UNIVERSITI PUTRA MALAYSIA

IN VITRO PROPAGATION OF Vanilla tahitensis Moore AND THE ROLE OF PLANT GROWTH PROMOTING RHIZOBACTERIA ON CROP SURVIVAL AND GROWTH IN THE FIELD

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By

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Vanilla is one of the most expensive spices after saffron and cardamon. Vanilla propagation is through stem cutting but this method is slow to induce shoot. *In vitro* propagation technology is a technique for easy propagation on a large scale and is quick to produce large number of planting materials.

In this study, *Vanilla tahitensis* Moore was used. Nodes from 5 months old seedlings of *V. tahitensis* Moore were used as explant and cultured in NDM + 0.2 activated charcoal. Explants were divided into two parts based on maturity, Explant 1 (1st to 4th node) and Explant 2 (5th node and below). Each part of the explant was sterilized in three treatments. The treatments were Sterilization 1 (60% Clorox®), Sterilization 2 (60% Clorox®, 40% Clorox®) and Sterilization 3 (60% Clorox®, 40% Clorox®, 20% Clorox®) at 15 minutes each. Parameters (% contamination-free, % survival, % shoot formation) were recorded after 30 days. Experiments on shoot regeneration (Experiment 1) and shoot multiplications (Experiment 2) were conducted using nodes from the *in vitro* derived shoot as explant. In Experiment 1, explant was cultured on NDM + 0.2% activated charcoal + Benzylaminopurine (BAP) (0 mg/L, 0.05 mg/L, 1 mg/L, 3 mg/L, 6 mg/L and 9 mg/L). Parameters were % shoot regeneration, days to shoot, the number of shoots per explant and shoot length, with all parameters recorded after 60 days of culture. In Experiment 2 hormone combination were used with BAP (0.5 and 1mg/L) + Naphtaleneacetic acid (NAA) (0, 0.5, 0.1, 0.2, 0.3mg/L). Data were recorded after 60 days including days to shoot development, number of shoots and shoot length. Thereafter, the new shoot was cut and cultured again in the new media with the same treatments. The number of shoots and shoot length were recorded after 60 days. For the experiment of rooting, nodes from the *in vitro* derived shoots were cultured in NDM (0.2% activated charcoal) +Indole-3-butyric acid (IBA) and NAA (0, 0.3, 1and 2 mg/L). Data on the number of roots

and roots length were recorded after 60 days of culture. In the next experiment, PGPR was used as a treatment, and plantlets around 6 months old were transferred to an *ex-vitro* environment (field). Plantlets were transferred to polybags and placed under 50% shading for 24 hours. Thereafter, plants were inoculated with 5 mL of bacterial suspension (UPMB10, *Pseudomonas sp.*, *Bacillus pumilus,* and *Paenibacillus sp.*) at a concentration of 10^8 cfu mL^{-1}. Data were recorded at 80 days after transplanting for percentage of survival, length of vines, number of nodes, length of internodes, number of leaves, length of leaves, width of leaves, number of aerial roots, length of aerial roots, number of roots, length of roots and stress measurement (POD and CAT).

In the sterilization experiment, the results showed no significant interaction between explant parts and treatment sterilization for all parameters, hence only single factor is discussed. The results of sterilization and the explant section for all parameters showed significant differences except for the shoots initiation. Sterilization protocol 3 and Explant 1 had the highest percentage for % contamination-free and survival. Subsequently, in Experiment 1 there were significant differences between BAP treatments on the number of days to shoot initiation and the length of the shoots. 1 mg/L BAP treatment was significantly quick in producing shoots and had the longest shoot length. Meanwhile, in experiment 2, the explant in the media produced only a single shoot and a combination of 1 mg/L BAP and 0.3 mg/L NAA produced the shoot in the shortest number of days. Meanwhile, explant in the media combination of 1 mg/L BAP and 0.2 mg/L NAA produced the longest shoot length. The result of the second culture shows that multiple shoot production is possible with the highest number of shoots obtained in the media combination of 1 mg/L BAP and 0.2 mg/L NAA. Result from the length of shoot showed that the explant in the media combination of 0.5 mg/L BAP and 0.3 mg/L NAA produced the longest shoot. For root experiments, the results of the number of roots showed no significant difference between the concentration of IBA and NAA. Meanwhile, root length measurements showed significant differences between concentrations of IBA and NAA. Media containing 1 mg/L of IBA produced the longest root compared to other treatments. Finally, field establishment of the seedling using PGPR, resulted in enhanced stem elongation, internode length, number of roots, root length, and POD activity with significant difference between treatments. Plants inoculated with UPMB10 had the longest internode while *B. pumilus* showed the highest results on the length of vine, the number of roots, root length, and POD activity. The results of the number of nodes, the number of leaves, the length of the leaf, the width of the leaf, the number of aerial roots, the number of roots, the root length, and the activity of the CAT showed no significant difference between the treatment.

