

**EFFECTS OF SELECTED ADDITIVES FOR GROWTH  
EFFICIENCY ON *in vitro* GROWN**

***Vanilla planifolia* Andrews**

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**UNIVERSITI SAINS MALAYSIA**

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***Vanilla planifolia* Andrews**

**by**

**GOH FOONG JING**

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## LIST OF ACRONYMS AND ABBREVIATIONS

°C	Degree Celsius
\$/kg	Dollar per kilogram
%	Percent/percentage
2iP	6- $\gamma$ - $\gamma$ -dimethylaminopurine
ANOVA	Analysis of variance
BAP	6-benzylaminopurine
c	Chloroplast
cm	Centimeter
CRD	Completely random design
CW	Coconut water
ed	Endodermis layer
EDTA	Ethlenediaminetetra acetic acid
ep	Epidemic layer
FAA	Formaliine-acetic acid-alcohol
FESEM	Field emission scanning electron microscope
g/L	Gram per litre
HCL	Hydrochloric acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
kgf/cm <sup>2</sup>	Kilogram-force/square centimetre
LL	Leaves length
LN	Leaves number
M	Molar
mg/L	Milligram per litre

MS	Murashige and Skoog
n	Nucleus
N69	Nitsch basal medium
NAA	Naphthalene acetic acid
NaOH	Sodium hydroxide
N	Normality
nm	Nano metre
p	Phloem
PH	Plant height
PLBs	Protocorms-like bodies
r	Raphides
rh	Root hair
RL	Root length
RN	Root number
SEM	Scanning electron microscope
SN	Shoot number
st	Sieve tube
TBA	Tertiary-butyl alcohol
v	Vein
vb	Vascular bundle
v/v	Volume/volume

**KESAN BAHAN TAMBAHAN TERPILIH UNTUK KEBERKESANAN  
PERTUMBUHAN *Vanilla planifolia* Andrews YANG TUMBUH SECARA *in vitro***

**ABSTRAK**

*Vanilla planifolia* Andrews ialah ramuan makanan yang mempunyai nilai ekonomi dan kedua paling mahal di dunia. Dalam kajian yang lepas, bahan tambahan organik telah menunjuk potensi meningkatkan perkembangan dan pertumbuhan pokok tumbuhan tetapi kajian yang teliti terhadap *V. planifolia* terhad. Maka, dalam kajian ini, kesan bahan tambahan organik berbeza yang dipilih telah dikaji terhadap pertumbuhan dan perkembangan *V. planifolia* Andrews. Peningkatan protokol yang lebih baik untuk pertumbuhan tumbuhan dan perkembangan vanila telah dijalankan dan akhirnya mengkaji perbezaan morfologi anak pokok yang ditanam secara *in vitro* dan *ex vitro*. Pucuk *V. planifolia* dikultur dalam kekuatan medium MS yang berbeza, kepekatan BAP dan sukrosa yang berbeza, dan jenis ejen gel yang berlainan untuk mengkaji medium pertumbuhan yang terbaik. Pertumbuhan anak pokok *V. planifolia* direkodkan selepas dirawat selama lapan minggu. Hasil Kajian menunjukkan pembekalan  $\frac{1}{2}$  kekuatan medium MS dan 20 g/L sukrosa didapati cukup berkesan untuk menggalakkan pertumbuhan pokok. Penambahan 1.0 mg/L BAP dapat meningkatkan purata  $4.11 \pm 0.36$  pucuk setiap eksplan. Keempat-empat jenis ejen gel tidak menunjukkan kesan yang signifikan terhadap pertumbuhan *V. planifolia*. Sebanyak tujuh jenis bahan tambahan organik telah dipilih untuk mengkaji kesan setiap bahan tambahan organik terhadap pertumbuhan dan perkembangan pucuk eskplan. Penambahan 10% air kelapa ke dalam medium itu didapati paling berkesan untuk meningkatkan pertumbuhan pokok *V. Planifolia*. Dengan penambahan 30% air kelapa atau 2 g/L ekstrak daun teh hijau, penggandaan



anak pokok *V. Planifolia* menghasilkan keputusan yang agak sama dengan  $4.26 \pm 0.43$  (air kelapa) dan  $4.00 \pm 0.86$  (ekstrak daun teh hijau) pucuk setiap eksplan berbanding dengan medium yang ditambah dengan 1 mg/L BAP. Walau bagaimanapun, kombinasi 1 mg/L BAP dan 10 % air kelapa dapat meningkatkan penghasilan pucuk baru sebanyak 30%. Pertumbuhan akar *V. planifolia* dapat mencapai 100% tanpa penambahan hormon dan bahan tambahan organik. Lumut sphagnum didapati merupakan medium berpasu yang paling sesuai untuk aklimitasi *V. planifolia* dengan mencapai kadar kemandiran 83.33 %. Histologi dan imbasan mikroskop elektron telah digunakan untuk mengkaji perubahan morfologi *V. planifolia* di bahagian daun, batang dan akar yang ditanam secara *in vitro* dan *ex vitro*. Berdasarkan kajian histologi, perbezaan struktur dalam anak pokok *in vitro* dan anak pokok *ex vitro* telah ditunjuk. SEM menunjukkan stomata anak pokok *in vitro* yang kurang lengkap dan kewujudan lilin epikutikular disekeliling stomata. SEM juga menunjukkan kewujudan raphid dalam *V. planifolia*. Kajian ini telah menunjukkan potensi bahan tambahan organik yang kurang didedahkan sebagai penyelesaian alternatif untuk meningkatkan pertumbuhan dan perkembangan pokok serta penyesuaian morfologi daripada keadaan *in vitro* terhadap keadaan *ex vitro* demi kepentingan kemandirian pokok.

