Faculty of Resource Science and Technology

EFFECTS OF DIFFERENT BASAL MEDIA AND COMPLEX ADDITIVES INTERACTION ON *IN VITRO* SEED GERMINATION AND SEEDLING GROWTH OF PETAI BELALANG (*LEUCAENA LEUCOCEPHALA*)

Jackson Wong Pak Seng

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JACKSON WONG PAK SENG

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DECLARATION

I hereby declare that this thesis is based on my original work and effort and it has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged.

Jackson Wong Pak Seng

Resource Biotechnology Programme

Department of Molecular Biology

Faculty of Resource Science and Biotechnology

Universiti Malaysia Sarawak

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LIST OF ABBREVIATION

°C	Degree Celsius
ANOVA	Analysis of variance
BH	Banana homogenate
Biotechcorp	Malaysian Biotechnology Corporation
cm	centimeter
CW	Coconut water
DAC	Days after culture
DMRT	Duncan’s multiple range tests
ECERDC	East Coast Economic Region Development Council
g/L	Gram per Liter
HCI	Hydrochloric acid
kg	kilogram
kJ	kilojoule
kPa	kilopascal
m	Metre
m3	Cubic metre
M	Molarity
MS medium	Murashige and Skoog’s medium
NaOH	Sodium hydroxide
NH4+	Ammonium ion
NO3−	Nitrate ion
PGR	Plant growth regulator
PH	Potato homogenate
pH	potential hydrogen
Psi	Pound-force per square inch
TDZ	Thidiazuron
UV	Ultraviolet
WPM	Woody plant medium

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EFFECTS OF DIFFERENT BASAL MEDIA AND COMPLEX ADDITIVES INTERACTION ON *IN VITRO* SEED GERMINATION AND SEEDLING GROWTH OF PETAI BELALANG (*LEUCAENA LEUCOCEPHALA*)

ABSTRACT

Leucaena leucocephala or petai belalang has been identified as one of the best nitrogen fixing, standard sized fast growing leguminous tree that grows well in Malaysia. Their wood, which have a high density and energy value together with various biochemical compounds, make it an excellent biomass and feedstock to manufacture a variety of valuable products; caused it has a high market demand. However, conventional propagation method of *L. leucocephala* does not guarantee a high seed germination rate and the seedling growth as exhibition of natural seed dormancy and the seedling is susceptible to *Phytophthora drechsleri* and *Fusarium semitectum.* Thus, this research is performed to determine the interaction of different basal media and complex additives on *in vitro* seed germination and the seedling growth of *L. leucocephala* with the purpose to enhance the seed germination and the seedling growth of *L. leucocephala*. To address this, seeds were undergo sterilization followed by seed pre-treatment. The seeds were then cultured onto various basal media (half and full strength MS medium and Gamborg B5 medium) with or without the supplementation of complex additives (banana homogenate, potato homogenate and coconut water) with different concentrations. The results showed that Gamborg B5 media gave the highest seed germination whereas MS media showed the least seed germination efficiency. It was found that supplementation of complex additives had no effect on seed germination. However, media with complex additives showed the positive effect on seedling growth of *L. leucocephala.* 10% coconut water was found to give the most significant effect towards the seedling growth. By the end of this study, the *L. leucocephala* seedlings are able to be produced in vast amount to meet the high market demand.

Key words: *Leucaena leucocephala, in vitro* seed germination, seedling growth, interaction of basal media, complex additives

INTERAKSI KESAN MEDIA ASAS DAN KOMPLEKS ADITIF TERHADAP *IN VITRO* PERCAMBAHAN BIJI BENIH, PERKEMBANGAN DAN PERTUMBUHAN PETAI BELALANG (*LEUCAENA LEUCOCEPHALA*)

