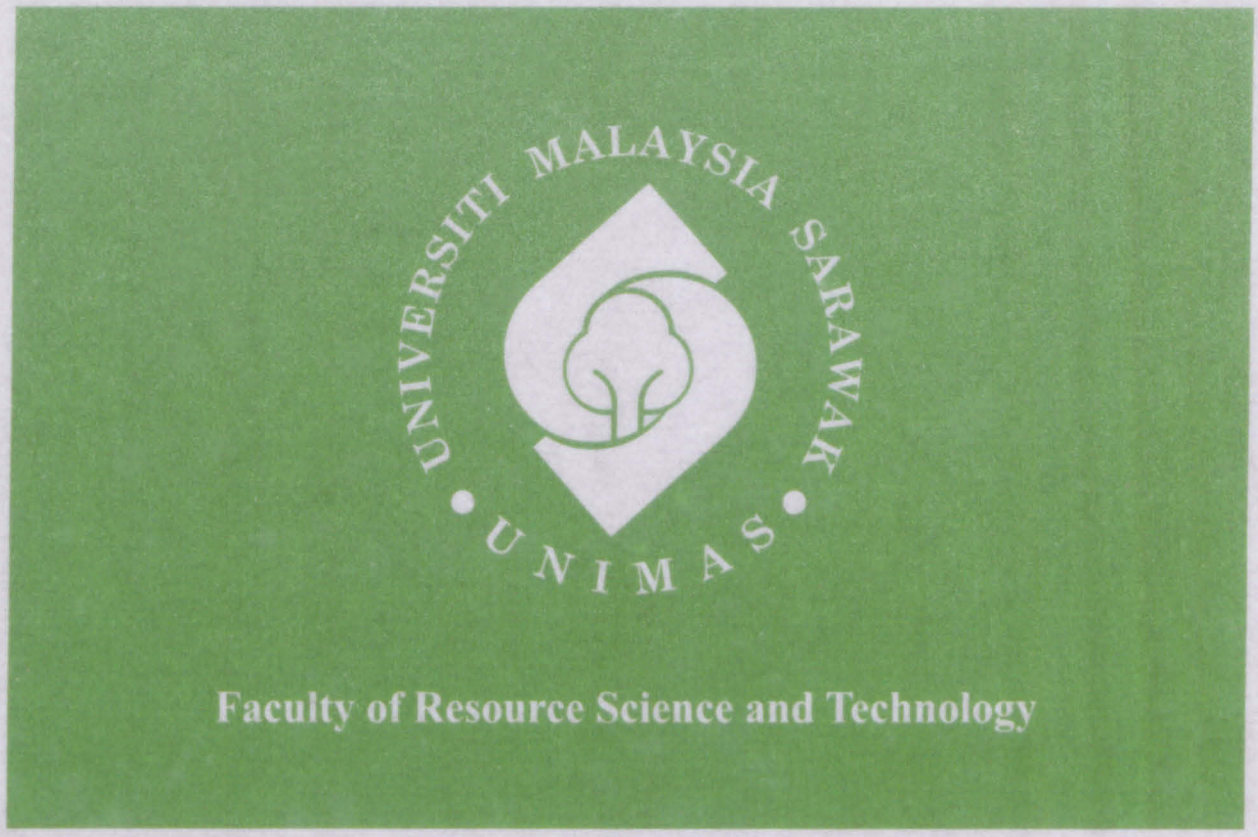

THE ESTABLISHMENT OF SURFACE STERILISATION FOR *MACARANGA GIGANTEA*

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Bachelor of Science with Honors
(Resource Biotechnology)
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The Establishment of Surface Sterilization for *Macaranga gigantea*

Ku Nur Azwa binti Ku Aizuddin (56337)

A project proposal submitted is a partial fulfilment of the
Final Year Project 1 (STF3013)

Supervisor : Associate Professor Dr. Ho Wei Seng

Resource Biotechnology Programme

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December 2019

Programme of Resource Biotechnology

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The Establishment of Surface Sterilisation for *Macaranga gigantea*

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ABSTRACT

The demand of wood has increased over time to fit the people's demand. However, this contributes to the logging activity that eventually affects the tropical forest. Thus, planting a fast-growing tree like *Macaranga gigantea* is one of the approaches that can reduce the dependency on timber production from natural forest. Hence, this plantation tree species has the potential to become one of the alternatives to overcome this problem. *Macaranga gigantea* is locally known as the 'giant Mahang' due to its capability of fast-growing up to 15 m tall. This study aims to use the *in vitro* propagation and ex vitro propagation technique to culture the *Macaranga gigantea*. Surface sterilisation to establish an axenic culture and the application of the pre-treatment method is used to overcome the physical dormancy of the seeds.