**EFFECTS OF SELECTED ADDITIVES FOR GROWTH EFFICIENCY ON *in vitro* GROWN *Vanilla planifolia* Andrews**

**ABSTRACT**

*Vanilla planifolia* Andrews is the economically important and second most expensive food ingredients in the worldwide. In previous studies, organic additives have shown their potential on promoting plant growth and development, however the detail studies of organic additive on plant growth and development of *V. planifolia* was limited. Thus, in this research, the effects of different selected organic additives were studied on the plant growth and development of *V. planifolia* Andrews. Improvement on the protocol for plant growth and proliferation of vanilla were carried out and finally to study the morphological differences between the *in vitro* and *ex vitro* plantlets. *V. planifolia* plant shoots were initially cultured on different strengths of MS, different concentrations of BAP, sucrose and different types of gelling to determine the best growth medium. The *V. planifolia* plantlets growth were recorded after eight weeks of treatment. Supplemented of ½ strength MS medium and supplementation of 20 g/L were efficient enough to promoted plant growth. Addition of 1.0 mg/L BAP was found to induce an average of  $4.11 \pm 0.36$  shoots per explant. The four gelling agents used were found to have no significant effect on the plant growth of *V. planifolia*. There were seven types of organic additives were selected to study the effect of each additive towards the plant growth and development of the shoots explant. Inclusion of 10% coconut water into the medium was found to be the most effective for enhancement of *V. planifolia* plant growth. With an addition of 30% coconut water or tea leaf extract, the multiplication of *V. planifolia* plantlets was found to produce similar results with  $4.26 \pm 0.43$  (coconut water) and  $4.00 \pm 0.86$  (tea leaf extract) shoots per explant as compared to

the basal medium with addition of 1 mg/L BAP. However, with the combinations of 1mg/L of BAP and 10% of coconut water, multiple shoots formation was able to increase about 30%. *V. planifolia* was able to perform 100% rooting without any plant growth regulator and organic additive. Sphagnum moss was found to be the more suitable potting medium as the survival rate of acclimatization of *V. planifolia* were able to archived 83.33% of survival rates. Histological studies and scanning electron microscopy were carried out to determine the morphological changes of *V. planifolia* on both *in vitro* and *ex vitro* plant leaves, stems and roots of *V. planifolia*. According to the histological analyses, there were structure differences in *in vitro* plantlets and *ex vitro* plants. Scanning electron micrograph displayed the under-develop stomata of plantlets and existent of epicuticular wax surrounding the stomata. SEM also displayed the existent of raphides in *V. planifolia*. This study had shown the potential of organic additive that are not fully discovered as an alternative solution for enhancement of plant growth and development and also the morphological adaptation changes from *in vitro* condition to *ex vitro* condition which is vital for plant survival.

## CHAPTER 1

### INTRODUCTION

Vanilla (*Vanilla planifolia* Andrews) is the second most expensive spice after saffron. It is a tropical climbing orchid from the Orchidaceae family which consist of 25,000 different species with nearly 110 species in vanilla genus. In the genus of Vanilla, there is a large leaf species and also species without leaf (Medina, 2009). *V. planifolia* is originated from Caribbean Islands, South and Central America. During the Spanish conquest, vanilla was acquired from Aztecs and brought back to Europe in 1510. Since then it had become a highly demanding spice required from all over the world (Lubinsky *et al.*, 2008; Kull *et al.*, 2009; Odoux and Grisoni, 2011).

*Vanilla planifolia* is a perennial, and herbaceous plant with have thick stemmed vine. All vanilla species produces flowers under optimum condition. Vanilla plant produces 12 to 20 yellowish-green flowers in a cluster. The fruits of the plants are similar to green bean in the form of a 6 to 10-inch-long pod with thousands of minuscule black seeds inside each pod. This plant is a climber that has tendrils like roots and is able to grow up to 60 to 80 feet tall. In vanilla's origin habitat, the flowers are pollinated by melipona bees and hummingbirds. The flowers will remain open for 24 hours and will wilt and drop off if they are not pollinated. Hence, when the natural pollinators were absent in others part of the world for vanilla flowers pollination, human hand-pollination become very essential in order to get vanilla seed pods (Kull *et al.*, 2009, Havkin-Frenkel and Belanger, 2011).

Vanilla plant is the only source that produces natural vanillin. Fresh vanilla pods do not produce any flavour. The vanillin is bound and has to undergo a series of processes to allow the vanillin to be released. Pure vanilla extract consists of

hundred different chemicals that is unable to be synthesized in laboratory. The vanilla bean which is the sources of the vanillin is used in varies kind of food industries including chocolates, ice-cream, soft drinks, liquors, sweets, cakes, biscuits and others food products. In addition, vanilla also been used in the perfumes and pharmaceuticals industries (Raghavan, 2006; Shinha *et al.*, 2008; Kull *et al.*, 2009; Havkin-Frenkel and Belanger, 2011; Glass, 2011).

The vanilla plant requires specific soil and climate to grow. It is also very susceptible to pests and diseases (Odoux and Grisoni, 2011). Vanilla plant is conventionally propagated by using vegetative propagation such as stem cutting technique. However, this method is slow and requires scarification of mature plant in order to get the stem cut. Due to these disadvantages, micropropagation technique was introduced as a better solution improves the cultivation of vanilla. This method had been proven to be more efficient than conventional vegetative propagation method which ensures constant high multiplication rate with sterile and disease free planting materials (Havkin-Frenkel and Belanger, 2011).

