Establishment of Axenic Culture and Induction of Somatic Embryogenesis in Kapur Bukit (*Dryobalanops beccarii* Dyer)

NORSHAHIDAH BINTI OSMAN

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Resource Biotechnology Programme
Department of Molecular Biology

Faculty of Resource Science and Technology (FRST)
Universiti Malaysia Sarawak (UNIMAS)

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APPROVAL SHEET

Name of Candidate: Norshahidah Binti Osman

Title of Dissertation: Establishment of Axenic Culture and Induction of Somatic embryogenesis in Kapur Bukit (*Dryobalanops beccarii* Dyer)

_____

(Dr. Mohd Hasnain Md. Hussain)

Supervisor

_____

(Dr. Awang Ahmad Sallehin Awang Husaini)

Coordinator of Resource Biotechnology Programme

Department of Molecular Biology

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak (UNIMAS)

DECLARATION

I hereby declare that Final Year Project Report 2010 is based on my original work except for quotations and citations, which have been duly acknowledged also declare that it has not been or concurrently submitted for any other degree at UNIMAS or other institutions of higher learning.

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Norshahidah Binti Osman

Resource Biotechnology Programme

Department of Molecular Biology

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak (UNIMAS)

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LIST OF ABBREVIATONS

CRD	Completely Randomized Design
PGRs	Plant Growth Regulators
PPM	Plant Preservative Mixture
TET	Tetracycline
WPM	Woody Plant Medium
2, 4-D	2, 4-Dichlorophenoxy Acetic Acids

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Universiti Malaysia Sarawak

ABSTRACT

Kapur Bukit (*Dryobalanops beccarii* Dyer) a medium hardwood in Dipterocarpaceae has been identified as one of the timber species recommended for forest plantation in Sarawak. Establishment of forest plantation requires mass quantity of the planting stocks. Since clonal propagation of this species by the conventional methods is difficult and slow, micropropagation using the *in vitro* culture technique has been considered as an alternative method to mass produce clonal material for planting. Micropropagation using field-derived stock plant required effective measures to establish contamination-free and viable culture and successful regeneration of plant regeneration *in vitro.* The first objective of this project is to establish axenic and viable explants in culture. The second objective is to induce somatic embryogenesis. Results of this study showed that immersing in 0.3% Benomyl (w/v) for one hour after surface sterilization could reduce fungal contamination to certain extent. Browning problem that lead to death of explant can be overcome by trimming explants in acid solution and incorporating of PVP, citric acid and ascorbic acid in WPM medium. It was found root developed from compact and friable callus induced by 2, 4-D. To date, no somatic embryogenesis was observed.

Keywords: axenic culture, surface sterilization, contamination, browning, somatic embryogenesis.

ABSTRAK

Kapur Bukit (*Dryobalanops beccarii* Dyer) adalah kayu keras sederhana dalam Dipterocarpaceae yang telah dikenalpasti sebagai salah satu spesies balak yang sesuai untuk perladangan hutan di Sarawak. Penubuhan perladangan hutan memerlukan stok tanaman dalam kuantiti yang besar dan juga bahan baka adalah pilihan utama. Propagasi vegetatif konvesyen adalah tidak begitu berjaya bagi speseis tersebut. Teknik *in vitro* telah difikirkan sebagai alternatif bagi pengeluaran bahan bakaan. Dalam usaha untuk menuju ke pembangunan protokol mikropropagasi dan langkah pertama bagi *D. beccarii*, objektif pertama dalam projek ini adalah untuk menubuhkan kultur yang bebas daripada kontaminasi dan berhidup. Objektif kedua adalah untuk memulakan induksi embrio somatik. Keputusan daripada kajian terkini menunjukkan rendam di dalam 0.3% benomil dalam masa 1 jam selepas pensterilan permukaan telah mengurangkan kontaminasi daripada kulat dalam jangkaan tertentu. Keperangan kultur yang mengakibatkan kematian eksplan dapat diatasi dengan mencantas eksplan dalam cecair asid dan memasukkan PVP, asid sitrik dan asid askorbik ke dalam media WPM. Akar dihasilkan daripada kalus kompak dan friabel yang diinduksikan oleh 2, 4-D pada eksplan. Tiada embriogenesis somatik yang dapat dilihat pada masa terdekat ini.