ABSTRAK

Leucaena leucocephala atau lebih dikenali sabagai petai belalang telah dikenal pasti sebagai pokok kekacang bersaiz sederhana yang tumbuh baik di Malaysia. Ia mempunyai ketumpatan kayu dan nilai tenaga yang tinggi serta mempunyai pelbagai kandungan biokimia, menjadikan ia sebagai biojisim yang baik dan bahan mentah untuk menghasilkan pelbagai produk yang berharga. Ini menyebabkan pokok tersebut mendapat permintaan pasaran yang tinggi. Tapi kaedah propagasi konvensional L. leucocephala tidak memberikan kadar percambahan biji benih dan pertumbuhan anak benih yang baik kerana pengwujudan dormansi benih yang semula jadi dan anak benih terdedah kepada Phytophthora drechsleri dan Fusarium semitectum. Oleh itu, kajian ini dijalankan untuk mengenalpasti interaksi media asas dan organik kompleks aditif dengan kaedah percambahan biji benih dan pertumbuhan anak benih secara in vitro dengan tujuan untuk meningkatkan percambahan biji benih dan pertumbuhan anak benih L. leucocephala. Eksperimen ini dimulakan dengan pensterilan biji benih diikuti dengan pra-rawatan biji benih. Biji benih yang dirawat kemudian dikultur ke pelbagai media asas (kepekatan setengah dan penuh media MS serta B5 Gamborg media) dengan atau tanpa penambahan bahan aditif kompleks (homogenat pisang, homogenat kentang dan air kelapa) pada kepekatan yang berbeza. Hasil kajian menunjukkan media B5 Gamborg memberikan percambahan biji benih yang tertinggi manakala media MS menunjukkan percambahan biji benih yang kurang baik. Disamping itu, hasil kajian menunjukkan bahawa penambahan organik kompleks additif tidak mempunyai kesan terhadap percambahan biji benih manakala media dengan penambahan bahan aditif kompleks menunjukkan kesan positif terhadap pertumbuhan anak benih L. leucocephala. Air kelapa 10% (v/v) telah didapati memberikan kesan yang baik terhadap pertumbuhan anak benih. Selepas menjalankan kajian ini, ia diharap dapat menghasilkan anak benih L. leucocephala dalam kuantiti yang banyak supaya dapat memenuhi permintaan pasaran yang tinggi.

Kata kunci: *Leucaena leucocephala, percambahan benih in vitro, pertumbuhan anak benih, interaksi media asas, bahan aditif kompleks*

CHAPTER I

INTRODUCTION

Leguminosae are the third largest family of flowering plants, with roughly 650 genera and 18,000 species. They have an unique characteristic, at which their fruits or seeds are contained within a pod or a shed. Under this family is categorized into another three subfamilies, namely *Papilionoideae, Mimosoideae* and *Caesalpinioideae*. *Leucaena leucocephala* is one of the species belong to this family (Hall, 2007; Rodd & Stackhouse, 2008).

Leucaena leucocephala or locally known as petai belalang is an evergreen shrub or tree that are able to adapt well in a wide range of climate and are cultivated throughout the world, especially in tropical and subtropical regions. It has been spotted to grow well in Philippine, Indonesia, Malaysia and Thailand (Masafu, 2006). In ancient time, it has been nicknamed as "miracle tree" due to its wide assortment of uses, for instance, firewood, green manure, soil improvement, reforestation, shade and erosion control, forage, pulp and paper industry (Pal *et al*., 2012; Somasegaran & Martin, 1986).

But recently, the use of the tree is advanced when it is fully occupied as a feedstock to manufacture a variety of valuable products including L-methionine, an important nutritional additive in the diet supplement industry. Besides that, it is fully utilized as a biomass in generating energy due to its superior characteristics of high wood density (500-600 kg/m^3) and energy value (19,250 kJ/kg). This is in line with government will to replace the use of fossil fuel. Therefore, the government has assigned 30,000 hectares for the purpose to cultivate *L. leucocephala* and this biomass plantation project is expected to require thousands seedlings of *L. leucocephala*, at which the demand is predicted to increase gradually (Heng, 2013).

However, conventional propagation method of *L. leucocephala* does not guarantee a high seed germination rate and seedling growth as exhibition of natural seed dormancy and the seedling is susceptible to *Phytophthora drechsleri* and *Fusarium semitectum*, a type of seedling rots (Hanum & Maesen, 1997). Thus, an alternative method is proposed, which is *in vitro* seed germination and development and it is supported by Kull and Arditti (2002), at which they mentioned the most appropriate propagation method which can support large quantity production of seedlings for conservation and commercial use is through *in vitro* seed germination.