Medium formulation plays an important role for optimum multiplication of explants in micropropagation technique. The basal culture medium that commonly use is MS medium, formulated by Murashige and Skoog (1962). Plant growth regulators are essential under *in vitro* sterile environments where they act as a stimulator for the initiation of adventitious shoots, roots formation or even callus formation. For example, cytokinin is used for induction of multiple shoot formation. Cytokinin is able to stimulate axillary shoot formation. However, in high concentration, it will inhibit the root formation and even retard the plant from maturing. Indeed, a specific concentration of plant growth regulator provided to the plants is essential for enhance of growth efficiency (George *et al.*, 2008).

Different organic additives also influence the growth of the plantlets. Some organic additives can be used as natural plant growth regulator, extra nutrient provider, anti-oxidant or even growth promoter. The commonly used organic additives included banana homogenate, charcoal and coconut water for enhancement of growth or absorption of phenolic compound which enhance the growth efficiency (George *et al.*, 2008). However, there are plenty of organic additives that potentially benefit to the plants growth and development.

Histological techniques are method to study the anatomy of selected sample which has been widely used in many research areas (Dolphin, 2001; Jain and Gupta, 2005). The major histological studies were focus on the vanilla beans instead of the plant itself (Odoux *et al.*, 2006; Mariezcurrena *et al.*, 2008; Nishimura and Yukawa, 2010). Palama *et al.* (2010) had conducted study on histological study on shoot differentiation from protocorm callus cultures of *V. planifolia*. However, it did not cover the other plant parts that are vital for the plant survival in the natural environment.

Hence, this study provides the opportunity to assess the effect of organic additive as an alternative additive for enhancement of plant growth by using *V. planifolia* as a model which is important by providing sustainable and cost efficient method for enhancement of plant growth. This study also provides the opportunity to understand the morphological, anatomical and physiological information of *V. planifolia* plant adaptation from *in vitro* environment to the *ex vitro* environment. This is vital for understanding the effect of micropropagation factors that cause impact on the plant growth and development and also provide the information for enhancement of micropropagation method in the future.

## 1.1 Objectives

The objectives of this study are:

- i. To induce multiple shoot formation of *Vanilla planifolia* Andrews and optimise the plantlets growth under *in vitro* condition,
- ii. To determine the effect of different organic additives towards improvement of the number of the vanilla plantlets,
- iii. To establish acclimatization protocol for vanilla plantlets,
- iv. To study the histological difference between *in vitro* plantlets and *in vivo* plants.

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Orchids

Orchids are monocotyledon plants under the Orchidaceae family which also the largest and most evolved within the plant family. They make up to 10 percent of the flowering plants and consisted of about 30000 species and 850 genera. They colonized almost every country except true desert and frozen Antarctica. Orchids are well known for its ornamental value because of its exotic, complex beauty flowers (Arditti, 2008). At present, orchids have become million-dollar business, primary due to floriculture cut flowers and potted plants trades (Roberts and Dixon, 2008; Chugh *et al.*, 2009; Hossain, 2011). Orchids are not only grown as ornamentals plants; some also are used as medicinal herbs. Many species belong to genera of *Anoctochilus* and *Dendrobium* has been reported to have medicinal properties and they are commonly used in Chinese traditional folk medicines (Pant, 2013).

##### 2.1.1 Vanilla genus

Vanilla genus consists of about 110 species belongs to the orchid family which ranges from tropical to sub tropic regions. Tropical America has the most diverse species of vanilla followed by south-east Asia and New Guinea. Vanilla genus is a vine-like plant with climbing characteristic. They can grow up to 35 m with alternate leaves along the stems. Vanilla leaf is commonly oblong in shape and dark green in colour. However, there are some species that their leaves have reduced to scales or totally leafless. Aerial roots grow from each node to allow the plant to climb (Bory *et al.*, 2008; Kull *et al.*, 2009).



### 2.1.2 *Vanilla planifolia* Andrews' history and distribution

*Vanilla planifolia* is native to Mexico and is cultivated in almost every tropical region all over the world for the production of vanilla pod. Vanilla was first used by the Aztecs to flavour their chocolate drinks. The Spanish brought back the vanilla in 1510 and introduced to Europe (Ramachandra Rao and Ravishankar 2000; Havkin-Frenkel and Belanger, 2007; Odoux and Grisoni, 2011).

The vanilla plant is a perennial herbaceous climber plants with thick stemmed vine (2n=32). The plant produces 12 to 20 yellowish-green flowers in a cluster. The fruit capsules which look like green bean were produced after pollination of the flowers. The thick tendril-like roots opposite each leaf enable the plant to climb. The wild types can even climb up 60 to 80-foot-tall which makes them the tallest of all orchids. The roots can also be used for absorbing water and nutrients. The cluster flowers open one at a time for twenty-four hours before the flowers drop off. Hand pollination must carry out in the morning for fertilization. The beans are collected before it completely matures. The beans are then undergoing a series process of curing for 3 to 6 months before shipped for extraction (Havkin-Frenkel and Belanger, 2007; Lubinsky, 2008; Kull *et al.*, 2009).

Vanilla production can be affected to climate and rainfall. The average temperature for the vanilla plant growth is about  $26 \pm 4^{\circ}\text{C}$ . Warm, moist, tropical climates with averages 200 cm annual rainfall is the suitable condition for the plantation of vanilla. However, vanilla can have root rot and others disease problems due to excessive rain and long duration of drying section can kill the plant. Hence, for the cultivation of vanilla, soils that can prevent water logging should be applied (Havkin-Frenkel and Belanger, 2007; Odoux and Grisoni, 2011).