ABSTRAK

Produksi dalam industri kayu telah meningkat dari masa ke masa untuk memenuhi permintaan rakyat. Walau bagaimanapun, ini menyumbang kepada aktiviti pembalakan yang akhirnya menjejaskan hutan tropika. Oleh itu, menanam pokok yang berkembang pesat seperti *Macaranga gigantea* adalah salah satu pendekatan yang dapat mengurangkan pergantungan pada pengeluaran kayu dari hutan semula jadi. Oleh itu, spesies pokok perladangan ini berpotensi menjadi salah satu alternatif untuk mengatasi masalah ini. *Macaranga gigantea* secara tempatan dikenali sebagai 'Mahang gergasi' kerana kemampuannya untuk tumbuh dengan cepat hingga 15 m. Kajian ini bertujuan untuk menggunakan teknik pembiakan *in vitro* dan *ex vitro* bagi penanaman pokok *Macaranga gigantea*. Penyucian permukaan bagi mewujudkan pembiakan yang bersih dan penggunaan kaedah pra-rawatan digunakan untuk mengatasi benih yang mempunyai dorman fizikal.

Keyword: *Macaranga gigantea, in vitro* propagation, *ex vitro* propagation, surface sterilisation and pre-treatment

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List of Abbreviations

Abbreviations	Descriptions
P. berolinensi	*Papyrus berolinensi*
PD	Physiological
MD	Morphological
MPD	Morphophysiological
PY	Physical
MS	Murashige and Skoog
%	percentage
°C	degree Celcius (temperature)
h	hours
g	gram
mg/L	milligram per litre
p	probability

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CHAPTER 1

INTRODUCTION

One of the essential natural resources for humans is wood (Warman, 2014). The demand for wood products has increased in the market, due to its potential for making furniture, building materials and papers. To fulfill the high demand, some of the wood production could give an adverse impact on the tropical forest. The increase of logging activity could further damage this area. As this problem arises, planting activity using fast-growing species should be taken into account. This approach could reduce the dependency on timber production from the natural forest. Thus, *Macaranga gigantea* has the potential to overcome this problem.

One of the largest family in the plant kingdom is the Euphorbiaceae family, as it comprises about 322 genera and 8910 species that consist of big woody trees to weeds (Mwine and Damme, 2011). Hence, *Macaranga gigantea* is one of the species that belong to this family. This particular species is widely spread and commonly found in Southeast Asia and New Guniea, followed by Africa, Madagascar, continental Asia, and other places in the Pacific Islands and Australia (Siregar and Sambas, 2000).

The unique features of the *Macaranga gigantea,* including the seeds, leaves, and stem, offers a lot of benefits that can be utilised by humans. This soft-wooded tree could be used as raw material and has the potential for bioethanol production in the future (Susanto, 2016a). Not only that, the pharmacological properties that comprise the *Macaranga* species could be used as a part of traditional medicine (Joseph, 2014). Thus, *Macaranga* genus consists of other beneficial properties including anticancer (Yoder et al., 2007), and antioxidants (Phommart et al., 2005) due to its pharmacology and chemical compound. However, despite having a high economic value, this invasive species is still not recognised as an essential commodity display specific medical effects.

