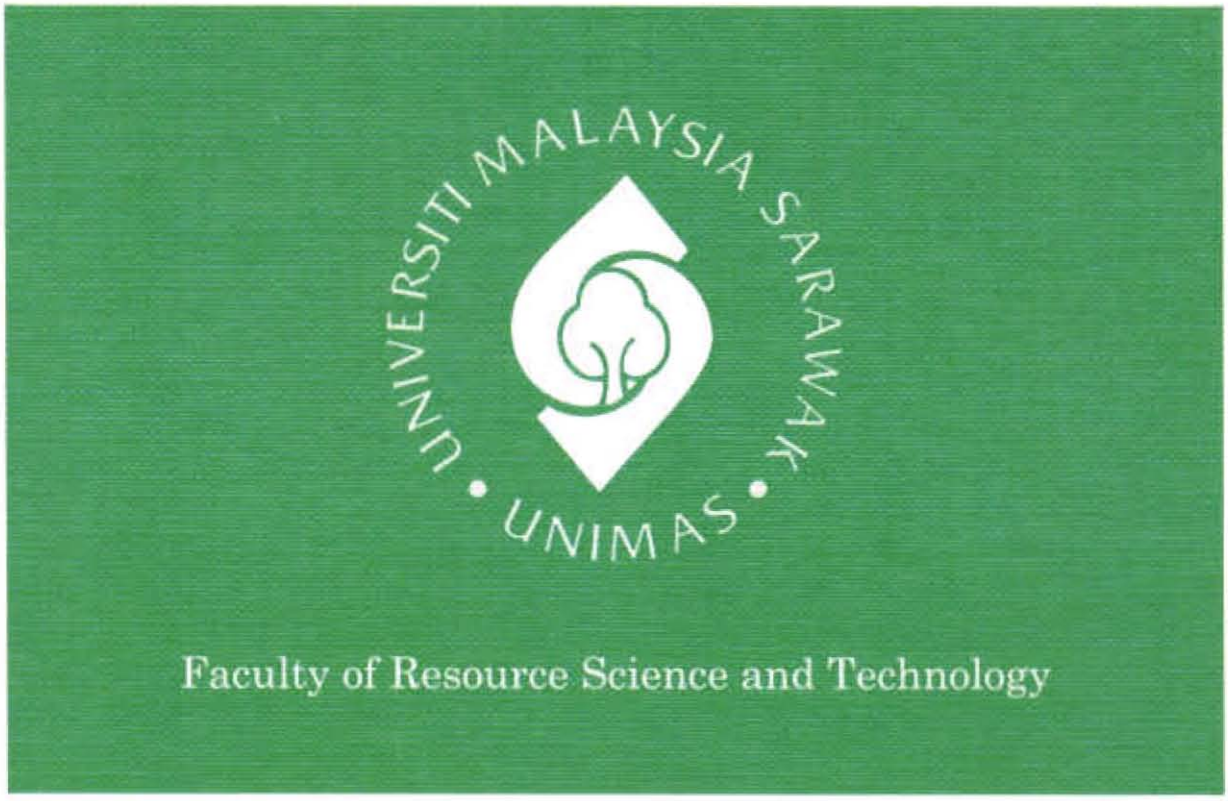

ESTABLISHMENT OF AXENIC CULTURE AND CALLUS INDUCTION OF *(Morinda citrifolia* L.) BY USING LEAF EXPLANT

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Bachelor of Science with Honours
(Plant Resource Science and Management)
2017

ESTABLISHMENT OF AXENIC CULTURE AND CALLUS INDUCTION OF (*MORINDA CITRIFOLIA* L.) USING LEAVES EXPLANT

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This report is submitted in partial fulfillment of the requirement for the degree of Bachelor of Science with Honours in Plant Resources Science and Management