The bean extraction, the complex vanillin compound is used to flavour chocolate, cakes, cookies and ice cream. Vanillin is also used as a fragrance for perfume, hand lotion, shampoo and room freshener (Augstburger *et al.*, 2000). Vanilla was also added into the recipe of Coca-cola as one of the essential ingredients of invented by Atlanta chemist John S. Pemberton which went on sale in 1886 (as been cited by Glass, 2011). Besides that, it also been used in the perfumes and pharmaceuticals industries (Kull *et al.*, 2009; Havkin-Frenkel and Belanger, 2011).

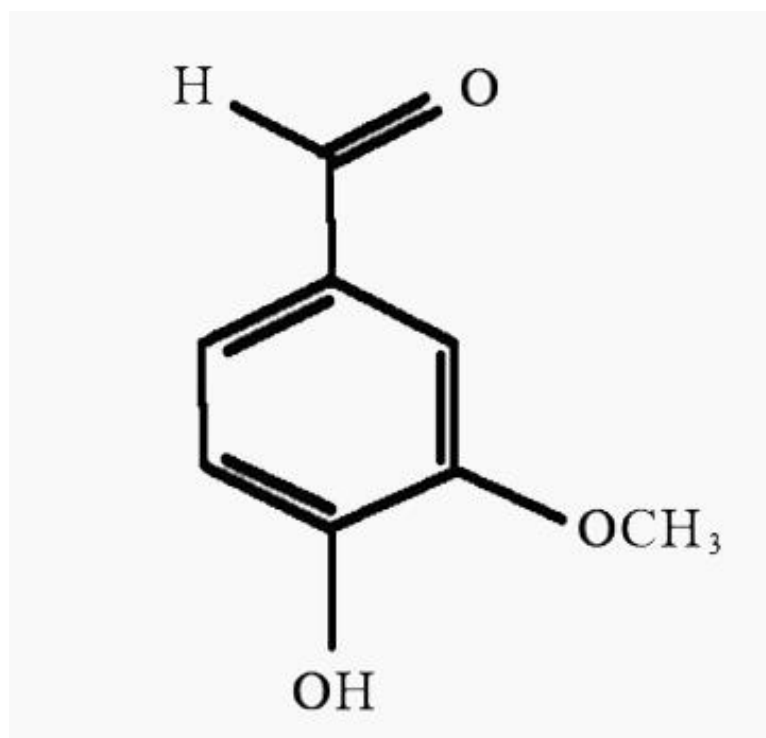
Vanilla bean was an expensive vanillin sources toward the end of the 50's until it replaced by synthetic vanillin. Synthetic vanillin is cheaper compare to natural vanillin and it sold 15000 metric tons per year with the price 15 \$/kg compare to nature vanillin that produce around 2000 tons annually with 1200 \$/kg (Gallage and Møller, 2015).

### **2.1.3 Vanillin and its uses**

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the major component in cured vanilla pods that is used as flavouring. Only 1%-2.5% of the vanillin is obtained from a cured vanilla pods. Vanillin is white crystalline powders which belong to the C<sub>6</sub>-C<sub>1</sub> phenolic compounds as shown at the Figure 2.1 (Shinha *et al.*, 2007; 2008).

Chemical derived vanillin has serious issue where the usage of poison or hazardous chemicals for purification of vanillin which could cause pollution to the environment. Hence, the researches for the environmental friendly pathway for producing synthetic vanillin have been continuing expanding (Dignum *et al.*, 2002;

Havkin-Frenkel and Belanger, 2011; Gallaga and Møller, 2015). Due to its desirable flavour, vanillin has been commonly used as primary flavour or as a component of another flavour in the industry. The products that used vanillin as flavouring agent include household products (cleansers, laundry detergent, and dish washing liquid), dairy food products (ice cream, yogurt, flavoured milk, coffee creamers, chocolate and etc.), baked goods (cupcakes, breads, cakes and etc.), beverages (carbonated drinks, coffee, chocolate drinks, alcoholic drinks and etc.), pet products, pharmaceutical products, perfume, and even toys (Havkin-Frenkel and Belanger, 2011).



**Figure 2.1: Vanillin compound structure**

## 2.2 Plant tissue culture system

Micropropagation is an *in vitro* asexual propagation using pieces of selected plant called explants which can be cells, tissues, or organs isolated from the mother plant cultured on an aseptic culture medium. In micropropagation, explants mainly derived from shoot tips, axillary buds, stem sections, leaf sections or seeds. The explant will be placed into the sterile nutrient medium which provides the essential nutrients for plant growth and differentiation. The plant growth will be monitored under control environmental condition with specific illumination, temperature and humidity (Thorpe, 2007; George *et al.*, 2008).

Plant tissue cultures enable studies to be conducted in controlled environment without seasonal affection. There are four major stage of plant tissue culture. The first one stage is establishment. The plant parts or explants from the selected plants were taken from *in vivo* condition into sterile *in vitro* condition. The explant will be surface sterilized accordingly before placed into the growth medium. The second stage is multiplication. The sterile explant from the first stage will be multiplied by using different protocol. Usually, plant growth regulators will be used to ensure the explant to produce more plantlets, calli or cells. The third stage was pre-transplant where it involves the plantlets which produce from the *in vitro* condition undergo a series of hardening process in order to survive the natural growth environment. The last stage is the acclimatization or transfer from culture to the *in vivo* condition or the natural growth environment (Arditti, 2008; George *et al.*, 2008; Kärkönen *et al.*, 2011).