Kata kunci: kultur axenic, pensterilan permukaan, kontaminasi, keperangan, embriogenesis somatik

1. INTRODUCTION

Kapur Bukit (*Dryobalanops beccarii* Dyer), a medium hardwood belongs to family Dipterocarpaceae is widely distributed throughout tropical rain forest (Noraini, 2008) and has been identified as one of the timber species recommended for forest plantation in Sarawak (Thai, 2002). This species is of economic value for its timber products (Noraini, 2008) and important in producing camphor oil (Soerianegara, 1994). As reported in New Sunday Times (2010), Dr. Lilian Chua of the Forest Research Institute Malaysia (FRIM) stated Dipterocarpaceae is a charismatic family which is very important to be conserved as this family has been the major contributor to the country's economy (Chai, 2010). *Dryobalanops beccarii* is the fastest growing tropical timber species in Dipterocarpaceae (Wong, 1997). According to Ashton (2004), the suitable habitats for *D. beccarii* are well-drained sandy soils on hills and ridges below 700 m altitude (Soerianegara, 1994).

The demand for timber began to exceed the supply since late 1970's due to the international trade in tropical timber (Thai, 2002) and this valuable timber also threatened by development and overexploited (Noraini, 2008). In order to sustain the high demand in the market, the development of forest plantation is introduced as an alternative source of timber supply other than from natural forest (Thai, 2002). Establishment of forest plantation requires mass quantity of the planting stocks. As reported in Bernama (2007), the timber industry is believed to be expanded through forest plantation in Sarawak where about 200 cubic metres per hectare could be harvested from planted forest rather than only 30 to 40 cubic metres from the native forest.

Dryobalanops beccarii planting stock could be produced in large quantity through seeds or vegetative propagation. However, the supply of seedlings is not reliable as flowering and

fruiting of *Dryobalanops beccarii* is irregular and the seeds are recalcitrant. It may take about sixty years before a Kapur Bukit tree flowers for the first time and then it may take about three to seven years to flowering again (Veevers, 1984). Storing the seeds also posed a problem because the seeds are recalcitrant (Marzalina, 2002) and the seed would not be viable when the moisture was lost. Recalcitrant seed also have tendency to get fungal infection and dehydration (Lei, 2009) if stored for long term storage.

Conventional vegetative production by rooting of cutting has not been very successful and the plants propagated by rooting of cutting tend to form plagiotropic growth of shoots (Kandasamy, 2005). Since clonal propagation of this species by the conventional methods is difficult and slow, micropropagation using the *in vitro* culture technique has been considered as an alternative method to mass produce clonal material for planting. Micropropagation using field-derived stock plant required effective measures to establish contamination-free and viable culture and successful regeneration of plant regeneration *in vitro.*

According to George & Sherrington as cited in Samantaray *et al*. (1994), plant tissue culture, an *in vitro* manipulating technique is an efficient tool for rapid clonal propagation for propagating plants which are difficult-to-propagate by conventional methods of vegetative propagation (Lineberger, 1998; Ajay, 2006). Tissue culture would be used as the way to solve the problems in propagating large scale woody plants including *Dryobalanops beccarii* Dyer. Somatic embryogenesis, one of the pathways in plant regeneration to form embryo from vegetative cells and tissues within *in vitro* system to regenerate into a complete plant is a highly efficient process for plant multiplication (Agnieszka & Rybezynski, 2008).

Establishment of contamination-free culture is a pre-requisite for *in vitro* culture and it is important to obtain reliable measurements of plant responses in absence of microbial organisms (Amelia *et al*., 2005). The maintenance of bacterial and fungal contamination-free culture is critical (Lineberger, 1998) especially in long-term axenic culture (Amelia *et al.*, 2005). In the previous attempts on micropropagation of this species a disinfection protocol which can achieve 70% aseptic explants has been established. This project will adopt the protocol and improve it further to achieve a percentage of axenic explants of higher than 70% and to attempt *in vitro* plant regeneration through somatic embryogenesis.