Thus, it is necessary to culture this species using the approach of micropropagation technique. There are three stages of micropropagation as defined by an early researcher named Toshio Murashige in 1974, but later it had been redefined as four stages (Chen and Jianjun, 2008). The micropropagation stages start from the zero stage. In this stage, the selection and preparation of the mother plant are carried out. The environment for growing the stock plants must be hygienic (Kumar and Reddy, 2011). This stage is then followed by the first stage, which is the initiation of the axenic culture. This stage aims to initiate microshoots. This cultivation process must be done under the aseptic condition as well. Then, the second stage for the micropropagation technique is the shoot multiplication. This stage is crucial as masses of tissues will be repeatedly subcultured in a new culture media that enhance the propagule to proliferate further. The third stage of the micropropagation would be the root formation and followed by the last stage, which is the transferring of the developing plantlets to *ex vitro* condition.

Nonetheless, the physical dormancy that exhibits by the seeds must be broken to successfully cultured through the micropropagation technique. Most hard seeded plants experience physical dormancy which is imposed by the seed coat (Rodrigues and Rodrigues, 2014). The structure features of the seed coats for the *Macaranga gigantea* is to further its embryo and acts as a physical defence for the seeds itself. Hence, the pre-treatment method must be applied to break the dormancy of the seeds.

This study aimed to establish a protocol for producing the cleaned axenic plant under the process of surface sterilization and to identify the optimum concentration of gibberellic acid for the pre-treatment method.

CHAPTER 2

LITERATURE REVIEW

2.1 *Macaranga gigantea*

Macaranga gigantea is a fast-growing pioneer species that is easily recognized along the roadside of Malaysia. This plant is locally known as the giant Mahang, belongs to the family of Euphorbiaceae (Joseph, 2014). With over 300 species comprises in the Euphorbiaceae family, *Macaranga* is the only genus under the subtribe of Macaranginae and classified as the large genus of 'Old World' trees within the Euphorbiaccea family (Lim et al., 2009). Naturally, this giant Mahang would grow in lowland tropical forest, particularly in gaps that are affected by the timber harvesting, forest fires, and shifting cultivation (Lawrence, 2005). Furthermore, it is commonly found in Thailand, Peninsular Malaysia, Singapore, Sumatra and Borneo. There are about 40 species reported where this *Macaranga sp.* were found growing in Malaysia, mostly in the secondary forest and newly cleared areas (Siregar and Sambas, 2000).

This plant has the ability to grow up until 15m tall and can either become a shrub or tree (Mohd Johari et al., 2019). Within a short period interval between the expansion of successive leaves, this giant Mahang tree can produce big leaves on a one erect stem. Thus, it can be seen that the petioles of this tree are much shorter for the newer leaves compared to the older leaves (Yamada et al., 2000). As for the stem itself, *Macaranga* plants appear to produce a threadlike wax crystal in their natural state (Joseph, 2014). The symbiotic relationship of the plants and insect are further maintained by the presence of terpenoids that comprises a large part of the wax bloom content which has been indicated by the chemical analysis (Markstädter et al., 2000). The wood itself is not suitable for construction purposes as this particular plant has a soft and light-weighted (Susanto, 2016a). Nevertheless, this wood managed to produce a

large amount of reducing sugar content due to its enzymatic hydrolysis process compared to other species such as *Paraseriantes falcataria* and *Acacia mangium.*

Agricultural activity relies heavily on the fertility of the land to increase their crop production. The nutrients status and the pH of the soil plays a huge role in the growth of the plant. Hence, this particular species can become a land fertility indicator for the cropping season (Susanto, 2016b). The land indicates that it is fertile enough for the cropping season as these species are found growing dominantly in that particular area. Not only that, this plant also has the capability of being a raw material for bioethanol products (Susanto, 2016a). The development of the biofuel industry has been increasing due to the global fuel crisis. Thus, the utilization of liquid bioethanol derived from the wood is sustainable, as long as the forest that grows the plant is preserved. *Macaranga* species consist of various pharmacological features with a variety of chemical structure, and this species has been practised in traditional medicine (Joseph, 2014).

