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PROTOCOL DEVELOPMENT FOR MICROPROPAGATION OF Phyllanthus niruri linn. *(DUKUNG ANAK) VIA* IN VITRO *CULTURE*

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By

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PROTOCOL DEVELOPMENT FOR MICROPROPAGATION OF *Phyllanthus niruri* Linn. (DUKUNG ANAK) VIA *IN VITRO* CULTURE

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Chairman : Mohd Hakiman Mansor, PhD
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Phyllanthus niruri, commonly known as Dukung Anak or Gale of Stone, is an herbaceous plant belonging to the family Phyllanthaceae that has commonly been distributed in tropical countries, including Malaysia. Even though this plant is considered a problematic weed for farmers, it has been used traditionally to treat various ailments such as diabetes, flu, and cough. Overexploitation in Malaysia, on the other hand, has resulted in wide destruction of this plant from its natural habitat. Besides, there is no information on the production of *P. niruri* in Malaysia. This study has provided a solution to this problem through *in vitro* technology approach. Specifically, this study's objectives were to study the growth efficiency of *P. niruri* with different surface sterilants, to study *in vitro* growth responses of *P. niruri* towards different growth factors, and to determine the factors affecting *in vitro* rooting and acclimatization of *P. niruri*. *P. niruri* explant's nodal segment was prepared for surface sterilization using Clorox® and nanosilver with 10, 20, 30 (% and ppm, respectively). The aseptic culture was established with the lowest percentage of contamination, 4.44% using 30% Clorox®. The explant from the previous experiment was multiplied by different plant growth factors, which were different types of basal media and its strength, different cytokinin and its concentrations, different carbon sources and its strength, and different initial pH of basal media. Murashige and Skoog (MS) media and Gamborg B5 (B5) with the strength of half, full and double were used in the first experiment. Full-strength MS media produced a decent number of shoots with 2.33 shoots, 3.11 cm length of shoots and 27.91 number of leaves. In a subsequent experiment, MS media was supplemented with different types of cytokinin, which were 6-benzylaminopurine (BAP), kinetin (Kn), zeatin (Zn), and 2-isopentenyl adenine (2iP) with different concentrations of 2.5, 5.0, 7.5, and 10 µM. The best treatment was obtained from MS media without cytokinin

(control), which produced 5.00 number of shoots, 3.68 cm length of shoot, and 27.33 number of leaves. In the next experiment, basal media were manipulated with different carbon sources like sucrose, fructose, and glucose at different concentrations of 20, 25, 30, 35, and 40 g/L. The result showed the explant in media enriched with 30 g/L of sucrose has the highest number of shoots with an average of 3.2 shoots and shoot elongation of 2.27 cm. Lastly, the initial pH media was tested at 5.2, 5.4, 5.6, 5.8, 6, and 6.2. The highest number of shoots was recorded at a pH level of 5.6 (2.93). In the last experiment, the explant was used for root induction using different auxins at different concentrations. There were indole-3-butyric acid (IBA), 1-naphthalene acetic acid (NAA), and indole-3-acetic acid (IAA) at 1.25, 2.5, and 5 µM, respectively. Media with 2.5 µM IBA produced the highest number of the shoot and the most extended length of the root with 17.92 shoots and 1.29 cm root length. Lastly, the explant was acclimatized *ex vitro* in different potting media; peat moss: cocopeat, peat moss: vermiculite, peat moss: perlite at a ratio of 1:1 w/w and mixed of all media peat moss: cocopeat: vermiculite: perlite at a ratio of 1:1:1:1 w/w. The plantlet in potting media consisting of peat moss: cocopeat (1:1 ratio) showed the best performance with 88% plant survival. In conclusion, protocol for micropropagation of *P. niruri* has been developed in this study.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

PEMBANGUNAN PROTOKOL BAGI MIKROPROPAGASI *Phyllanthus niruri* Linn. (DUKUNG ANAK) MELALUI KULTUR *IN VITRO*