In addition, in line with objective of plant tissue culture which is large scale propagation of plantlet within short period of time and cost effectively, a lot of researchers added different types of organic additives into basal medium to determine its effect on seed germination and seedling growth of many plant species. Complex organic additives such as banana homogenate, coconut water and potato extract have undefined compositions, but do contain different kind of amino acids, vitamins and PGRs, which can promote the growth of plants (Sudipta *et al*., 2013).

Till date, there is no report on the effect of different basal media and organic complex additives on *in vitro* seed germination and seedling growth of *L. leucocephala* has been reported. Hence, this research is conducted to identify the requirements needed for the seed germination and seedling growth of *L. leucocephala* in term of basal media and complex additives.

Objectives of this study are:

1. To develop a new protocol for seed germination and seedling growth of *L. leucocephala*;

2. To determine the interaction of different basal media and complex additives on *in vitro* seed germination and the seedling growth of *L. leucocephala*;
3. To identify the most appropriate basal medium and complex additive for *in vitro* seed germination and the seedling growth of *L. leucocephala* in addition to develop a cost effective protocol for *in vitro* seed germination of *L. leucocephala*, and
4. To establish an efficient *in vitro* plant regeneration system of *L. leucocephala* through the induction of callus and the shoot organogenesis.

CHAPTER II

LITERATURE REVIEW

2.1 *Leucaena leucocephala* (Lam.) de Wit

2.1.1 Taxonomic classification (Itis report, 2015).

Kingdom	:	Plantae
Subkingdom	:	Viridiplantae (Tracheobionta)
Infrakingdom: Streptophyta		
Superdivision	:	Embryophyta (Spermatophyta)
Division	:	Tracheophyta (magnoliophyta)
Subdivision	:	Spermatophytina
Class	:	Magnoliopsida
Subclass	:	Rosidae
Superorder	:	Rosanae
Order	:	Fabales
Family	:	Fabaceae/leguminosae
Genus	:	*Leucaena*
Species	:	*Leucaena leucocephala* (Lam.) de Wit
Subspecies	:	*Leucaena leucocephala* ssp. *leucocephala* (Lam.) de Wit

: *Leucaena leucocephala* ssp. *Glabrata* (Rose) S. Zárate

2.1.2 Vernacular names

L. leucocephala has a variety of common names. Below are some common names of *L. leucocephala* (Pasiecznik, 2007).

Table 2.1: Common names for *L. leucocephala*

Common name	Countries
Leadtree; jumbie bean; cow tamarind	Australia
Faux-acacia	Brazil
Kanthum theet; kratin	Cambodia
Yin ho huan	China
Aroma blanca; soplillo	Cuba
Barba de leon	El Salvador
Tangan-tangan	Guam
Ekoa; koa haole	Hawaii
Frijol guaje	Honduras
Kubabul; subabool	India
Lamtoro; petai cina	Indonesia
Klandingan	Java
Kathin; koong khaaw	Laos
Petai belalang; lamtoro	Malaysia
Calguaje; guache tierra caliente; huaxin	Mexico
Kunai	Papua New Guinea
Arabisca	Peru
Ipil-ipil; elena; kariskis	Philippines
Reuse wattle	South Africa
Kra thin; to bao	Thailand
White lead tree; leucaena	USA
Keo dau; bo chet	Vietnam

2.1.3 Distribution and habitat

L. leucocephala is a woody plant species that is originated from the Northern Central America or more specifically to the Southern of Mexico and had been widespread throughout the world. It was first distributed to the Philippines by Spanish at 17^{th} century with the aim as a fodder. It was then widespread throughout South-East Asia (Hanum & Maesen, 1997). In Malaysia, it has been widely planted at the state of Terengganu (Heng, 2013).

According to Aung (2007), *L. leucocephala* grows best under high humidity (650 – 3000 mm rainfall) with long warm temperature (25 to 30 °C) and sunshine weather. Therefore, it is escaped as a wild plant in equatorial region. It can be found at an area above 1000 meters and grow well up to 1,900 meters above sea level. However, at higher altitudes, it shows a reduced in growth since it cannot withstand even light colds. A heavy frost has a very high potential to cause *L. leucocephala* leaves to shed or more seriously can inhibit or even cause death of the whole plant (Aung, 2007).