### 2.2.1 Orchid micropropagation

Plant tissue culture technique has been accepted as a breakthrough for orchid propagation, especially for the purpose of conservation of endangered orchids. This technique has used to conserve many species of wild orchids, subsequently reduces the number of collection from the wild. Many economically important orchids grow, develop protocorms and propagate slowly naturally. This led to the development of orchid tissue culture to enhance the productivity to fulfil the demand of orchid as cut flower, pot flower and even medicinal purpose (Arditti, 2008; Kull *et al.*, 2009; Deb and Pongener, 2012; da Silva, 2013; Pant, 2013).

Development of different plant parts as explant for micropropagation of orchids is very essential. The selection of explant plays an important role for the succession of micropropagation of orchids. Shoot tips have been reported to be effectively used for induction of shoot and protocorm-like bodies (PLBs) of many orchids. Seeni and Latha (2000) had been reported the use of shoot tip culture technique for successful establishment and multiplication of *Vanda coerulea* from the forest of Western Ghats. Geetha and Shetty (2000) reported the development of a large-scale micropagation protocol for *Vanilla planifolia* using both shoot tips and nodal buds. Accordingly, they used two types of media, the establishment medium - MS + 1 mg/L BAP + 3% sucrose and the multiplication medium - N69 (Nitsch basal medium; Nitsch, 1969) + 0.5 mg/L BAP + 0.5 mg/L biotin + 0.5 mg/L folic acid and 2% sucrose. According to their report, these two medium were able to produce up to 100,000 plants in about 15 subcultures from single explant. However, Kalimuthu *et al.* (2006) reported that by using a single type of medium using MS + 1 mg/L BAP + 150 mg/L of coconut water for initiation, multiplication, elongation and rooting of *Vanilla*. Gonzalez-Arno *et al.*, (2009) had reported successfully develop *in vitro*

fragmented explants (IFEs) technique by using the remaining base of the vanilla plant clusters as explant producing up to 15 plantlets after 4 months of culture in ½ MS medium supplemented with 1 mg/L of BAP, and 0.5 mg/L of indole-3-butyric acid (IBA).

### 2.2.2 Culture medium

Naturally, plants obtained nutrients from the soil. Culture medium is a source of nutrients designed to provide necessary nutrients to the plant cells to grow. Indeed, culture medium is one of the most important factors for *in vitro* culture technique is the plant culture medium. Plant propagation is greatly influenced by the formulation of the culture medium used for a successful micropropagation (George *et al.*, 2008; Kärkönen *et al.*, 2011). *In vitro* culture media are generally made up from these components: macronutrients, micronutrients, vitamins, amino acids or other nitrogen compounds, sugar, other organic compounds, solidifying agents, and growth regulators. For orchid's plant tissue culture purpose, the common micropropagation mediums are MS medium [1962], Vacin and Went medium [1949] and Knudson C medium [1946] (Murashige and Skoog, 1962; Arditti, 2008; George *et al.*, 2008; Pant, 2013).

Carbon source in the form of carbohydrates are supplied to the *in vitro* plantlets as a source of carbon dioxide and osmotic agent. The carbon source is supplied to the plant for assimilation of CO<sub>2</sub> during photosynthesis process. Hence, *in vitro* plants depend on the supplied carbohydrates for energy supply in order for the plant to grow. There are many carbohydrates available to supply into culture medium, however sucrose is the most common used as it is cheap and easily obtained (Caponetti *et al.*, 2000; Arditti, 2008; George *et al.*, 2008). This carbon source

required for plant growth was varying according to plant species. Sucrose concentration at 65 g/L produced optimum level in-term of multiplication rate of plantlets, and root numbers in date palm (Abdulwahed, 2013).

Plant's growth required large amount of macronutrients and comprise at least 0.1 % of the dry weight of plants are included ions of nitrogen (N), potassium (K), calcium (Ca), phosphorus (P), magnesium (Mg) and sulphur (S) (Caponetti *et al.*, 2000; George *et al.*, 2008). These macronutrients are required as major component of amino acids, plant energy metabolism, cofactor for plant enzymes, element in osmotic potential of cells, component of cell walls, component of chlorophyll, and etc. Lacking of these elements could cause stunted plant growth and unhealthy plant growth. These nutrients are usually required in higher amount compared to micronutrients (Mezzetti *et al.*, 1991; Caponetti *et al.*, 2000; Arditti, 2008; George *et al.*, 2008; Taiz and Zeiger, 2010).

The trace elements or micronutrients which are needed for small amounts and necessary for plant growth and developments are included iron (Fe), nickel (Ni), chlorine (Cl), manganese (Mn), zinc (Zn), boron (B), copper (Cu), and molybdenum (Mo). They act as element in photosynthesis, break down urea, activation agent for enzymes, component of alcohol dehydrogenase, and even responsible in cell elongation, nucleic acid metabolism, hormone responses, and membrane function (Mezzetti *et al.*, 1991; George *et al.*, 2008 Taiz and Zeiger, 2010).

Plants absorb these inorganic nutrients in ions form. The medium is supplemented with these inorganic nutrients as salts that would dissolve in the medium to form into cations and anions. Normal plants produce their own vitamins as catalysts in different metabolisms. However, plant cells and tissues that are grown



*in vitro* will have difficulty of producing their own vitamins. Hence, vitamins such as thiamine (B<sub>1</sub>), nicotinic acid (niacin) and pyridoxine (B<sub>6</sub>) are added to the culture medium. Amino acids are a source of nitrogen that is rapidly taken up by the cells for stimulation of cell growth. Amino acids are more easily absorbed by the plant compare to inorganic nitrogen ion sources. The most commonly used amino acids in culture medium are casein hydrolysate, L-glutamine, L-asparagine, and adenine (George *et al.*, 2008 Taiz and Zeiger, 2010).

