UNIVERSITI PUTRA MALAYSIA

IN VITRO PROPAGATION OF *Eurycoma longifolia* JACK AND COMPARISON OF GENETIC FIDELITY AND ANTIOXIDANT ACTIVITY IN *IN VIVO* PLANT

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FBSB 2020 4

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By

ANNOR GEBRIL ANNOUR ALTTAHER

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chairman : Associate Professor Noor Azmi Shaharuddin, PhD
Faculty : Biotechnology and Biomolecular Sciences

Eurycoma longifolia Jack or Tongkat Ali as locally known in Malaysia is traditionally used as aphrodisiac and health supplement for various diseases. Due to its low rate germination, poor flowering, and its potential commercial value as a plantation crop as well as to conserve its germplasm, it is necessary to establish a suitable protocol of *in vitro* propagation as a better alternative for mass production of true-to-type plants. Hence, this study was conducted to develop an efficient protocol for Tongkat Ali micropropagation. Specific objectives were to induce *in vitro* adventitious shoot from the leaf and cotyledon explants and production of *in vitro* root biomass, to investigate the effect of different concentration of cytokinins on formation of multiple shoot from cotyledonary node explants, to assess the genetic fidelity of *in vitro* propagated plantlets, and to determine the total antioxidant content in *in vitro* and *in vivo* root extracts. Leaf and cotyledon explants were excised from *in vitro* seedlings for shoot regeneration; either indirectly through the callus or directly from the explant. The results showed the highest frequency of callus induction was 100% obtained from leaf explants on Murashige and Skoog (MS) medium supplemented with 1mg/L 2,4-dichlorophenoxy acetic acid (2,4-D) followed by 90% and 80% with 3.0 mg/L Dicamba and 1.0 mg/L Naphthaleneacetic acid (NAA) respectively. On the contrary, cotyledon explants produced the lowest percentage of callus at the all concentrations of auxins tested compared to leaf explants. Additionally, shoots were directly regenerated from leaf and cotyledon explants. The results revealed that 1.5 mg/L of 6-benzylaminopurine (BAP) gave an excellent response in shoot regeneration from both explants with an average of 1.8 ± 0.5 and 2.2 ± 0.4 shoots per explant respectively. On the other hand, root biomass was successfully produced from leaf explants in half-strength MS liquid medium containing various concentrations of indole-3-butyric acid (IBA) in combination with NAA. The result indicated that the highest fresh weight of root biomass was 9.18 ± 0.1g/L in a medium contained 0.5 mg/L IBA + 0.5 mg/L NAA within 6 weeks of culture. However, formation of multiple shoots was achieved by using cotyledonary node as an explant. The highest number (3.53 shoot per explant) obtained on MS medium

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supplemented with 1.0 mg/L BAP after 3 weeks. Besides, optimum rooting (3.20 roots per shoot) was achieved in half-strength MS medium containing 0.1 mg/L IBA. Platelets were successfully acclimatized to *ex vitro* conditions with 85% of survival percentage. Simple Sequence Repeat (SSR) and Inter Simple Sequence Repeat (ISSR) markers were tested to assess the genetic fidelity of the *in vitro* raised clones of *E. longifolia.* Out of the 12 SSR primers screened, nine primers produced 15 amplicons (1.7 bands in average) ranging from 100 to 800 bp, whereas eight ISSR primers generated 27 bands with an average of 3.4 bands ranging between 300 to 1000 bp. The monomorphic banding pattern confirmed the clonal homogeneity of the tissue culture-raised *E. longifolia* plantlets and reliability of the multiplication system used. Furthermore, different solvents were used for root extraction of *in vitro* and *in vivo* plants to determine the total antioxidant activity, total phenolic and flavonoid contents. Results of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging method showed that *in vitro* root extract at 25°C exhibited maximum antioxidant activity with 0.114 mg TE/g DW. Meanwhile, Ferric Reducing Antioxidant Potential (FRAP) showed the highest antioxidant content with 0.075 mg TE/g DW in *in vivo* root extracts at 25ºC. On the other hand, *in vivo* root extracts with 0.09 mg GAE/g DW had maximum phenolic content at 4°C. Whereas, *in vitro* root extract at 25°C showed the highest total (0.31 mg GAE/g DW and 0.10 mg RE/g DW) of both polyphenol and flavonoid content respectively. From this study, the successful *in vitro* propagation of *E. longifolia* could provide a potential of large-scale production of planting materials in meeting the industrial and domestic demands.