Oleh

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Phyllanthus niruri, dikenali sebagai Dukung Anak atau *Gale of Stone* ialah satu pokok herba dari keluarga Phyllanthaceae yang kebanyakan tumbuh di negara tropika termasuk Malaysia. Walaupun pokok ini dikenali sebagai rumpai yang mengundang masalah di kalangan petani, pokok ini telah digunakan secara tradisional untuk merawat pelbagai penyakit seperti kencing manis, selesema dan batuk. Oleh kerana pokok ini telah dieksploitasi secara berlebihan, ia menyebabkan kemusnahan pokok ini di alam semulajadi. Tambahan pula, tiada sebarang maklumat mengenai pengeluaran herba ini di Malaysia. Justeru, kajian ini dicadangkan untuk menyelesaikan masalah berkenaan dengan menggunakan pendekatan kaedah teknologi *in vitro*. Secara khasnya, matlamat kajian ini adalah untuk mengkaji kecekapan pertumbuhan *P. niruri* dengan pensteril permukaan yang berbeza, untuk mengkaji tindak balas pertumbuhan secara *in vitro P. niruri* terhadap faktor pertumbuhan yang berbeza dan untuk menentukan faktor yang memberi kesan terhadap pengakaran *in vitro* dan aklimatisasi pokok *P. niruri*. Segmen nod pokok *P. niruri* telah digunakan untuk pensterilan permukaan menggunakan Clorox® dan nanosilver dengan kepekatan 10, 20, 30 (% dan ppm, masing-masing). Kultur aseptik telah dihasilkan dengan peratusan kontaminasi yang terendah, 4.44% menggunakan 30% Clorox®. Eksplan daripada eksperimen sebelumnya digandakan dengan faktor tumbesaran tumbuhan yang berbeza iaitu jenis media basal dan kepekatannya yang berbeza, sitokinin dan kepekatannya yang berbeza, sumber karbon dan kepekatannya yang berbeza dan nilai pH awal media basal yang berbeza. Di dalam eksperimen pertama, media Murashige and Skoog (MS) dan Gamborg B5 (B5) dengan kepekatan separuh, penuh dan seganda telah digunakan. Hasil kajian menunjukkan media MS dengan kepekatan penuh menghasilkan bilangan pucuk iaitu 2.33 pucuk, 3.11 sm kepanjangan pucuk dan 27.91 jumlah daun. Dalam eksperimen seterusnya, media MS telah dibekalkan

dengan jenis sitokinin yang berbeza iaitu 6-benzilaminopurina (BAP), kinetin (Kn), zeatin (Zn) dan 2-isopentenil adenina (2iP) dengan kepekatan yang berbeza iaitu 2.5, 5.0, 7.5 dan 10 µM. Hasil terbaik yang didapati ialah daripada media MS yang dibekalkan tanpa sebarang sitokinin (kawalan), iaitu menghasilkan 5.00 bilangan pucuk dan menghasilkan 3.68 sm panjang pucuk bersama 27.33 bilangan daun. Dalam eksperimen berikutnya, media basal dimanipulasi dengan sumber karbon yang berbeza seperti suckrosa, fruktosa dan glukosa pada kepekatan yang berbeza iaitu 20, 25, 30, 35 dan 40 g/L. Keputusan mendapati bahawa eksplan di dalam media yang dibekalkan dengan 30 g/L sukrosa mempunyai bilangan pucuk tertinggi dengan nilai purata 3.2 pucuk dengan kepanjangan pucuk yang tertinggi iaitu 2.27 sm. Akhirnya, nilai awal pH bagi media basal telah dikaji pada nilai 5.2, 5.4, 5.6, 5.8, 6 dan 6.2. Bilangan pucuk yang tertinggi dicatatkan pada nilai pH 5.6 iaitu 2.93 pucuk. Pada eksperimen terakhir, eksplan digunakan untuk induksi akar menggunakan jenis auxin dan kepekatannya yang berbeza. Terdapat asid indol-3-butirik (IBA), asid 1-naftalenaasetik (NAA) dan asid indol-3-asetik (IAA) pada 1.25, 2.5 dan 5 µM, masing-masing. Media yang mengandungi 2.5 µM IBA menghasilkan bilangan pucuk dan kepanjangan pucuk yang tertinggi iaitu 17.92 dan 1.29 sm, masing-masing. Seterusnya, eksplan diaklimatisasi secara *ex vitro* di dalam media pengerasan berbeza; lumut gambut: gambut coco, lumut gambut: vermikulit, lumut gambut: perlit pada nisbah 1:1 dan campuran ke semua media lumut gambut: gambut coco: vermikulit: perlit pada ratio 1:1:1:1. Pokok di dalam media yang mengandungi lumut gambut: gambut coco (ratio 1:1) menunjukkan prestasi yang terbaik dengan 88% peratus hidup. Kesimpulannya, protokol untuk membiakkan *P. niruri* secara mikropropagasi telah dibangunkan dalam kajian ini.

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

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2,4-D	2,4-dichlorophenoxy-acetic acid
2iP	6-(γ,γ-Dimethylallylamino) purine
ANOVA	Analysis of variance
B5	Gamborg B5
BA	Benzyladenine
BAP	6-benzylaminopurine
CRD	completely randomized design
HCl	hydrochloric acid
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
Kinetin	6-Furfurylaminopurine
LSD	least significant difference
MS	Murashige and Skoog
NAA	naphthalene-acetic acid
NaOCl	sodium hypochlorite
NaOH	sodium hydroxide
NS	Nano silver
PGRs	Plant growth regulators
RAPD	random amplified polymorphic DNA
RH	relative humidity
UPM	Universiti Putra Malaysia
Zeatin	6-(4-hydroxy-3-methylbut-2-enylamino) purine

CHAPTER 1

INTRODUCTION

1.1 Background of study

Over the past several years, medicinal plants have gained greater attention and recognition in Malaysia, particularly as demand for alternative medicine and natural health products increases. A total of 351 hectares of Malaysian land has yielded 1317 metric tonnes of herbs in 2007. Since then, Malaysia's cultivated herbaceous area has grown from 1,198 hectares in 2011 to 2317 hectares in 2017, an annual increase of roughly 14% (Department of Agriculture, 2017). The Malaysia Economic Transformation Program, through the National Key Economic Areas Agriculture sector, has identified numerous high potential herbs that can be commercially utilized as a new economic growth source in the herbal industry. One of these herbs is *P. niruri*, identified locally as Dukung Anak (Farizah et al., 2015).