Plant Resource Science and Management

FACULTY OF RESOURCES SCIENCE & TECHNOLOGY
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LIST OF ABBREVIATIONS

M. citrifolia L.	*Morinda citrifolia* Linn.
PGR	Plant Growth Regulator
MS media	Murashige and Skoog Media
2, 4-D	2, 4-Dichlorophenoxy acetic acid
NAA	Naphthaleneacetic acid

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ESTABLISHMENT OF AXENIC CULTURE AND CALLUS INDUCTION OF (*MORINDA CITRIFOLIA* L.) USING LEAVES EXPLANT

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ABSTRACT

Morinda citrifolia (Mengkudu) is from the family Rubiaceae which has high demand in medicinal use and has industrial importance. It is used to treat asthma, cough, cold, heartbun, fever, rheumatism and prevent diseases like stroke, cardiovascular disease and cancer. The objective of this study were to determine the best method for axenic culture of young leaf of *M. citrifolia* and to induce callus of *M. citrifolia* from leaf explants by using different concentration of 2,4-Dichlorophenoxy acetic acids (2,4-D) and Naphthaleneacetic acid (NAA). Results showed that the best method of surface sterilization of leaf explants *M. citrifola* is when pre-treated with 70% of Ethanol (30 Sec) followed by the surface sterilization method from treatment with 5% Clorox® concentration with 5 minutes time of exposure. This method was used to sterilize the leaf explants for callus induction. The leaf explants showed the best response to produce callus when supplemented into MS medium containing 2.0 mg/l and 3.0 mg/l of 2,4-D. Different approach can be developed in further studies for callus induction and shot regeneration of *Morinda citrifolia.*

Keywords: *Morinda citrifolia* (Mengkudu), axenic cultures, callus induction, 2,4-Dichlorophenoxy acetic acids (2,4-D), Naphthaleneacetic acid (NAA), surface sterilization

ABSTRAK

Morinda citrifolia (Mengkudu) adalah dari famili Rubiaceae yang mempunyai permintaan yang tinggi dalam penggunaan ubat-ubatan dan mempunyai kepentingan industry. Ia digunakan untuk merawat asma, batuk, selsema, pedih hulu hati, demam, sakt sendi dan mengelakkan penyakit seperti strok, penyakit kardiovaskular dan kanser. Objektif kajian ini adalah untuk menentukan kaedah yang terbaik untuk menghasilkan daun muda yang bersih dari penyakit dan kulat. Objektif kajian yang seterusnya adalah untuk menghasilkan prosedur yang terbaik untuk induksi kalus daripada daun muda dengan menggunakan konsentrasi 2,4-Dichlorophenoxy acetic acids (2,4-D) dan Naphthaleneacetic acid (NAA) yang berbeza. Keputusan menunjukkan kaedah yang terbaik untuk mengahsilkan daun muda yang bersih dari penyakit dan kulat adalah apabila dikulturkan dengan 5% konsentrasi Clorox® selama 5 minit. Kaedah ini juga digunakan untuk menghasilkan daun muda yang bersih untuk pertumbuhan kalus. Daun menunjukkan tindak balas yang terbaik apabila di kultur kan didalam media yang mempunyai 2.0 mg/l and 3.0 mg/l of 2,4 D. Pendekatan yang berbeza boleh dihasilkan dalam kajian lanjut untuk induksi kalus dan pertumbuhan pucuk Morinda citrifolia.

Kata kunci: *Morinda citrifolia* (Mengkudu), axenic cultures, callus induction, 2,4-Dichlorophenoxy acetic acids (2,4-D), Naphthaleneacetic acid (NAA)

1.0 INTRODUCTION

1.1 Background study

Plant is natural organism that have been used for traditional medicine and also use for treatment among various diseases since thousands of years (Rao *et al.*, 2004). According to Cragg and Newman (2004), plants are the main sources which highly effective conventional medicine that could be used for treatment of various type of cancer. Nowadays, more than 60% currently used anti-cancer agent are produced from natural resources as reported by Cragg *et al,* (2005) and Newman *et al.* (2003). More, 80% of the world's population depends on traditional medicines and use plant extracts in traditional treatments (WHO, 1993).