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

PEMBIAKKAN *IN VITRO Vanilla tahitensis* MOORE DAN PERANAN PLANT GROWTH PROMOTING RHIZOBACTERIA KE ATAS KELANSUNGAN HIDUP DAN PERTUMBUHAN TANAMAN DI LAPANGAN

Oleh

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Vanilla adalah salah satu rempah yang paling mahal selepas safron dan pelaga. Pembiakkan vanila adalah melalui keratan batang tetapi kaedah ini lambat untuk menginduksi pucuk. Teknologi pembiakan *in vitro* adalah teknik pembiakan mudah untuk menghasilkan sebilangan besar bahan penanaman dalam skala besar dan cepat.

Dalam kajian ini, *Vanilla tahitensis* Moore digunakan. Nod dari anak benih *V. tahitensis* Moore berumur 5 bulan digunakan sebagai eksplan dan dikultur dalam NDM + 0.2 % arang aktif. Eksplan dibahagikan kepada dua bahagian berdasarkan kematangan, Explant 1 (nod 1 hingga 4) dan Explant 2 (nod ke 5 hingga ke bawah). Setiap bahagian eksplan disterilkan dalam tiga rawatan. Rawatannya adalah Sterilisasi 1 (60% Clorox®), Sterilisasi 2 (60% Clorox®, 40% Clorox®) dan Sterilisasi 3 (60% Clorox®, 40% Clorox®, 20% Clorox®) masing-masing pada 15 minit. Parameter (% bebas pencemaran,% kelangsungan hidup,% pembentukan pucuk) dicatatkan setelah 30 hari. Eksperimen pada regenerasi tunas (Eksperimen 1) dan pengandaan tunas (Eksperimen 2) dilakukan dengan menggunakan nod dari tunas *in vitro* sebagai eksplan. Dalam Eksperimen 1, eksplan dikultur pada NDM + 0,2% arang aktif + Benzylaminopurine (BAP) (0 mg / L, 0,05 mg / L, 1 mg / L, 3 mg / L, 6 mg / L dan 9 mg / L). Parameter adalah % regenerasi pucuk, hari untuk pucuk tumbuh, jumlah pucuk per eksplan dan panjang pucuk, dengan semua parameter dicatat setelah 60 hari kultur. Dalam Eksperimen 2 kombinasi hormon digunakan dengan BAP (0,5 dan 1mg / L) + Naphtaleneacetic acid (NAA) (0, 0,5, 0,1, 0,2, 0,3mg / L). Data direkodkan selepas 60 hari termasuk hari untuk pertumbuhan pucuk, jumlah pucuk dan panjang pucuk. Selepas itu, pucuk baru dipotong dan dikultur lagi di media baru dengan rawatan yang sama. Jumlah pucuk dan panjang pucuk dicatatkan setelah 60 hari. Untuk eksperimen pengakaran, nod

dari pucuk *in vitro* diletakkan dalam NDM (arang aktif 0.2%) + Asid Indole-3-butyric (IBA) dan NAA (0, 0.3, 1 dan 2 mg / L). Data mengenai jumlah akar dan panjang akar dicatatkan setelah 60 hari kultur. Dalam eksperimen berikutnya, PGPR digunakan sebagai perawatan, dan plantlet berusia sekitar 6 bulan dipindahkan ke lingkungan *ex-vitro* (ladang). Plantlet dipindahkan ke polibeg dan diletakkan di bawah naungan 50% selama 24 jam. Selepas itu, tanaman diinokulasi dengan 5 mL suspensi bakteria (UPMB10, *Pseudomonas sp., Bacillus pumilus*, dan *Paenibacillus sp*.) Pada kepekatan 10^8 cfu mL-1. Data direkodkan pada 80 hari selepas pemindahan untuk peratusan kelangsungan hidup, panjang pokok , jumlah nod, panjang nod, jumlah daun, panjang daun, lebar daun, jumlah akar udara, panjang akar udara, jumlah akar, panjang akar dan pengukuran 'stress' (POD dan CAT).