In this study, the petiolule of the leaflets was used as the explants for culture. The disinfection protocol developed earlier mainly for shoot-tip explants were adopted with modifications to improve its efficiency. Explants were collected from stock plants grown under a shaded house in an attempt to alleviate browning problem. Measure like handling the disinfection process under cool condition to slow down production of phenolic compounds was taken. The study of the effectiveness of different disinfectants was done and found that the disinfectant also have a role in maintaining the quality of cell to form callus. *In vitro* plant regeneration via somatic embryogenesis pathway would be attempted by inducing more callus formation and encouraging the somatic embryogenic callus by transferring the callus formed explant on media containing 2, 4- D. By the end of this study the percentage of aseptic and viable explants could be increased to more than 70% and positive result could be obtained in the induction of somatic embryogenesis.

The objectives of this project thus is to further improve the protocol for establishment of contamination-free (axenic) culture. The second objective is to attempt *in vitro* regeneration of *Dryobalanops beccarii* through somatic embryogenesis.

2. LITERATURE REVIEW

2.1 Botanical Description

Dryobalanops beccarii Dyer is a large and tall timber tree, belongs to family Dipterocarpaceae. It also have synonym name, i.e. *Dryobalanops oocarpa* v. Slooten (Soerianegara, 1994). *Dryobalanops beccarii* Dyer has different vernacular names according to the country where it is found, i.e. Kapur Merah (Sabah), Kapur Bukit (Sarawak) and Keladan (Indonesia). This tree species can grow up to 65 m tall (Soerianegara, 1994 & Ashton 2004) with a straight bole up to 2 m in diameter (Ashton 2004). Its bark is dark yellowish tawny, prominently irregularly flaky but rarely shaggy (Ang, 2009). The density of the wood can reach up to 600 to 710 kg/m^3 at 15% moisture content (Soerianegara, 1994). The leaves are ovate and lanceolate, thinly and coriaceous, 5-8 x 1-3 cm with an up to 17 mm long acumen and the bark and leaves are aromatic, but not producing dammar (Soerianegara, 1994). According to Ashton (2004), *D. beccarii* are glabrous and had slender, terete and smooth twigs to 1 mm diameter apically and irregularly doubly branched. Fruit calyx lobes with obconical cup up to 6.5 cm x 0.8 cm, narrower than nut. The trees are locally abundant in leached sandy soils on hills and ridges, also along streams and seasonal swamps.

Dipterocarps have high economical value of wood (Purwaningsih, 2004) that contributed to country's economy (Chai, 2010) and high demand in the domestic and world trade as they are the major supply of tropical timber (Zabala, 1993; Noraini, 2008) to produce wood, paper and furniture and also non timber products or as a part of dynamic biodiversity and ecosystems in the tropical rainforest (Noraini, 2008). *Dipterocarpaceae beccarii* Dyer is one of the fastest growing among the family. However, the excessive logging,

development, agriculture (Noraini, 2008) and shifting cultivation had caused the Dipterocarpaceae disappearing rapidly (Kandasamy, 2005).

2.2 Problems of propagation by seed and conventional methods

According to WWF (2006), forests in Malaysia are mostly dominated by trees from Dipterocarpaceae. In this dipterocarp mixed forest that is important as tropical timber source, most of the species produce recalcitrant seeds (Purwaningsih, 2004). The seeds are desiccation sensitive (Ajay *et al*., 2006), metabolically primed for immediate germination once they reached maturity, instead of undergoing dormancy (Marzalina, 2002) and metabolically active (Berjak, 2005). This characteristics of seeds caused the plant hard-to propagate. Furthermore, the flowering of this species is also very irregular which only occurs in the intervals of two to ten years (Appanah, 1993). According to Marzalina (2002), forest plantation by seeds would be facing problem as the seeds must be sown immediately once collected or they would be perished. The seeds also will tend to be injured if dried below 35% moisture content (King & Roberts, 1979 as cited in FAO, 2002). Feeding of the dipterocarp seeds by small rodents (Ashton, 2004) and insects (Nair & Sumardi, 2000), short storage period of the seeds (Phartyal *et al.*, 2002; Berjak, 2005), sensitiveness of the dipterocarp seedling to its environments (Adjers & Otsamo, 1996) and the limited seeds dispersal (Ashton, 2004) are the other problems that also make the seed not feasible for use in large-scale propagation. Berjak (2005) also reported that most species of recalcitrant seed would have potential of fungal infection.