### **2.2.3 Gelling agent**

The growth of shoots or roots is greatly affected by the physical stability of the culture medium. The solidification of the medium has acts as a support system to ensure the plant tissues and organs to stay above the nutrient medium as well as supporting the plant root growth. Many gelling agents are used for plant culture media, e.g., agar, agarose, phytigel and Gelrite (George *et al.*, 2008). There are various brands and grades of agar, with different price, and composition. The choice of agar band mostly determines by actual use and experience for a plant species. For industrial scale micropropagation, cheaper brands of agar are more economically practical where higher grade agar is unnecessary (Prakash *et al.*, 2004; George *et al.*, 2008). Mengasha *et al.*, (2012) has reported to use enset starch as an alternative gelling agent for *Vanilla planifolia* culture medium. This enset starch has reported have low charity that can affect contamination detection; however, it also reported that it able to enhance root proliferation. Zimmerman *et al.* (1995) had reported using corn starch gelled medium for tissue culture of six cultivars of apple together with two red raspberries. Compared to MS medium gelled with agar (7.5 g/L), the fruit

crops were reported to have equal or better shoot proliferation on MS medium gelled with a mixture of corn starch (50 g/L) and Gelrite (0.5 g/L). However, the medium appeared to be gray-white in colour causing the difficulty for the detection of contamination.

Agar as a gelling agent remains the popular choice for solidification of plant culture media since 25 years ago. It has been used for solidified the culture medium used for higher plants. However, it had been reported that agar may cause growth inhibition in plants (Prakash *et al.*, 2004). Scholten and Pierik (1998) even reported within different brand of agars affected major differences in the grow rate of plantlets.

Gelrite has become the preferred gelling agent after the agars due to its purity and consistent quality. Relatively smaller quantities of Gelrite able to produce the same gelling property as compare to agar (Harris, 1985). The cucumber somatic embryos had higher germination rate when the culture media were solidified with Gelrite (Ladyman and Girard, 1992). However, Gelrite had also been reported that could cause hyperhydricity in *Aloe polyphylla* as compared to agar-solidified medium (Ivanova and Van Staden, 2011).

‘Isabgol’, the *Plantago ovata* seeds’ husk had been reported to be used as a gelling agent in tissue culture medium. The large quantity of mucilage present in the husk is colloidal and polysaccharidic which is able to soluble in hot water, forming viscous liquid and sets to gel when cooled. The medium can be gelled with 30 g/L of ‘Isabgol’. The plant growth on media gelled with ‘Isabgol’ were no significant difference to that on media gelled with agar (Babbar and Jain, 1998). Agrawal *et al.* (2010) in another hand used 35g/L of ‘Isabgol’ to solidified the medium for *in vitro* micropropagation of banana.

#### **2.2.4 Cytokinin**

Cytokinins are growth regulators that are used for stimulating cell division and other plant growth. It is the most commonly used hormone or plant growth regulators for stimulating the respond of multiple shoot formation in plant tissue culture. Cytokinins that widely used nowadays are 6-benzylaminopurine or 6-benzyladenine (BAP, BA), 6- $\gamma$ - $\gamma$ -dimethylaminopurine (2iP), N-(2-furanylmethyl)-1H-puring-6-amine (kinetin), and 6-(4-hydroxy-3-mehty-trans-2-butenylamino) purine (zeatin) (George *et al.*, 2008).

Cytokinins were able to promote proliferation of shoots and inhibit shoot elongation. Elongation of *Arabidopsis thaliana* was retarded after sprayed with 22.5 mg/L BAP (Greenboim-Wainberg *et al.*, 2005). Higher concentrations of cytokinins caused abnormal growth where too many small shoot is produced and fail to elongate eventually. Higher level of cytokinins may cause the leaves of some species became abnormal in shape and the shoot will become hyperhydricity (George *et al.*, 2008).

#### **2.2.5 Organic additives**

Organic additives are undefined composition materials which can be used for establishment of the cultures. These organic additives may act as extra carbon sources, nitrogen sources, and even as plant growth regulators. These organic additives included coconut water (CW), orange juices, banana pulp, tomato juice, charcoal, potato extract, yeast extract and malt extract (Agarwal *et al.*, 2004; Aktar, 2008; Daud *et al.*, 2011). These organic additives had been used to enhance plant growth, PLBs regeneration, PLBs proliferation, enhance callus growth and etc (Rahman *et al.*, 2004; Aktar *et al.*, 2008; George, 2008). These complex additives

can be very effective in enhances the *in vitro* plant growth of orchid seedling with its undefined organic nutrients and growth factors. Tharapan *et al.* (2014) reported that potato extract able to enhances the shoots growth of *Dendrobium* orchids which soy milk able to enhance the roots growth. These organic additives were simple, efficient, effective, and convenient to enhance culture media for commercial production. Optimization and adjustment are required for optimum growth of the culture. These enhancement effects of the organic additives work differently on different plant. Some organic additives were reported enhances plant growth but some were reported inhibit plant growth (Arditti, 2008).