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PROPAGASI *IN VITRO Eurycoma longifolia* JACK DAN PERBANDINGAN FIDILITI GENETIK DAN AKTIVITI ANTIOKSIDAN DENGAN TUMBUHAN *IN VIVO*

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Eurycoma longifolia Jack atau di Malaysia dikenali sebagai Tongkat Ali, secara tradisinya digunakan sebagai suplemen afrodisiak dan kesihatan kepada pelbagai penyakit. Oleh kerana kadar percambahannya yang rendah, kurang penghasilan bunga dan potensi nilai komersilnya sebagai tanaman perladangan serta untuk memelihara plasma germanya, adalah perlu untuk membangunkan protokol yang sesuai dalam pembiakan *in vitro* sebagai alternatif untuk pengeluaran besar-besaran. Oleh itu, kajian ini dijalankan untuk membangunkan protokol mikropropagasi Tongkat Ali. Objektif spesifik adalah untuk meransang pertumbuhan pucuk secara *in vitro* dari eksplan daun dan kotilidon serta pengeluaran biojisim akar secara *in vitro*, untuk mengkaji kesan kepekatan sitokinin yang berlainan pada percambahan pucuk dari eksplan daripada nod kotilidon, untuk menilai fidiliti genetik dalam tumbuhan propagasi *in vitro*, dan untuk menentukan jumlah kandungan antioksidan dalam ekstrak akar *in vitro* dan *in vivo*. Eksplan daun dan kotilidon telah dikeluarkan dari anak benih *in vitro* untuk regenerasi pucuk; sama ada secara tidak langsung melalui kalus atau langsung dari eksplan tersebut. Hasil eksperimen menunjukkan frekuensi tertinggi induksi kalus adalah 100% diperolehi daripada eksplan daun di dalam medium Murashige dan Skoog (MS) yang ditambah dengan asid asetik 1mg / L 2,4-dichlorophenoxy (2,4-D) diikuti oleh 90% dan 80% dengan 3.0 mg / L Dicamba dan 1.0 mg / L asid Naphthaleneacetic (NAA). Sebaliknya, eksplan kotilidon menghasilkan peratusan kalus yang paling rendah pada semua kepekatan auksin yang diuji berbandingkan dengan eksplan daun. Selain itu, pucuk dihasilkan secara langsung daripada eksplan daun dan kotilidon. Sebanyak 1.0 mg / L 6-benzylaminopurine (BAP) memberikan tindak balas yang sangat baik dalam regenerasi pucuk dari kedua-dua eksplan dengan purata masing-masing menghasilkan sebanyak 1.8 ± 0.5 dan 2.2 ± 0.4 pucuk setiap eksplan. Biojisim akar juga telah berjaya dihasilkan daripada eksplan daun di dalam medium cecair MS kekuatan separuh yang mengandungi pelbagai kepekatan asid indole-3-butyric (IBA) dan NAA. Keputusan menunjukkan bahawa berat biojisim akar tertinggi adalah 9.18 ± 0.1g / L dalam medium mengandungi 0.5 mg / L IBA + 0.5 mg / L NAA yang dikultur selama 6 minggu. Walau bagaimanapun,