Vegetative propagation, the alternative mass production method of which the plants are propagated asexually through rooted cuttings (Zabala, 1993), has been studied and found successful to some dipterocarps species. However, the success of this method is depending

on physical condition. Vegetative propagation of *D. beccarii* by stems cutting has been done but the successful rate is not satisfactory since rooting of *D. beccarii* is very difficult (Edward, 2005). According to Kandasamy *et al*. (2005), production of cuttings from mature trees is difficult and commonly tends to have plagiotropic growth of shoots from cuttings. This may produce planting stock with undesired characteristics and not suitable in forest plantation.

2.3 Micropropagation

Establishment of tropical forest plantation could be achieved by *in vitro* multiplication of forest species, through micropropagation. This technique is used for clonal mass propagation (Okazaki, 2008), reversion to juvenility and uniformity of stands of forest species (Adriana, 2003). The availability of good quality planting materials for timber species used in forest plantation is the main problem in tropical country that encounter high rate of deforestation (Surdarnowati, 2000). The important characteristic of micropropagation include the possibility of mass propagation of elite, endangered or difficult-to-propagate species, in relatively small areas and time period (Adriana & Wagner, 2003). In addition, micropropagation can have significant impact not only in the multiplication of important genetic material, but also for the *in vitro* maintenance of germplasm (Adriana & Wagner, 2003).

The culture medium is important for the success of an *in vitro* process. The most commonly used culture medium is MS medium (Murashige & Skoog, 1962). Other culture media commonly used in micropropagation of woody species are WPM (Lloyd and Mc Cown, 1980), de Fossard (1974), Quoirin and Lapoivre (1977) and Goncalves (1980), which are considered as having a medium total ionic concentration (Adriana & Wagner,

2003). WPM usually used as the basal medium for the salt- sensitive woody plant (Pierik, 1997).

2.3.1 Problem of juvenility and explants selection

Several factors are responsible for the success of micropropagation including the selection of suitable plant growth regulators, type of explants, physical state of culture medium, culture condition, addition of various and specific substances and the levels of nutrients in the culture medium (Adriana & Wagner, 2003). Shoot tip culture is commonly used for the mass propagation of woody species (Lineberger, 1998).

Using of pre-existing meristems which can easily develop into shoots are important characteristics of good explant, thus axillary buds and shoot tips are recommend to succeed in micropropagation (Mohammad *et al*., 2003). According to Hudson *et al*., (1990), shoot-tip culture utilizing axillary shoot proliferation for multiplication is the most important commercial technique for micropropagating plants. This is because it provides the most genetic stability and gives the almost true-to-type plants. According to Edward (2005) as cited in Kwan (2008), only shoot-tip explants of *D. beccarii* showed response in induction of multiple shoot formation via callus. However, shoot-tip explants have high risk of bacterial and fungal contamination and browning problem. Ang (2009) reported that petiolule has low contamination problem than shoot tips. In addition, petiolule explant had potential to form callus, while explants from lamina and lamina tip of leaflets were not responsive. Pradeep (2000) also found that very young petiole explants exhibit a higher regeneration potential rather than the leaf explants. Besides, the regeneration potential of woody plants *in vitro* was influenced by the maturation of explants materials, as proved by Papafotiou and Martini (2009) that petiolule explants showed the highest morphogenic

response to plant growth regulators compared to other explant. Juvenile materials are more responsive in *in vitro* regeneration. Therefore, the newly emerged petiole and petiolule is recommended for used as explants.