L. leucocephala is able to adapt well in all kinds of soil except acidic soil. It prefers to grow especially in alkaline soil without waterlogged conditions. In lowland or wasteland where the soils are insufficient of nutrients, *L. leucocephala* also is able to propagate since it is belonging to nitrogen fixing species (Aung, 2007; Nelson *et al*., 2007)

2.1.4 General morphology

Varieties of *leucocephala* species have been identified where two most commonly found are “Salvador” type, of which is able to grow up to 16 m and a “common” shrubby type, which is shorter (Hanum & Maesen, 1997).

In general, it is a small to medium-sized evergreen tree which is able to grow up to a height of 20 m, with a basal diameter of 10 – 30 cm. It has a characteristic of high growing rate and able to perform nitrogen fixation with the aid of nitrogen fixing bacteria such as rhizobium (Pandey & kumar, 2013).

L. leucocephala is a thornless shrub or tree with a long taproot. The main root is come with many fine tertiary roots and is deeply rooted into the soils (Pandey & kumar, 2013). Come toward the stem, according to Pandey and Kumar (2013), they stated "The stem of *L. leucocephala* is woody, erect, cylindrical, solid, branched rough with shallow, rusty orange-brown vertical fissures and deep red inner bark while their branches are smooth, stout, woody, and dark grey-brown in colour" (p. 1166).

L. leucocephala wood is belonging to soft wood. However, the wood is very tough and has a high wood density (500-600 kg/m3) and energy value (19250 kJ/kg); make them an excellent firewood and charcoal **(**Bhat *et al.,* 2003; Hanum & Maesen, 1997).

L. leucocephala has a very unique leaves. Their leaves are bipinnate with 6-9 pairs of pinnae and each pinna bears around 20 pairs of leaflets. The leaflets are 0.9 – 1.6 cm long with 0.2 – 0.45 cm wide. The leaves are high in protein (C/N ratio 9.80), minerals and vitamin content and often used as forage (Pandey & Kumar, 2013).

2.1.5 Flowering and fruiting

Fruit of *L. leucocephala* is referred to its pod, which always found in clusters form. The pod is flat and oblong in shape with 11 – 19 cm long, 1.5 – 2.1 cm wide and contains around 8 to 20 seeds. The pod is orange-brown in color when mature (Aderibigbe *et al*., 2011; Bhat *et al*., 2003; Maity, 2014; Pandey & Kumar, 2013; Pollard, 1996).

The flowers of *L. leucocephala* are yellowish-white or pink in colour and grow crowd together in globosely heads. The flowers roughly have a diameter of 2.54 cm and will only form from May to July and November to February (Nelson *et al.*, 2007).

2.1.6 Seed dormancy and pre-treatment

L. leucocephala reproduces mainly via seed, at which the seeds are stored in their pods. The seed is dark brown with a hard shinning waxy seed coat, and is about 0.7 cm long in the main axis with an ovoid shape **(**Bhat *et al.*, 2003).

L. leucocephala seed are consisted of three major tissues; embryo, endosperm and testa. The testa (seed coat) is a dead protective tissue that surrounds the embryo and endosperm with the function to attach the seed to the pea pod via the stalk-like funicle. It has a composition of lignin, tannins, suberin, callose and cutin that impermeable to water and acts as a mechanical barrier to protect embryo against extreme heat from sunlight, physical damage, dispersing animals or severe drought (Górecki, 2001). The presence of the seed coat causes the seed to exhibit dormancy, at which the mechanism involved is known as "hardseededness". This mechanism is very important as it only allows the seed to germinate when all the criteria for seed germination are fulfilled (Tadros *et al*., 2011).