Dalam eksperimen pensterilan, hasilnya tidak menunjukkan interaksi yang signifikan antara bahagian eksplan dan pensterilan rawatan untuk semua parameter, oleh itu hanya satu faktor yang dibincangkan. Hasil pensterilan dan bahagian eksplan untuk semua parameter menunjukkan perbezaan yang signifikan kecuali untuk permulaan tunas. Protokol pensterilan 3 dan Explant 1 mempunyai peratusan tertinggi untuk % bebas pencemaran dan kelangsungan hidup. Selepas itu, dalam Eksperimen 1 terdapat perbezaan yang signifikan antara rawatan BAP pada jumlah hari untuk memulakan tumbuh pucuk dan panjang pucuk. Rawatan BAP 1 mg / L sangat cepat menghasilkan pucuk dan mempunyai pucuk terpanjang. Sementara itu, dalam eksperimen 2, eksplan dalam media hanya menghasilkan satu pucuk dan gabungan 1 mg / L BAP dan 0.3 mg / L NAA menghasilkan pucuk dalam jumlah hari terpendek. Sementara itu, eksplan dalam kombinasi media 1 mg / L BAP dan 0.2 mg / L NAA menghasilkan pucuk terpanjang. Hasil kultur kedua menunjukkan bahawa pengeluaran pucuk berganda adalah signifikan dengan jumlah pucuk tertinggi yang diperoleh dalam gabungan media 1 mg / L BAP dan 0.2 mg / L NAA. Hasil dari panjang pucuk menunjukkan bahawa eksplan dalam kombinasi media 0.5 mg / L BAP dan 0.3 mg / L NAA menghasilkan pucuk terpanjang. Untuk eksperimen akar, hasil bilangan akar tidak menunjukkan perbezaan yang signifikan antara kepekatan IBA dan NAA. Sementara itu, ukuran panjang akar menunjukkan perbezaan yang signifikan antara kepekatan IBA dan NAA. Media yang mengandungi 1 mg / L IBA menghasilkan akar terpanjang berbanding dengan rawatan lain. Yang terakhir, penanaman benih di lapangan menggunakan PGPR, menghasilkan pemanjangan batang , panjang internode, jumlah akar, panjang akar, dan aktiviti POD dengan perbezaan yang signifikan antara rawatan. Tumbuhan yang diinokulasi dengan UPMB10 mempunyai internode terpanjang sementara *B. pumilus* menunjukkan hasil tertinggi pada panjang batang, jumlah akar, panjang akar, dan aktiviti POD. Hasil bilangan nod, jumlah daun, panjang daun, lebar daun, jumlah akar udara, jumlah akar, panjang akar, dan aktiviti CAT tidak menunjukkan perbezaan yang signifikan antara rawatan.

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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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PGR	Plant growth regulator
BAP	6-Benzylaminopurine
NAA	1-Naphthaleneacetic acid
IBA	Indole-3-butyric acid
IAA	Indole-3-acetic acid
TDZ	Thidiazuron
NOA	Naphthoxy acetic
p-CPA	para-chlorophenoxy acetic acid
2-ip	isopentenyladenine
PLBs	Protocom like bodies
PGPR	Plant growth rhizobacteria
NDM	New Dogashima Medium
MS	Murashige and Skoog
DNA	Deoxyribonucleic acid
$HgCl_2$	Mercury (II) chloride
RCBD	Randomized complete block design
ANOVA	Analysis of variance
DMRT	Duncan's multiple range test
PPFD	Photosynthesis poton flux density
POD	Peroxidase
CAT	Catalase
ROS	Reactin oxygen species

cm	centimeter
mM	milliMolar
kg	kilogram
ATP	adenosine triphosphate
g/L	gram per litre
µmol m^{-2} s^{-1}	Micromole per second and square meter
cfu	Colony Forming Unit
nm	Nanometre
U/mg	Unit per miligram

CHAPTER 1

INTRODUCTION

The family orchidaceae is one of the largest family of flowering plant (Lubinsky *et al.,* 2008). This family consist of 880 genera with some 35 000 species distributed worldwide. Vanilla belongs to the *Orchidaceae* family, Vanilloideae sub-family, and Vanillinae tribe (Njoroge *et al.,* 2005); Cameron, 2011; Azofeifa-Bolaños *et al.,* 2017). This genus comprises about 110 species and is distributed in the tropical area of the world (Divakaran *et al*., 2006). Only three species of vanilla, namely *Vanilla planifolia Andrew, V. tahitensis* Moore, and *V. pompano* Schiede are commercially cultivated (Tan *et al*., 2011).