Rubiaceae, the family consist of 609 genera and about 31,357 species is a taxon of dicotyledonous flowering plants. This family are tree, shrub and most of them are herbs. According to Morton (1992), *Morinda Citrifolia* belong to the family Rubiaceae. It is also known as Noni and locally this plant is known Burma phal, pongee phal, lorang, etc. by the tribals of Andaman and Nicobar Islands (Singh *et al*., 2005a, b). According to the Nelson (2006), the young leaves and fruit of the *Morinda citrifolia* are evergreen and the surface of both leaves and fruits are waxy and glossy. More, it is widely grow through pacific region and one of the most important sources of traditional medicines (McClatchey, 2002; Nelson, 2001).

Noni also can adapt to extreme environmental conditions and can grows well in acidic, alkaline and even infertile soils. According to Cambie and Ash (1994), this plant can be found in open areas near the shoreline, pastures, coconut plantations, disturbed forest, dry to mesic forests, littoral forest and in waste lands. *M. citrifolia var. citrifolia, M. citrifolia var. bracteata and M. citrifolia var. potteri ar*e the commonly grown commercial varieties.

Even these varieties have different morphological aspects but their chemical properties are similar (Wang and Su, 2001; Nelson, 2005). The example of the chemical properties are phenolic compounds, organic acids and alkaloids. There are many findings support that *Morinda citrifolia* have a medicinal values and a broad range of health benefits for cancer, asthma, hypertension, infection, arthritis and pain (Whistler, 1992).

1.2 Problem statement

Morinda citrifolia is mainly propagated by seed but the seed have a problem of seed dormancy or hard seed coat (Kochuthressia & Jaseentha, 2015). Furthermore, seed propagation are very slow which takes longer time in germination process (Kochuthressia & Jaseentha, 2015). Tissue culture technique is the most popular technique and only the way in producing a mass number of mature plants in a short time. Besides, it also increase the rate of propagation of a new cultures. Hence, this project is designed to study and evaluate the establishment of axenic culture and callus induction of (*Morinda citrifolia* L.) by using leaf explant.

1.3 Objectives

The specific objective of this study are:

1. To establish an effective surface sterilization protocol for producing axenic culture of *Morinda citrifolia* by using young leaf explants.
2. To induce callus formation from the young leaf explants of *Morinda citrifolia* by using 2,4-Dichlorophenoxy acetic acids (2,4-D) and Naphthaleneacetic acid (NAA).

2.0 LITERATURE REVIEW

2.1 The plant

Morinda citrifolia is a member of the Rubiaceae family which comprises of 609 plant genera according to the statistic (The Plant List, 2013) and about 31,357 species but only 13,673 species accepted (The Plant List, 2013) and it is also a taxon of dicotyledonous of flowering plants. This family mostly are tree, shrub and most of them are herbs. At family level, they can be easily recognized morphologically by their simple, opposite or whorled, entire leaves, and stipules united and large as leaf blades.

Figure 2.1: *Morinda citrifolia* L. plant

Flower usually small, bisexual, actinomorphic, sometimes slightly zygomorphic and ovaries inferior. *Morinda citrifolia* has various common names. It is known as Mengkudu (Malaysia), Bengkudu (Indonesia), Noni (Hawai), Ungcoikan (Myanmar), Cheesefruit (Australia), Yor ban (Thailand). The common English name is Great Morinda and Indian Mulberry (Figure 2.1).

Morinda citrifolia has various synonyms, there are *Morinda citrifolia* var. *citrifolia*, *Morinda citrifolia* cv. Potteri and *Morinda citrifolia* var. bracteata (Will.C.McClatchey, 2003). These three species of *Morinda citrifolia* are used traditionally as herbs in Southeast Asia.

2.2 Botanical description

The taxonomic classification and nomenclature of *Morinda citrifolia* (National Plant Database, 2003).

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Subclass: Asteridae

Order: Rubiales

Family: Rubiaceae

Genus: Morinda L.