Phyllanthus niruri (Phyllanthaceae) has traditionally been used to treat several ailments such as jaundice, kidney stone, flu, fever, and diabetes (Unander and Blumberg, 1991; Calixto et al., 1998; Karthikeyan et al., 2008). Several researchers discovered that *P. niruri* possesses anti-tumor, anti-oxidant, anti-carcinogenic, and hepatoprotective properties (Chatterjee and Sil, 2006; Harish and Shivanandappa, 2006; Rajeshkumar et al., 2002). In addition, certain phenolic compounds such as gallic acid, epicatechin, gallocatechin, epigallocatechin, epicatechin 3-O-gallate, epigallocatechin 3-O-gallate have gained significant interest from the researcher (Ishimaru et al., 1992). Furthermore, a novel compound named niruriside was isolated from methanol extract of *P. niruri* that are capable of inhibiting the binding of human immunodeficiency virus Rev protein (HIV REV) (Qian-Cutrone et al., 1996). These secondary metabolites have a wide range of therapeutic activities that have essential pharmacological effects on humans. Hence, many products were made from this medicinal plant have been successfully commercialized.

The main issue for the commercialization of herbal-based products is the uniformity and consistency of the material. In contrast, the growing demand for medicinal plants would undoubtedly decrease the sustainable supply of raw materials in the future. This might occur due to overharvesting and unsustainable agriculture practices, which lead to many restrictions, such as a limited supply of herbs. Moreover, to extract elevated amounts of secondary metabolites, large quantities of raw materials are expected.

Plant micropropagation may also serve as an alternative solution to ensuring the sustainable supply of plant materials as it is an excellent technique to produce plants in a large amount in a short period. For example, 10000 *Ornithogalum* plants could be grown from a single leaf in eight months using micropropagation, instead of the conventional method that takes ten years to collect the same number (Thiart, 2003). Micropropagation is the *in vitro* aseptic culture of cells, tissues, or even organs of plants grown under a controlled environment and nutrient media for growth and multiplication. This technology can be used to eliminate diseases, secondary metabolite production, plant improvement, and conservation of threatened and endangered species (Hussain et al., 2012). Plant micropropagation consists of four major stages 1) surface sterilization of explant, 2) induction and multiplication of shoot, 3) *in vitro* root induction, and 4) acclimatization. The correct protocol should therefore be used to perform the plant micropropagation technique.

Appropriate selection of chemical sterilization is essential in the first stage of plant micropropagation. All microorganisms that potentially could contaminate the culture should be removed and eliminated. This step will establish an aseptic culture to be carried out in the following phase of micropropagation. On the other hand, numerous factors affect the explant's development in the growth and multiplication phase (Read, 2007). For instance, the type of basal media and its concentrations (Abobkar and Elshahed, 2016), type of carbon source (Sul and Korban, 2004), and its concentrations and initial pH value of culture medium also play an essential role in influencing the shoot induction of *P. niruri* explant. Culture medium supplemented with plant growth regulators; cytokinin and auxin were used in many plants to propagate via *in vitro* techniques (Preil, 2003). Commonly, cytokinin contributes to shoot induction (Schaller et al., 2014), whereas auxin helps in root induction (Woodward and Bartel, 2005), both functioning by promoting cell division and differentiation (Trigiano and Gray, 2011). *In vitro* plantlets will then be transferred to potting media during acclimatization, the final stage. In this stage, the survival rate of plantlets is also influenced by the type of potting media.

1.2 Problem statement

Due to its high medicinal values and properties, *P. niruri* has captured the attention of people all over the world. This plant is harvested with minimal commercialized production, and no sanitary consideration is taken on the sample collected. Besides, the Herbs and Spices Statistic in Malaysia stated that the total production of local herbs was 2312.42 Mt however, there is no information on the production of *P. niruri* (Jabatan Pertanian Malaysia, 2016). This suggests that the plant has not been thoroughly utilized for commercialization, especially in Malaysia. Herbs, particularly for medicinal

purposes, are in high demand right now. However, the herbal sector faced many obstacles due to lack of raw materials and knowledge in associated herbs. Thus, this study may propagate *P. niruri* rapidly and produce quality and safe *P. niruri* for human consumption. Hence, plant micropropagation is the most suitable approach to solve these problems.

1.3 Objectives of study

The general objective was to develop protocol for *in vitro* culture of *P. niruri* via micropropagation technique.

Besides, the specific objectives of this study were:

1. To determine the efficiency of different types and concentrations of chemical sterilants on surface sterilization of *P. niruri* explants.
2. To examine the growth responses on multiplication of *P. niruri* explant towards different growth factors.
3. To evaluate the effect of different types and concentrations of auxins on *in vitro* rooting and determine the suitable potting media for acclimatization of *P. niruri.*

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