#### **2.2.5.1 Coconut water (CW)**

Coconut plant (*Cocos nucifera* L.) has long been recognized an important crop for human life. The coconut water (CW) which is the liquid endosperm has assumed the most nutritious beverage provided by the nature (Khan *et al.*, 2003). In plant tissue culture, coconut water is a common organic additive that has long been used as a growth promoting component which contained many uncharacterized biochemical compounds than directly or indirectly enhances the plant growth rate. Coconut water can induce plant cells to develop and differentiate rapidly. According to Ge *et al.* (2004; 2005; 2006), they had identified few types of natural occurrence cytokins present in coconut water which includes zeatin, zeatin riboside, trans-zetin, O-glucoside, dihydrozeatin O-glucoside, ortho-topolin, kinetin free base and kinetin riboside. Coconut water had reported containing natural indole acetic acid (IAA) which used to induce adventitious rooting of *Dracaena purplecompacta* L. (Agampodi and Jayawardena, 2009). The overall phosphorus content of the media

was reported to be increased when coconut water was supplemented (Mezetti *et al.*,1991).

#### **2.2.5.2 Coconut sugar**

Coconut sugar is made from the sap from coconut tree (*Cocos nucifera* L.) blossoms. Generally, the sap collected will be boiled to produce brown solid sugar block. This sugar is widely used in Malaysian's foods and drinks preparation (Apriyantono *et al.* 2002). It has been reported that commercial sugars had total sugar content between 761.96 – 841.23 mg/g dry weight with 0.15-122.07 mg/g of reducing sugar content. Thus, comparing to commercial sugars, it have been reported that coconut sugar has lower total sugar content but higher in reducing sugar content. According to Kongkaew *et al.* (2014), some amount of calcium, iron, sodium, magnesium, and potassium were detected. They were found slightly higher compared to commercial sugar. Besides that, it has also found to have slightly higher antioxidant activity compared to commercial sugars (Kongkaew *et al.*, 2014). It had been reported that sucrose can be replaced by 6% date syrup for culturing the *in vitro* somatic embryogenesis of date palm (Alkhateeb, 2008).

#### **2.2.5.3 Activated charcoal**

Activated charcoal has been widely used in many industrial due to its ability to absorb and removal of the harmful or unwanted substances which included food, beverage, pharmaceutical, chemical and even biotechnology industries. It has been widely used to treat drinking water and wastewater which had been proven to have

the ability to absorb organic pollutant (Elhussien and Isa, 2015). Activated charcoal has included animal and plant charcoal, but plant charcoal is preferable in plant tissue culture as additive in the medium to enhance the growth performance such as seed germination, somatic embryogenesis, protoplast culture and others micropropagation technique (Thomas, 2008).

For *in vitro* micropropagation, it is very common for the plant to exudate its phenol compound and it often affect the plant growth. Polyphenols released by *Aristolochia indica* had reported affecting the shoot growth when culture in *in vitro* condition (Soniya and Sujitha, 2006). Many *in vitro* explants would have explant browning problem. In order to encounter this type of situation, activated charcoal was added into the media to avoid the explant browning (Wang *et al.*, 2005; Feyissa *et al.*, 2005). In addition, activated charcoal combined with TDZ supplemented medium also able to promote and enhance the multiple shoot formation of the seedling of *Rhynchosyilis retusa* (Thomas and Michael, 2007).

#### **2.2.5.4 Pineapple juice**

Pineapple (*Ananas comosis*) is widely grown in tropical countries. As being one of the organic additives, it was not being use as frequent as coconut water. Pineapple fruits, the edible portion, contain about 85% water, 0.4% protein, 14% sugar, 0.1% fat and 0.5% fibre. In the total 14% of sugars content – half of these is sucrose with another half of glucose and fructose. It is also rich in calcium, phosphorus, iron, calcium, vitamins A and B together with 7-9% of citric acid (Vaughan and Judd, 2006; Pua and Davey, 2007, Patil *et al.*, 2011). The growth promoting activity of the pineapple is still yet to be completely understood.

Santiago-Silva (2011) reported that detection of several polyamines in the pineapple fruits. Wisziewska *et al.* (2013) confirmed that pineapple pulp was able to promote root development in *Daphne* plants. They suggested that this might be due to the presence of rooting cofactors and polyamines stimulates formation of the rooting.

#### **2.2.5.5 Dragon fruit (pitaya) juice**

Dragon fruits/pitayas (*Hylocereus* spp.) are perennial climbing cactus origin from tropical regions of America. It has been commercially available in Malaysia. However, due to its new to the market, information from the research conducted was limited (Lim *et al.*, 2007).

The fresh fruit contains about 86% of water, with 5-8% of total sugars content. These sugars content mainly derived from reducing sugar such as glucose and fructose where sucrose was only found about 3-8% of total sugars (Le Bellec, 2006). Charoensiri *et al.* (2009) reported that dragon fruits contain some amount of beta-carotene, lycopene, and vitamin E. According to Lim *et al.* (2006), dragon fruit has relative low total phenol compound and ascorbic acid content compared to others Malaysia's local fruits. Dragon fruit contain high proline content which range from 1.1 to 1.6 g/L of juice and proline is well-known of helping plant species to adapt to the environment stress. It also contains high amount of potassium, magnesium and calcium (Le Bellec, 2006, Szabados and Savoure', 2009).

### 2.2.5.6 Banana pulp

Bananas (*Musa* spp.) are common fruits that grown throughout the tropical countries. In 100g of fresh fruits, banana contained about 15g of starch and 12 g of total sugars. It also contains various vitamin and trace minerals particularly high amount of potassium, calcium, phosphorus and also tryptophan that can promote the plant growth (Sharaf *et al.* 1979; Gnasekaran *et al.*, 2010; USDA, 2015). Addition of banana into the culture medium for orchid growth became widely used to enhance growth of the plants. Due to the dark colouration, banana additive culture media are easily recognized. It has been reported that ripe banana pulp is able to stimulate the growth of vanilla seedling and immature embryos (Withner, 1955). However, the mechanisms of the enhancement of banana towards the *in vitro* plantlets are not clear (Arditti, 2008). It has been reported that banana could stabilize the pH when supplemented into the medium. However, high concentration of banana pulp can act as inhibitor to the plants growth (Gnasekaran *et al.*, 2010).

