, which might be beneficial to the horticulturists and gardeners for rapid plants growth and cloning. People also make use of the methods unused by plants, which include tissue culture, stem cuttings, grafting, and air layering. The production of the plants takes place by using material from a single parent; hence, no exchange of genetic material is present in the method of vegetative propagation almost produce plants with the same parents. Vegetative propagation involves using the part of plants, for examples, roots, stems, and leaves. Some plants, however, are capable of producing seeds without fertilization as well as seeds carrying genetic material of the parent plants. Thus, asexual reproduction involves propagation through asexual seeds or apomixes but not vegetative propagation.

Micropropagation refers to new plants multiplication under aseptic conditions in an artificial medium. Usually very small plants tissues are used for multiplication which include embryos, seeds, shoot and root tips, callus, single cells, and also pollen grains. The applications of these propagation techniques are to establish pathogen-free planting materials, to separate genetically distinctive cells or cell lines with the tendency to become new variants of plant, and for rapid multiplication under disease-free conditions.

The process of micropropagation begins by selecting the plant material to be propagated and the removal of plant tissues from an intact plant under a sterile condition. Stock materials that are clean and free from viruses and fungi are significant in producing the healthiest plants. Once the plant material has been selected to be cultured, the collection of explants takes place and it relies heavily upon the type of tissue to be used, such as stem tips, petals, anthers, pollen, and other plant tissues. Next, the explants material is surface sterilized in repetitive processes of bleach and alcohol washes before they are finally rinsed with sterilized water. Small portion of plant tissue or solely a single cell is placed on a growth medium that usually contains sucrose as the energy source and one or more plant growth regulators as plant hormones. The medium is commonly thickened with agar which creates a gel for supporting the growth of the explants. While some plants can easily be grown on simple media, successful growth of other plants however require more complicated media; the growth of plant tissue and its differentiation into new tissues rely heavily upon the medium. For instance, branched shoots from plant buds are created through media that contains cytokinins.

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*., 1995; Fujiwara & Kozai, 1995). However, there should be similarity between the two culture 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).

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 cannot be met with conventional methods either generative or vegetative (Triwibowo, 2006).

Asparagus officinalis L. has a low rate of multiplication through conventional methods. Other than having a short post-harvest life, it is also extremely perishable due to its high respiration rate (Saito & Akiba, 2003). Garden asparagus (*Asparagus officinalis L.*) is a vegetable that has high economic value. Resistant Asparagus cultivars to be recovered through conventional breeding is disabled due to the

ubiquitous property of Fusarium in the soil, perennial and dioecious traits of Asparagus, and the influence of polygenic control to disease resistance. The use of *in vitro* selection techniques has alleviated the disease resistant plants generation in other pathosystems (Pontaroli and Camadro, 2005).

1.4 Objectives of Study

i. To identify and establish the most efficient regeneration system for *Asparagus officinalis* L. in tissue culture.
ii. To establish callus induction of *Asparagus officinalis* L.
iii. To produce the synthetic seed for *Asparagus officinalis* L.
iv. To introduce medium for acclimatization of *Asparagus officinalis* L. plantlet.

1.5 Importance of the Research

Plant breeding in biotechnology and molecular biology has ability to overcome some of the problems that occur in conventional breeding (Muhammad *et al*., 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, produced more healthy seeds and enabling genetic manipulation.

In the last 20 years, tissue culture techniques have assisted researchers, plant growers, and nursery industry in increasing a large quantity plants (Yusnita, 2004). The studies on plant propagation in tissue culture especially for *Asparagus officinalis* L. 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 *Asparagus officinalis* L. 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 *Asparagus officinalis* L. Aseptic seedlings parts such as roots and stems were utilized as explants sources. In this study, auxin and cytokinin comprising various combinations and concentrations were used for the explants. The experiment were also limited by plant contaminations. However it could be overcome with numbers of replications.