2.2 The seed of *Macaranga gigantea*

In plant physiology, one of the most extensive research areas is seed biology. The seed is the ripened ovule that contains the embryo. This reproduction unit of a plant has the capability to develop into a new plant. Hence, seed is the sign of beginning in the scientific agriculture field. In order to support the establishment of the seedlings, majority of the plant species' seeds must consist of ample nutrients which act as a rich source that are eaten by a wide group of animals (Kestring et al., 2009). As for pioneer trees in tropical Asia, including some of the subspecies in the *Macaranga* genus, the seeds are mainly disseminated by birds (Corlett and Hau, 2000). Furthermore, for this particular species, the mean of fecundity is about 19,000 seeds per year (Davies, 1998). Fecundity defined as the number of seeds produced by a plant. The life history and the adaptive strategy of the plant species will mainly determine its fecundity rate.

As for the seed shape of the *Macaranga gigantea*, it can be seen that it has a lenticular shape with shallow grooves. Thus, to preserve the seed from penetrated by the insects predator, the thickness, as well as the fracture-resistance of the enclosing structure, will act as the physical defence for the seed (Souza et al., 2011). The seed coat also served as a purpose to protect its embryo from the humidity and temperature fluctuation. However, different species of Mahang trees would have different shape of seeds. For example, *Macaranga hypolueca* would have a spheroidal shape with large shallow round pits while *Macaranga winkleri* would have an ovoid shape with a coarsely verrucose surface (Tiansawat et al., 2016). Moreover, the size of the seed itself is small that has an average weight of about 0.018g (Susanto, 2016a). As this smaller seed from tropical pioneer trees is planted at a suitable area, it will germinate in canopy gaps. Canopy gaps occur when there is an empty area within the forest canopies (Muscolo et al., 2014).

The seed of the *Macaranga gigantea* has a low water content which consists of about 8.23%. The lower water content of a seed is classified as orthodox (Suita and Nurhasybi, 2009). Orthodox seeds can be dried to low moisture content and able to withstand freezing temperatures (Chin et al., 1989). Hence, due to the drying maturation of the orthodox seed, the metabolism of the embryo will be suspended. Plus, the mature seeds that are associated with a low water content will not be able to germinate even though the seeds are not in the dormant state unless they are fully hydrated (Penfield, 2017). Examples of orthodox seeds are *Hibiscus esculentus* and *Vigna sesquipedalis.*

The success of regenerating a plant is frequently determined by its seed-stage (Tiansawat et al., 2016). For this reason, the seed must be nurtured in a favourable condition to induced germination rate and seedlings establishment. One of the critical regulators for seed germination in small-seeded plants is light. The seed of *Macaranga gigantea* is considered as the most light-demanding species for its genus. The germination percentage of small seed would increase when it is exposed to a light condition compared to a dark (Pearson et al., 2002). Germination of *Macaranga gigantea* seed only take place under complete light exposure and not even below 20% shading (Raphael et al., 2015) In order to control the water uptake as well as to avoid rapid desiccation, seed that appears to have a thicker seed coat will germinate in a drier high light environment ((Daws et al., 2005; Koizumi et al., 2008). On top of that, the rate of germination could also be increased by using wet extraction compared to the dry extraction process when it takes place in the laboratory (Susanto, 2016a). It can be seen that the rate of germination increases by 20% when the seed of *Macaranga gigantea* is soaked into a 0.2% potassium nitrate solution for 20 minutes rather than performing a dry extraction onto the seed which only gives about 2% to 10% of germination rate.

2.3 *In vitro* propagation technique

Over the years, not only technology has become an increasingly important element in our daily life, but it also gives a modern twist in the botany industry. Thus, the use of biotechnology application has successfully cultured the cells, protoplast and tissues (Pasqual et al., 2014). With the help of this advanced technology, the micropropagation technique has become an alternative mean of propagation that is used in mass multiplication of plants within a short period.

Micropropagation is a process whereby the axenic plant materials undergo a manipulation technique to produce new plants in a controlled environment (Alistock and Shafer, 2006). As the cells, tissues or organs of selected plants undergo the micropropagation technique, it can further differentiate into a whole plant. Cells that have the potential to grow and further develop into a multicellular organism is defined as totipotency (Kumar and Reddy, 2011). The micropropagation method involves the growth of plant cells under the *in vitro* condition. Meaning, these experimental units will be kept in a controlled surrounding.