According to Aktar *et al.* (2008), 10% w/v of Sabri banana pulp supplemented into ½ MS medium had significantly enhance the growth and development of *Dendrobium* orchid PLBs. PLBs proliferation of *Phalaenopsis violacea* orchid showed 10% increment with Pisang Mas (AA) pulp (Gnasekaran *et al.*, 2010). Obsuwan and Thepsithar (2014) reported that *Vanda* Tokyo Blue seedling cultured in Vacin and Went (VW) medium supplemented with banana pulp showed increased in fresh weight and root number. However, Nambiar *et al.* (2012) reported that banana pulp did not promote the proliferation of PLBs in *Dendrobium* Alya Pink.



#### **2.2.5.7 Green tea leaf extract**

Tea is one of the most popular drinks in the world. Green tea is prepared by drying the fresh tea leaf without fermentation. Green tea is rich in catechins, one of the flavanol which make up of 30% of dry leaf weight. There are also others flavonols compounds and their glycosides. Total caffeine content is about 3% of dry leaf weight. Tea leaf also is rich in potassium, calcium, magnesium and aluminum. Tea leaf has high antioxidant property due to its various flavonoids compound and bind with others molecules even proteins to form non-toxic component. Hence, it also reported to have the ability to inhibit enzymatic activity. It can also stimulate the modulation of receptors, enzymes, or regulatory proteins at low concentration (Graham, 1992; Balentine, 1997; Rani *et al.*, 2014). Jeszka-Skowron and Zgola-Grzekowial (2014) reported the optimized protocol for extraction green tea compound by infusing the green tea leaves in 95°C for 15 minutes. In animal tissue culture, tea leaf extract had been reported added into the tissue culture medium. Barakat *et al.* (2014) reported that 0.3 g/L of green tea leaves extracts able to enhance the sheep blastocyst formation. Green tea polyphenols also reported to be used in enhancement of cow oocytes development (Wang *et al.* 2012).

#### **2.2.6 Rooting**

Rooting of *in vitro* plantlets is one of the important stages in micropropagation system (Thiart, 2003). Plantlets grow inside *in vitro* environment are subsequently small and fragile where survival *ex vitro* will be typically difficult (Thiart, 2003). Rooting of plantlets ensure the plantlets develop the ability to obtain nutrients from the soil efficiently (Thiart, 2003). This ensures the plants to be able to

survive outside the sterile environment. Different plants required different conditions for rooting induction, hence different formulations of *in vitro* culture medium is require for different type of plantlets to establish their rooting (George *et al.*, 2008). Lee-Espinosa *et al.* (2008) reported that *V.planifolia* able to promote rooting when culture in ½ MS media supplemented with 0.44 µM naphthalene acetic acid (NAA). However, Zuraida *et al.* (2013) reported *V. planifolia* able to produce 100% rooting when culture with ½ MS without any plant growth regulators.

### **2.2.7 Acclimatization**

Acclimatization is the method which *in vitro* plantlets were transferred from *in vitro* condition to the greenhouse condition. High humidity and a low light intensity of the *in vitro* condition caused the plantlets to be very fragile. The stomata of the leaves may fail to close under low humidity conditions such as green house or field condition. The plantlets lose water rapidly through transpiration when they are moved out from *in vitro* environment. The plantlets would not be able to absorb enough water for the transpiration process. This eventually causes the plantlets to wilt. However, once the plants are slowly adapting the external condition for couples of few days, the plants will be able to survive in the green house or field. To facilitate this, the plantlets are kept in a place with high humidity and low light intensity for several days before transfer to greenhouse condition (George *et al.*, 2008).

Parthasarathy and Nagaraju (1999) reported the use of jars as acclimatization container for rooted Gerberawas found to be better than pots as jars able to maintain high humidity around the plant and reduce the chance for the plants to be dry out which increased the survival rates before transfer to the field. There are numerous

types of potting mediums which can be used in acclimatization such as peat, sand, perlite, soilrite and others soil mixtures. Sherif et al (2012) had used coconut coir, activated charcoal and commercial fertilizers in the ratio of 3:1:1 to acclimatize the *Anoectochilus elatus* Lindley in greenhouse. The rooted vanilla plantlets were transferred to hardening medium with top soil and compost mixture at the ratio of 2:1. The survival rate of the plantlets achieved 90% of total plantlets after 4 weeks' culture in glasshouse condition (Zuraida *et al.*, 2013).

Peat or sphagnum moss were commonly used as orchid potting material due to its characteristics of spongy fibrous texture, a high porosity with high water-retaining and low ash content (Zettler et al., 2007; Dutra *et al.*, 2008; Bunt, 2012). *V. planifolia* also reported to be acclimatized by potting in peat moss and agrolite (1:1) in the green house condition for 30 days with 90% survival rate before transplanted to soil in the field (Lee-Espinosa *et al.*, 2008).

### **2.3 Histological analysis**

In order to understand the inner and outer structure changes of the plants which undergo the acclimatization process, histological studies such as light microscopy study and scanning electron microscope (SEM) analysis should be carried out. These studies enable researcher to understand the microstructure changes and inner cellular changes of the plants from *in vitro* condition to *ex vitro* condition.

Histological changes occur due to the cellular process can be studied and provides information that allow further research study which improved the understanding of normal and abnormal functions of the plant (Yeung, 1998; Jain and Gupta, 2005). Niranjana *et al.* (2009) suggested that by studying the basic