2.3.2 Problem of contamination

Contamination is referred to internal and external pathogens that are present on or within the explants. *In vitro* contamination by bacteria, fungi or yeast is one of the major and serious problems associated with tissue culture (Michael *et al.,* 1998; Okazaki, 2008)). Contaminations are normally caused by systemic contaminants in the explant itself, laboratory environment, operators, mites and ineffective sterilization technique (Barbara & Piyarak, 1995). It is very difficult to control and establish viable *in vitro* culture especially with explant material obtained from the open field (Michael *et al*., 1998; Webster *et al*., 2003). According to Webster *et al*. (2003), selection of healthy stock plant and effective surface sterilization procedure will reduce percentage of contamination. However, controlling fungal contamination is extremely difficult in woody plant as fungal contamination is the main problem in *D. beccarii* culture (Michael & Randall, 2002; Kwan 2008; Okazaki, 2008).

According to Skirvin *et al*. (1999), the explant itself is the main source of fungal and yeast contamination. Most woody plant tissue cultures are established from branches that are brought from the field or greenhouse and stored in water to eliminate debris and hold them until they are ready to explanting (Skirvin *et al*., 1999). However, the storage water and its microorganisms can be a source of the internal (endogenous) contamination. The microorganisms can survive in the vascular tissue of plants and may not initially apparent, but may begin to proliferate around the base of the explant and appear as a halo of

contamination (Skirvin *et al.*, 1999). These contaminations have been given the name of 'white ghost' in the U.S.A. (Pierik, 1997). Pierik (1997) reported that the white ghost is often in the form of rod bacteria, particularly *Bacillus licheniormis* and *Bacillus subtilis*. Skirvin *et al*. (1999) also reported that fungal and yeast contamination generally can be seen within the first 1 or 2 weeks of culture.

In order to reduce contamination, several germicidal reagents such as Clorox, alcohol, mercuric chloride, fungicide and antibiotics can be used to disinfect and surface sterilize explants (Aminah, 2002). Webster *et al*. (2003) found that fungicide such as Benomyl could decrease internal fungal contamination and it was proven by Jose *et al.* (2003) that Benomyl at concentration at 1.0 g L^{-1} was effective at cleaning leaf segments. Soaking in fungicide Benomyl at 0.3 % for one hour after surface sterilization with Clorox was found to be effective in increasing the number of aseptic explants in Dabai (Chong, 2009). Ang (2009) found that immersing *D. beccarii* explants in 0.3% Benomyl solution for 15 minutes before transferred to solid media was quite effective in reducing of fungal contamination. In addition, as cited in Kwan (2008), Tie (2007) reported that spraying the explants of D. beccarii with 75% ethanol + 0.1 %. Benomyl and soaked in 0.1% Benomyl for 1 hour before surface sterilization was the best pre-treatment to obtain axenic explants. Besides, a wetting agent like Tween 20 is used to ensure good contact of sterilizing agents with explants. Mercuric chloride or Clorox that contains the active ingredient of 5.25% sodium hypochlorite are the most widely used disinfectant (Singh, 2005).

Some other chemicals are also used to control contamination. These include antibiotics and fungicides, alcohols and oxidizing biocides such as halogen compound i.e. chlorine, bromine and iodine (Michael & Randall, 1998). Plant preservative mixture (PPM), an extremely effective heat stable biocide contains isothiazolones, a class of industrial

biocides is an effective biocide to control microbial contamination (Michael & Randall, 1998; Okazaki, 2008; Ang, 2009). However, Tie (2007) has found that high concentration of PPM (4 mg/L) would probably be phytotoxic and killed the *D. beccarii* explants. In addition, prolonged exposure to PPM and antibiotic may affect response of explants. Kwan (2008) found that medium with 2 ml/L PPM was effective in the control of fungal contamination. Okazaki (2009) also reported that PPM affectively decreased the probability of fungal contamination with minimal effect to the explant survival, compared to other antifungal.