Species: *Morinda citrifolia*

Scientific name: *Morinda citrifolia* L.

Morinda citrifolia is an evergreen shrub or herbs that can grow up to 3-8(-10) m tall with a deep taproot. The bark are greyish or yellowish-brown. The leaves opposite and simple, elliptic to lanceolate (10)15-50cm x 5-17 cm (Orwa *et al.*, 2009). The leaves have apex acute to shortly acuminate, cuneate at base, and pinnately nerved (Orwa *et al.*, 2009). Petioles of the

species are 0.5-2.5cm long and stipules variable in size shape, broadly triangular (Orwa *et al.*, 2009) (Figure 2.2).

Figure 2.2: The fruits of *Morinda Citrifolia* L.

According to the Johansson (1994) and Wong (1989), the flower of *Morinda citrifolia* grow in a many flowered cluster known as a capitulum. The inflorescence is terminal which develop from the terminal bud not the axillary bud. The peduncle is 4-14 mm long. The capitula is solitary, each 7-20mm long and 7-15 mm wide with about 25-60 flowers. The flower are white and usually have 5 petal each and are bisexual. The corolla tube is 5-10mm long and the petals are 5.5-7mm long, 1.7-2.3mm wide and are ovate to triangular. The stamens are inserted in or just below the corolla throat. The filaments are 2-3 mm long and each of the inferior ovaries has 2 locule. Each locule with a solitary ovule.

2.3 Medicinal uses of *Morinda citrifolia*

M. citrifolia has been used traditionally and is important herbal medicine. Recently, scientific researches have been done to trace the advantages of *M. citrifolia*. However, the evidents are limited to state the effectiveness of the fruit juice to treat complication. Typical *M. citrifolia* products are commonly being used for the treatment of high blood pressure, menstrual cramps, arthritis, gastric ulcers, sprains, injuries, mental depression, senility, poor digestion, atherosclerosis, blood vessel problems, drug addiction, relief of pains (McClatchey, 2002).

According to Wang and his co-workers (2001), the extracts from *M. citrifolia* has been proven that this herb exhibit anti-viral properties to fight with diabetes, gout, cancer, and internal weaknesses. The fruit juice of *M. citrifolia* also contain Gamma aminobutyric acid (GABA) ligands which benefits to lower the blood pressure of numb dog (Davison, 1927; Moorthy and Reddy, 1970; Youngken, 1958; Youngken *et al*., 1960). Deng and his partners (2007) reported that sedative and anxiolytic effects will be generate when the receptors of GABA binding with these ligands in the neurotransmitter of mammals.

Further reports showed that individual who consumed M. *citrifolia* juice, especially among athletes, could generate more energy, faster the recovery time and increase the stamina after intensive physical training (Palu *et al*., 2008a; Wang and Su (2001). This showed that *M. citrifolia* have a good efficacy in short time due to the multiple antioxidants present in the fruit extracts. Besides, the ability of extraction from *M. citrifolia* was reported to possess analgesic and tranquilizing effects. The analgesic efficacy of the fruit juice extract is 75% as strong as morphine, which used in today's surgical use (Younos *et al*., 1990).

According Hirazumi *et al*., (1994), *Morinda citrifolia* also have an anti-tumor activity because this plant are rich in polysaccharides and an important substance known as noni-ppt. noni-ppt stimulates the release of TNF-α3, IL-1β, IL-10, IL-12 p70 and IFN-γ from the immune

system. From the juice of *Morinda citrifolia*, it was proven to play an important role in modulating immune cells to come about in times of immunosuppression. This statement supported by Hirazumi *et al*., (1996) in a later research done in the Lewis Lung Carcinoma.

2.4 Plant Tissue Culture

Plant tissue culture is growing pant cells, tissue or organ isolated from the mother plant into and artificial media such as MS media. It includes various technique and methods used in order to achieve the objectives. Plant tissue culture technique is usually performed under aseptic condition which is using laminar flow cabinet. Generally, living plant materials from the environment are contaminated with microorganisms. Thus, sterilization methods are needed for the starting material (explants).