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pembentukan pelbagai pucuk telah dicapai dengan menggunakan nod kotilidon sebagai eksplan. Ia menghasilkan jumlah 3.53 pucuk bagi setiap eksplan yang dicambah di dalam media MS yang ditambah dengan 1.0 mg / L BAP selepas 3 minggu. Selain itu, penghasilan akar yang optimum (3.20 akar setiap pucuk) dicapai dalam medium MS kekuatan separuh yang mengandungi 0.1 mg / L IBA. Tumbuhan ini telah berjaya disesuaikan dengan keadaan *ex vitro* dengan 85% peratusan hidup. Penanda *Simple Sequence Repeat* (SSR) dan penanda *Inter Simple Sequence Repeat* (ISSR) telah diuji untuk menilai fidiliti genetik klon *E. longifolia* yang dihasilkan secara *in vitro*. Daripada 12 pencetus SSR, sembilan pencetus menghasilkan 15 amplikon (1.7 garisan secara purata) dari yang bersaiz 100 hingga 800 bp, manakala lapan pencetus ISSR menghasilkan 27 garisan dengan purata 3.4 garisan yang bersaiz antara 300 hingga 1000 bp. Corak perbandingan monomorfik telah mengesahkan homogeniti klon tumbuhan *E. longifolia* dari kultur tisu yang dihasilkan dan kebolehpercayaan sistem multiplikasi yang digunakan. Selain daripada itu, pelarut yang berbeza telah digunakan untuk pengekstrakan akar tumbuhan *in vitro* dan tumbuhan *in vivo* untuk menentukan jumlah aktiviti antioksidan, jumlah kandungan fenolik dan flavonoid. Hasil kaedah 1,1-diphenyl-2- picrylhydrazyl (DPPH) menunjukkan bahawa ekstrak akar *in vitro* pada 25°C menunjukkan aktiviti antioksidan maksimum dengan 0.114 mg TE / g DW. Sementara itu, kaedah potensi penurunan ferik (FRAP) menunjukkan kandungan antioksidan tertinggi dengan 0.075 mg TE / g DW dalam ekstrak akar *in vivo* pada 25ºC. Sebaliknya, dalam ekstrak akar *in vivo* dengan 0.09 mg GAE / g DW mempunyai kandungan fenolik maksimum pada 4°C. Manakala ekstrak akar *in vitro* pada 25°C menunjukkan jumlah kandungan polifenol dan flavonoid tertinggi masing-masing (0.31 mg GAE / g DW dan 0.10 mg RE / g DW). Dari kajian ini, penghasilan *E. Longifolia* secara *in vitro* telah Berjaya dilakukan dan berpotensi untuk menghasilkan pengeluaran bahan-bahan penanaman secara besar-besaran dalam membantu memenuhi permintaan industri dan domestik.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
CTAB	Hexadecyltrimethylammonium bromide
DW	Dry weight
FW	Fresh weight
g	Gram
IBA	Indole-3-butyric acid
In vitro	In glass (test tube)
Kin	Kinetin
mg/L	Milligram per litre
mL	millilitre
MS	Murashige and Skoog
NAA	1-Naphthylacetic acid
NaOH	Sodium Hydroxide
EDTA	Ethylenediaminetetraacetic acid
nm	nanometer
P	Probability
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
PGRs	Plant growth regulators
S.E.	Standard Error
TDZ	Thidiazuron
v/v	Volume per volume
w/v	Weight per volume
x g	Times gravity
µl	Microliter
µM	Micro molar

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CHAPTER 1

INTRODUCTION

1.1 *Eurycoma longifolia*: An introduction

Plants are part and parcel of human beings' life; it is essential components of existence of humanity. Through the mankind history, different plant materials have been used for treating many diseases and enhance health. Plants have been known as an important depot of medicinal properties for thousands of years (Alsarhan et al., 2014; Mustafa et al., 2017). Over 21,000 plant species listed by the World Health Organization (WHO) are used for medicinal purpose around the world. Malaysia has been identified as one of the 12 mega-biodiversity countries in the world (Talaat et al., 2013; Abdullah et al., 2015). Numbers of medicinal plant species are rare or threatened with extinction and people are still relying on these plants as traditional remedies. Therefore, it is important for its germplasm to be conserved (Ekor, 2014).

Eurycoma longifolia Jack is one of the medicinal plants that have become medically targeted by local communities due to its aphrodisiac properties. *E. longifolia* belongs to the family Simaroubaceae, is one of the important medicinal plants that are found in the tropical forest of South-East Asia. It is commonly known by its vernacular name 'Tongkat Ali' in Malaysia (Low et al., 2013). Due to its diverse medicinal values, every part of the plant, especially the root is traditionally used as medicine (Rahmawati & Esyanti, 2014; Yahya et al., 2015). The roots are also prepared as additives in health supplements and beverages, e.g. isotonic drink, coffee and tea, to increase virility, libido and sexual prowess (Teh et al., 2010; Meng et al., 2014; Mohamed et al., 2015).

Phytochemical studies of *Eurycoma longifolia* have identified various chemical compounds in the root such as quassinoids, eurycomanone, eurycomaoside, eurycolactone, eurycomalactone, euryconolactone and an alkaloid, 9- thoxycanthin- 6-one (Lulu et al., 2015; Mohamed et al., 2015). These chemical compounds have been reported to have effective medicinal values in sexual enhancement property for males, as well as antipyretic, antimalarial, antibacterial and antitumor properties (Rahmawati & Esyanti, 2014; Rehman et al., 2016). In addition, many studies had proven that the *E. longifolia* has effectiveness as general health, aphrodisiac property, anti-malaria, anti-ulcer, anti-microbial and cytotoxic activities (Park et al., 2014).

*Eurycoma longifoli*a (Tongkat Ali) was most commonly propagated using their seeds that were produced once a year and had the lowest percentage of germination furthermore, the seed took a long time to germinate due to the extremely immature state of the zygotic embryo at the time of dispersal (Danial et al., 2011; Rosmaina et al., 2016). Like other woody plants, proliferation of Tongkat Ali through the seed germination was difficult due to the low rate of seed germination and slow growth, as well as the

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unreliability flowering habit and quick loss of viability. Furthermore, Tongkat Ali roots were harvested after 4-7 years of cultivation, so the production of its roots is time-consuming, and fluctuated depending on the seasons (Darus, 2012).