However, germination is thought to be enhanced when seeds are scarified or soaked in concentrated sulfuric acid or hot water. Various chemical and mechanical treatments had been tested to overcome hard or impervious seed coats so that the imbibition of water and exchange of gases necessary for germination can occur (Asl *et al.*, 2011). A research study conducted by Omran (2013) showed around 46% of seed germinated under normal condition, but after treating the seeds by mechanical scarification using sand paper to soften the seed coat, around 70% of the seeds were germinated. However, Shaik *et al*. (2009) conducted an experiment and proved imbibition of the seeds by soaking the seeds in sufficient amount of sterile distilled water overnight give a better result of seed germination, which is around 90%. Zayed *et al*. (2014) proved that seed treatment with hot water (100 °C) for 20 seconds followed by soaking in water at room temperature for 48 hours gave a result of 95.9% germination percentage. Another research carried out by Tadros *et al*. (2011) showed a germination percentage of 97% when soaked the *L. leucocephala* seeds in 70 °C water bath for 20 minutes followed by soaking in water at room temperature for 48 hours.


a
b
c
d
CUTTING MAT 30X22CM A4
e
f
0 CM 1 2 3 4 5 6
g

Figure 2.1: (a) Mature *Leucaena leucocephala* tree.
(b) The trunk of *L. leucocephala*.(Source: http://www.plantekey.com/plants/fabaceae/leucaena-leucocephala).
(c) The leaves of *L. leucocephala* are bipinnate with 6-9 pairs of pinnae.
(d) The green seed pods of *L. leucocephala.*
(e) The mature seed pods of *L. leucocephala.*
(f) The seeds of *L. leucocephala* are dark brown with a hard seed coat.
(g) The seed is about 0.7 cm long in the main axis with an ovoid shape.

2.1.7 Economic significance

In the old days, *L. leucocephala* are known as miracle tree due to its wide assortment of uses. In Western countries, it is mainly used as a forage and green manure due to its high nutritive value. It also used in alley cropping as it is a good nitrogen fixing species. In Eastern countries, it is mostly used as fuelwood to generate energy or building materials for furniture, plywood, parquet flooring and paper production (Hanum & Maesen, 1997). Nowadays, it is fully occupied as a feedstock and biomass to generate energy and to manufacture various potential products. It has an extremely high wood density (500-600 kg/m3) and energy value (19250 kJ/kg), and thus is able to generate energy to support and run the factories in Kertih Biopolymer Park, Terengganu and replace the use of fossil fuel (Hanum & Maesen, 1997; Malaysian Biotechnology Corporation, n.d; *López et al.*, 2008). In addition, cooperation between Terengganu State Government, BiotechCorp, and ECERDC had developed the Asia's largest biorefinery complex in objective to utilize *L. leucocephala* integrated with different advance technologies to produce a variety of valuable products. One of the main potential products produced from *L. leucocephala* is L-methionine, which is produced through fermentation process. L-methionine, an amino acid which has antioxidant properties is made possible as a nutritional additive in the diet supplement industry (Heng, 2013).

2.2 Conventional Propagation and Biotechnological Approach

L. leucocephala can be propagated by either using seeds (direct sowing of seeds in the field) or vegetative methods. However, Pandey and Kumar (2013) reported that only 50 -60% seeds are germinated using this conventional propagation method. Furthermore, the seedlings grow very slow in the first three months and are susceptible to seedlings fungal, *Fusarium semitectum* and *Phytophthora drechsleri*, which is responsible for the main loss of *L. leucocephala* seedlings (Ong, 2008). Besides that, *L. leucocephala* is a lowland growing species, at which lowland is mostly a phosphorus deficient soils. According to Slot *et al*. (2012), seed germination and plant growth are largely depend on two macronutrients, nitrogen and phosphorus. Thus, *in vivo* propagation method does not promise a high return of seed germination and seedling growth.

Since the previous method is not very suitable, a more appropriate method of propagation through *in vitro* is developed. *In vitro* propagation has more advantageous as it provides aseptic conditions and more complete nutrients for optimum seed germination and seedlings growth. In addition, seed pre-treatment can also be applied and factors affecting the plant development can be adjusted, for instance, light, humidity, temperature and nutrient. *In vitro* propagation method is weather and seasonal independent and able to yield the required quantity of seedlings in a targeted time and does not require extensive labor for weeding and watering (George *et al*., 2008).

2.3 *In Vitro* seed germination and seedling growth

Seeds are chosen to be an explant for propagation as they can maintain the viability after stored for long periods, easily distributed, and are able to be produced in huge quantities so the seedlings regenerated from them are individually low cost (George *et al*., 2008). According to Springfield (1968), seeds were considered germinated only when cotyledons