Interestingly, the above-mentioned vanilla produces an edible fruit or flavouring pods. Vanilla is one of the most expensive spice materials, known as the 'Prince of Spice' (Sasikumar, 2010). It is also the third most expensive spice in the world market after saffron and cardamom (Tan and Chin, 2015). A report from the International Trade Centre (ITC) showed that only 30% of the vanilla world market contains pure vanilla extract, while the remaining is synthetic vanilla extracts produced from chemical components (Zuraida *et al*., 2013). The demand for vanilla in the world market is very high, reaching 18000 metric tons per year (Utusan Malaysia, 28 January 2008). Vanilla is planted in Madagascar, Indonesia, India, Mexico, Reunion Islands, and The Comoro Islands, besides other countries such as Fiji, China, Guadeloupe, Malawi, French Polynesia, Tonga, Uganda, and Zimbabwe (Sasikumar, 2010).

Vanilla is mainly propagated through stem cuttings taken from mature vines, but this is a slow growth method and is labour intensive (Abebe *et al*., 2009). According to a farmer from Pahang, Malaysia (Ishak Musa, 2013), vanilla stem cutting requires 4 to 6 weeks to produce the shoot. This method also requires the use of mother plants as stock for plant material causing the loss in plants as the traditional culture of monopodial orchid such as vanilla requires the removal of the apical shoot, which retards the growth and development of the mother plant. Due to the limited production of the vanilla, propagation of vanilla plants via traditional culture is not sufficient to accommodate the demand for natural vanilla. Today, people prefer to use natural resources as pure natural vanilla, which contains not just vanillin but also other compounds. Thus, the natural vanilla is more valuable than its synthetic version, which only has vanillin compounds (Rao and Ravishankar, 2000).

To overcome the problem of insufficient production of planting materials, the tissue culture technique can be used to produce large number of plants in a shorter duration. The tissue culture technique has been widely used for the *in vitro* propagation of several commercially important orchids over the past decade. This technique used the totipotency nature of plant tissues (Gantait *et al.,* 2010). It has been used to grow and maintain plant cells, tissues, or organs

on a nutrient culture medium under clean and sterile conditions. Explant is an important component in the success of plant tissue culture. According to Tan *et al*. (2011), a number of parts of the vanilla plant can be used as explants, such as the node of stems, root tips, and shoot tips.

The use of appropriate explant on optimized medium plays an essential role in the development of plant growth. A medium contains plant growth regulator (PGR), singly or in combination and it acts as a simulator to produce adventitious shoots and roots under *in vitro* sterile environments. However, the type and concentration of PGR is often species specific and must be determined and provided to the plants to enhance growth efficiency (George et al., 2008).

Although micropropagation has the potential to produce large-scale planting material, however, a challenge faced is the difficulties in the plant to resume active growth after being transferred to the field. This phase is critical for propagating plants from *in vitro* to *ex vitro* cultivation (Muniz *et al.,* 2013). Significant losses can occur during acclimatization due to the plantlet's vulnerable physiological status (Vettori *et al.,* 2010). Therefore, the use of plant growth promoting rhizobacteria (PGPR) can help to overcome the difficulties during the establishment state upon transfer of the plants to the field and improve the survival rate.

PGPR is a beneficial bacteria that actively colonize plant roots and influences plant growth (Vacheron *et al.,* 2013). PGPR can produce iron-chelating siderophores, to fix nitrogen, to solubilize phosphates and other nutrients, to synthesize the auxin hormone (Indole-3-acetic acid), and to act as a biocontrol agent (Gupta *et al.,* 2015).

Some PGPR can improve plant enzyme activity which reduces the oxidative damage induced by environmental stress. When a plant is exposed to environmental stress such as drought, salinity, and temperature extreme after transplant from *in vitro* condition, the balance between the production of reactive oxygen species (ROS) and the quenching activity of antioxidants is disrupted, often resulting in oxidative damage (Kusvuran *et al.,* 2016). To control the level of ROS, plant tissues synthesize several enzymes such as Peroxides (POD) and Catalase (CAT) for scavenging ROS.

Thus, the objectives of this study were:

1. To determine a suitable sterilization protocol for *V. tahitensis* shoot explant.
2. To establish the type and concentration of plant growth regulator (PGR) for shoot regeneration, multiplication, and rooting.
3. To study the effect of plant growth promoting rhizobacteria (PGPR) on the growth of *V. tahitensis* under field condition.

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