As the demand of Tongkat Ail roots increases, this species had become endangered and is scarcely available on the forest fringe (Bhat & Karim, 2010; Abubakar et al., 2017). Recently Tongkat Ali has been declared a protected plant in most of the countries, including Malaysia, and its harvesting is highly restricted in nature. Meanwhile, the traditional cultivation method of Tongkat Ali propagation has not been able to supply the raw material used in pharmaceutical industry and an alternative production method is required to meet that growing demand (Lulu et al., 2015). Since the conventional propagation via seed germination was the most common method, albeit with a low rate of seed germination; therefore, plant tissue culture technique provides an alternative solution for mass production of Tongkat Ali plants which could be performed efficiently in a laboratory environment (Danial et al., 2011). Tissue culture is a technique for growing any explant such as; organs, tissues, and even cells under sterile *in vitro* environment to regenerate complete plantlets that are identical with stock plant (Varshney, 2014; Ikenganyia et al., 2017). This technique could overcome many agricultural issues that could not be resolved by using traditional propagation methods, including limited space, natural disasters such as floods, droughts, pests, and disease attacks. In addition, transformed plants could also be produced via this *in vitro* culture technique while valuable secondary metabolites could be accumulated in a controlled condition (Karuppusamy, 2009; Filová, 2014; Suman, 2017).

Based on literature review, several studies were conducted on the Tongkat Ali plant, especially, for pharmaceutical properties of its biological compounds. However, *In vitro* culture propagation research for improving the quality and production of Tongkat Ali has yet to be sufficient to meet the pharmaceutical industry demand. This emphasizes an urgent need to establish an efficient micropropagation system for high-quality plant production, in order to prevent this species from extinction. Hence, this current study was conducted to develop an efficient protocol for direct and indirect Tongkat Ali shoot regeneration. One of the major requirements of micropropagation was a production of uniform plantlets, but sometimes this could be hampered due to genetic variability. Therefore, in this research, molecular markers such as Simple Sequence Repeats (SSR) and Inter Simple Sequence Repeats (ISSR) were used to assess the genetic uniformity of the Tongkat Ali plantlets. Furthermore, different solvents were used to evaluate the antioxidant activity, flavonoid and total phenolic contents of *in vitro* roots and compare with a result obtained from wild plant. By using different solvent extractions, the content of total antioxidant from root extracts of *in vivo* and *in vitro* plant would be varied depending on its polarity and other factors and produce different flavonoid content.

1.2 Research Objectives

This study was generally conducted to establish an efficient *in vitro* regeneration protocol to produce high-quality Tongkat Ali plantlets.

1. To induce adventitious shoot from *in vitro* seedling explants and adventitious root biomass from *in vitro* plantlet explants.
2. To investigate the effect of cytokinins at different concentrations on multiple shoots formation from cotyledonary node explants.
3. To assess the clonal fidelity of micropropagated plantlets using SSR and ISSR markers.
4. To evaluate the total antioxidant content in *in vitro* and *in vivo* roots.

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PUBLICATIONS

Conferences and symposiums attended

Poster Presenter

Alttaher, A. G. A., Yusof, Z. N. B., Mahmood, M. & Shaharuddin, N. A. (2015). Plant Regeneration by *In vitro* Shoot Proliferation from Cotyledonary Node Explants of Tongkat Ali (*Eurycoma longifolia*). 2nd International Conference on Crop Improvement Sustainability Through Leading – edge Technology.2-3 December 2015. Auditorium Jurutera, Faculty of Engineering, Universiti Putra Malaysia, UPM Serdang, Selangor.

Journal Publication

Research articles

Alttaher, A. G. A., Yusof, Z. N. B., Mahmood, M. & Shaharuddin, N. A. (2020). HIGH-FREQUENCY INDUCTION OF MULTIPLE SHOOTS AND PLANT REGENERATION FROM COTYLEDONARY NODE EXPLANTS OF TONGKAT ALI (EURYCOMA LONGIFOLIA JACK). Accepted

Alttaher, A. G. A., Yusof, Z. N. B. & Shaharuddin, N. A. (2020). Direct Shoot Organogenesis of Tongkat Ali (*Eurycoma longifolia*) and Confirmation of the Clonal Fidelity using Simple Sequence Repeat and Inter Simple Sequence Repeat Markers. Submitted

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