Explants are usually placed on the solid cultured media which made up of inorganic salts, with few organic nutrients, vitamins, and plant hormone. There are two type of media which are solid and liquid media. Solid media are prepared from liquid media with addition of gelling agent, gelrite. Commonly used plat tissue culture media is Murashige and Skoog media (Murashige and Skoog, 1962).

The importance of tissue culture is to produce large quantities of plant and to increase production of new varieties into the market where large amount can be produced in a very short time compared to conventional methods. More, tissue culture importance in establish and maintain the contaminants free among the plant. Nowadays, plant tissue culture have been widely used to produce clones plants in a method which is known as micropropagation. Plant tissue culture plays a vital role in search for alternative to production desirable medicinal compounds from plants (Robert & Dennis, 2005).

Plant tissue culture have more advantage compare to traditional method which a mass number of mature plants can be produced at a short time and also increase the rate of propagations of new cultures. So, more endangered plant such as orchid can be clone safely and genetically identical plant can be produced in large quantity. Furthermore, plant tissue culture can producing plants which free from disease and pathogen. According to Mustafa (2002), plant that have problems or not known to germinate through seed can be easily manipulated to produce a new whole plant.

2.5 Tissue Culture Technique

2.5.1 Surface sterilization

The main important things for a success of establishment and maintenance of plant tissue culture is an axenic explants. The explant that are taken from the outside are usually highly contaminated with microorganisms and this will contaminants outgrow the explants and resulted in death. Thus, sterilization is the best method for make sure the process of making explants free without any contamination before the cultures being establish. Factor that affect the possibility of microorganism includes the explants, culture medium, apparatus and materials, environment of explant transfer area, technicians and tissue culture room (Dodds & Roberts, 1995). So, various sterilization agents can be used to sterilize the tissue. During the sterilization, the living materials should not lose their biological activity and only contaminants should be eliminated. (Oyebanji, 2009).

According to Hartmann *et al*. (1990), contaminants usually obtained externally such as fungi, molds, bacteria and other microorganism which are present everywhere either in the air or many surface of the plants, tables, hands and so on. So, a rapid production of axenic explant is the main objective in the plant tissue culture. Sodium hypochlorite, calcium hypochlorite,

mercuric chloride ethanol, hydrogen peroxide, bromine water and silver nitrate are sterilizing agents that are widely used.

Based on Elakkuvan and Manivannan (2015), the leaves, nodal segments and the shoot tip of the *Morinda citrifolia* which treat on 2% of sodium hypochlorite within 10 minute time exposure record the highest survival rate in surface sterilization process. More, 5% of calcium hypochlorite within 15 minutes time exposure also record the highest survival rate of explants. (Elakkuvan & Manivannan, 2015). For the sterilizing agent such as mercuric chloride, there is an evidence from the previous research of Elakkuvan and Manivannan (2015) which proved that 0.1 % of mercuric chloride within 4 minutes time exposure give the highest survival percentage of the explants.

According to Oyebanji (2009), ethanol is most powerful sterilizing agents but extremely phyto-toxic. Therefore, the explants is typically exposed to it for only a few seconds and minutes. In the research according to Elakkuvan and Manivannan (2015), the best percent of ethanol used in vitro culture of *Morinda citrifolia* is 70% followed by 1 minute's time exposure. To enhance effectiveness of sterilization procedure, a surfactant like Tween 20 is frequently added to the sterilizing solution. In general, the sterilizing solutions containing the explants are continuously stirred during the sterilization period.

2.5.2 Callus induction

According to Bottino (1981) callus is define as an unorganized tissue mass growing on solid substrate. Callus forms naturally on plants in responses to infestations, wounding, or at graft unions. Callus formation is a desirable prerequisite for plant regeneration because callus offers greatest opportunity for in vitro selection and production of genetic variations (Espinasse & Lay, 1989). Callus is formed through three developmental stage which is induction, cell