Antibiotics such as tetracycline and streptomycin are usually used to inhibit microbial growth in culture media. Young *et al*. (1984) found that tetracycline and rifampicin had broader range of bactericidal activity than others. Studies done by Kwan (2008) and Ang (2009) showed that medium incorporated with higher concentration of tetracycline showed higher percentage of axenic explants than low concentration of tetracycline. However, according to Jose *et al* (2003), ampicillin treatments at 1.0 to 4.0 mg/L were ineffective at bacterial contamination. These prove that not all antibiotics are effective in controlling bacterial contamination. Many antibiotics carry adverse effect on plant materials. According to Dodds and Robert (1981) as cited in Kwan (2008), antibiotic often inhibits morphogenesis and has phytotoxic effect to the explants.

According to Kwan (2008), surface sterilization of explant by submerging the explants in 20% Clorox combined with culturing in medium incorporated with PPM and tetracycline were effective in reducing percentage of contamination of explant. In contrast, studies done by Ang (2009) found that 20% Clorox caused damage to the shoot-tip explants whereas chlorine dioxide found to be less damaging to the explants. His study showed that chlorine dioxide was more effective than Clorox in reducing fungal contamination on the *D.*

beccarii petiolule explants. Aminah (2002) also found that Clorox with higher concentration i.e. more than 60% will damage the explants. Chlorine dioxide has strong oxidative power to inactivate single cell microorganisms (Ang, 2009) and have potential to control phenolic compound (Water Corporation, 1999). In addition, chlorine dioxide had been reported to be more effective against all kinds of germs and contaminants such as viruses, bacteria and fungi than other biocides (Olsen, *et al*., n.d as cited in Ang, 2009). As chlorine dioxide is a strong oxidation agent, using in low concentration is enough to be effective for disinfection. Therefore, using of 50 ppm Chlorine dioxide (ClO_2) for 15 minutes during surface sterilization found to be effective in controlling contamination (Ang, 2009).

2.3.3 Problem of browning

Browning is one of the problems usually hampered and lethal to *in vitro* culture especially for woody plants. Browning is generally considered a condition of explants turn dark brown upon oxidation of phenolic compounds by polyphenoloxidases, peroxidases or air released from the cut ends of the explants (Heldge & Kulasekaran, 1996; Bhat & Chandel, 1991). According to Bhat & Chandel (1991), the oxidized products, namely quinines are highly reactive and tend to inhibit enzyme activity, thus leading to the death of the cells and explants.

Since browning is another factor affecting the viability of explants, strategies and methods have to be developed to overcome the harmful effect of browning especially in explants in small volume of medium where the potential of explants being killed is greater than in larger volume of medium (Bhat & Chandel, 1991).

Several methods have been used to control and minimize the phenolic problems include subculture the explants every three to seven days (Aminah *et al*., 2002), culturing the explants in liquid medium for three to ten days (Heldge & Kulasekaran, 1996), keeping culture in dark condition (Aminah *et al*., 2002: Heldge & Kulasekaran, 1996) and using adsorbing agents such as activated charcoal to absorb polyphenols secreted in the medium (Aminah *et al*., 2002; Helgde & Kulasekaran, 1996: Bhat & Chandel, 1991). Choosing the juvenile explants, culture in liquid medium and soaking explants in antioxidants prior to inoculation such as PVP (polyvinyl-pyrrolidone), ascorbic acid, cysteine-HCl or citric acid (Aminah *et al*., 2002; Helgde & Kulasekaran, 1996; Bhat & Chandel, 1991) and choosing the low salt medium and proper growth regulator are strategies which have been proved to overcome browning problem.

The initial incubation was recommended to be carried out in dark environment and low temperature condition because light and temperature fluctuation will increase the activity of enzymes associated with the oxidation of phenolic compounds that will further lead to browning (Linington, 1991; Singh, 2005). However, the use of activated charcoal is not ideal when the culture medium was added with plant growth regulator or antibiotics as these substances will be absorbed by the activated charcoal making them less effective. Frequent subculture is the most common method to reduce browning. According to Kwan (2008), the damage part of the tissue have to be removed during sub culturing to prevent the accumulation of toxic oxidize phenolic compound in the medium.

Studies done by Ang (2009) showed that nearly 70% contamination-free culture and viable shoot-tip explants of *Dryobalanops beccarii* could be established by several important strategies such as the selection of young tree growing under the shade as the source for explant material could reduce browning. Maintaining cool condition during the process of