The use of these techniques has the following advantages. Firstly, micropropagation technique can produce a higher rate of new plants all year-round compared to other methods (Ailstock and Shafer, 2006). Due to the seasonal constraints, the traditional way like cutting and grafting would have a limited potential for cloning plants. Thus, this method could also produce in large quantity for genetically uniform of healthy plants. The aseptic technique is used when performing the micropropagation technique in order to obtain the whole plant. Hence, all of the axenic cultures are kept under a controlled environment.

As for growing a plant under the *in vitro* condition, there are a few factors that contribute to the development of the plant which includes the genotype, type of explant, plant growth regulators and the basal medium. Thus, the basal medium itself has it owns contribution for the *in vitro* multiplication and regeneration of plant which has been demonstrated using a lot of species, such as Easter lily (Ramsay et al., 2003), *Populus alba* × *P. berolinensis* (Wang et al., 2008), and *Aquilaria hirta* (Hassan et al., 2011).

The technique of micropropagation is also greatly affected by the use of the culture medium (George et al., 2008). The basal media that is used for this project is the Gamborg B5 basal media which was formulated by Gamborg O.L. It is designed for the cultivation of plant tissue. Hence, it is ideal for growing calluses and various plant organs. Gamborg B5 basal media consist of microelements, microelements, vitamins and carbohydrate. Thus, one of the determining factors for growth is the composition of the media itself. There are a few species of plants that germinate well using this particular media compared to other basal media which include Murashige and Skoog (MS) medium or Woody Plant Medium (WPM). The dry cell weight of *Vitex glabrata* increases as it is cultured using the Gamborg B5 medium compared to the MS medium (Duangjai et al., 2007). Not only that, the combination of low salt with boron deficiency as well as the limit amount of nitrogen in Gamborg B5 are found to be the most suitable basal for *Linum usitatissimum* to increase the callus biomass (Zahir et al., 2018)

2.4 *Ex vitro* propagation technique

Ex vitro propagation technique is also part of the micropropagation process. This technique is applied by culturing the plant in an environment outside the artificial tissue culture, usually in soil or potting mixture. The best survival condition for the transferred healthy explant can be achieved by implementing the optimization under the *in vitro* condition. Nevertheless, good *in vitro* conditions sometimes does not give an ideal *ex vitro* results in some settings (Bhatia, 2015). Hence, optimizing the variables which involve during *in vitro* growth and link with the *ex vitro* acclimatization results could further improve the tissue culture process.

There are a few advantages that involve using the *ex vitro* propagation method. It can be seen that growth of the root system for *ex vitro* rooted plant are greater and have a higher chance of survival compared to the *in vitro* rooted plant (Borkowska, 2001). This is because, *ex vitro* rooted plant is associated with lateral roots which equivalent to the natural root system with more root length in transplanting (Yan et al., 2010). Plus, *ex vitro* rooting has a lower probability of damaging the root during transplantation to soil (Arya et al., 2003). Moreover, the efficiency of micropropagation from both biological and economic perspective can be improved by using the *ex vitro* rhizogenesis approach (Borkowska, 2001). This situation could be achieved as the *ex vitro* propagation technique can simplify the procedure involve and minimize the cost of production as well.

Various types of woody plant species that are difficult to produce roots can be induced by rooting the microshoots using the ex vitro propagation technique (Benmahioul et al., 2012; Vibha et al., 2014). *Pistacia vera* is one of the plant species that implement the micropropagation system effectively that includes the rapid axillary bud proliferation and ex vitro rooting (Benmahioul et al., 2012).

CHAPTER 3

MATERIALS AND METHODS

3.1 *In vitro* propagation technique

3.1.1 Surface sterilisation and establishment of axenic culture

The seeds of *Macaranga gigantea* were used in this study. Surface sterilisation process was done by using 70% ethanol and five different concentration of commercial bleach, Clorox (Method 1: 10%, Method 2: 20%, Method 3: 30%, Method 4: 40% and Method 5: 50%) for about 10, 15 and 20 minutes interval. The seed coat of the *Macaranga gigantea* was removed to release the embryo. This step was continued by culturing the seed's embryo in the Gamborg B5 basal media and will be kept in the growth chamber at the temperature of 22 ± 2°C. Further observation was recorded after 2-4 weeks in culture. The number of clean cultures was taken for later reference.

3.1.2 Basal Medium

Half strength of the Gamborg B5 basal medium was used to culture the seeds of *Macaranga gigantea*. The stock solution of macronutrients, micronutrients, chelating iron, vitamins, and distilled water were added into a glass sampling bottle. 20g of sucrose was added to act as a carbon source for the media. The pH was adjusted to 5.8 using sodium hydroxide, NaOH or hydrochloric acid, HCl. 8g of agar was added to solidify the media further. The media was autoclave at a temperature of 121°C.

3.1.3 Pre-treatment method

The pre-treatment method using the hormone treatment was used to break the physical dormancy of the *Macaranga gigantea* seeds. The half strength of Gamborg B5 supplemented with two different concentrations of gibberellic acid (GA: 2.5mg/l and 5.0mg/l) were prepared. The basal media that was not added with the gibberellic acid will act as a control. The culture was maintained at a temperature of 22 ± 2°C with 16 h light and 8 h dark. Further observation of the culture was recorded.

3.2 *Ex vitro* propagation technique

The seeds of Macaranga gigantea were used in this research. The seed coat was removed to release the embryo by breaking the seed coat using the bottom part of the sample glass bottle. The seeds' embryo was then placed in a petri dish that consists of a damped filter paper. Three replicates were prepared. Each of the replicates consisted of five seed embryos as an explant for the research. The observation was recorded after 2-4 weeks in culture.

CHAPTER 4

RESULTS

4.1 Surface sterilisation using different concentrations of Clorox

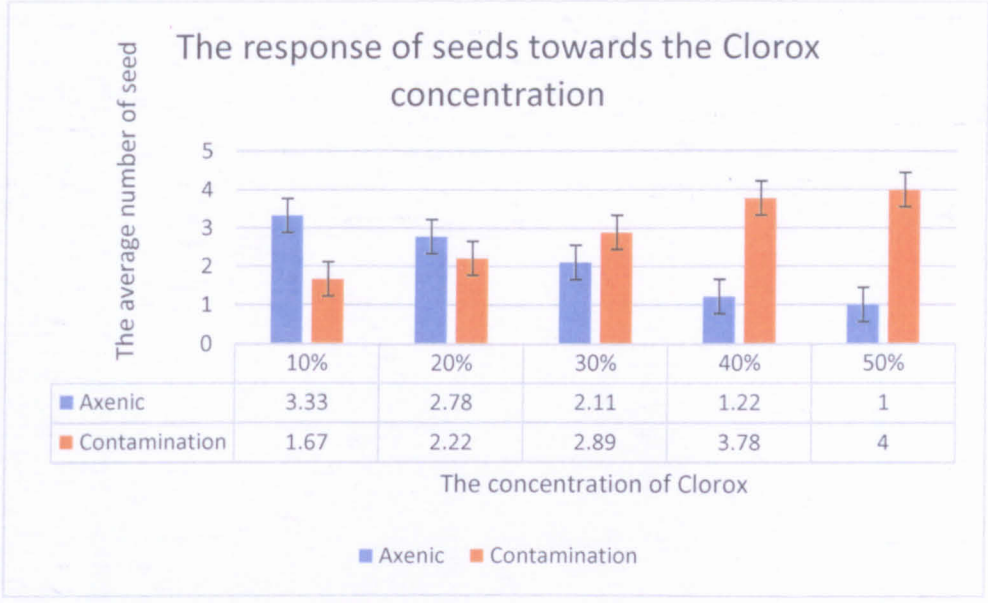

Figure 1. The response of seeds towards the Clorox concentration

Based on the result above, 10% of Clorox is the most suitable concentration of surface sterilisation for the *Macaranga gigantea* seeds. This is because the average number of contamination for seeds that were sterilised with 10% of Clorox concentration is the lowest compared to the other concentration. Moreover, it can be seen that a higher concentration of Clorox reduced the average number of axenic yet increase the average number of contamination for the seeds. It can be seen that the average number of seeds were still axenic after sterilised with 10% of Clorox concentration is at 3.33. However, the average number of seeds were still axenic after sterilized with 50% of Clorox concentration is only at 1.00.