MICROPROPAGATION OF MEYER LEMON

(*Citrus* x *meyeri*)

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

µL	Microlitre
µM	Micrometre
°C	Degree Celsius
2,4-D	2,4-Dichlorophenoxyacetic acid
ANOVA	Analysis of variance
BAP	6-Benzylaminopurine
cm	Centimetre
CRD	Complete randomized design
DMRT	Duncan multiple range test
DNA	Deoxyribonucleic acid
g/L	Gram per litre
GPx	Glutathione peroxidase
HCl	Hydrochloric acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IC_{50}	50% growth cell proliferation inhibitory concentration
ISNT	Isosinensetin
ISSR	Inter-simple sequence repeat
LDL	Low density lipoprotein
mg/L	Milligram per litre
mL	Mililitre
MS	Murashige and Skoog
NAA	Naphthaleneacetic acid
NaOH	Sodium hydroxide
nm	Nanometre
O_sO_4	Osmium Tetroxide

PCR	Polymerase chain reaction
PGR	Plant growth regulator
PMFs	Polymethoxyflavones
PON	Paraoxonase
RAPD	Random amplified polymorphic DNA
ROS	Reactive oxygen species
RPM	Revolutions per minute
SCoT	Start codon targeted
SE	Somatic embryogenesis
SEM	Scanning electron microscope
SO	Shoot organogenesis
SOD	Superoxide dismutase
T_m	Melting temperature
TBA	Tert-Butyl alcohol
TBE	Tris-Borate-EDTA
TDZ	Thidiazuron
v/v	Volume per volume
VW	Vacin and Went
w/v	Weight per volume
WPM	Woody plant medium

MIKROPROPAGASI LEMON MEYER (*Citrus* x *meyeri*)

ABSTRAK

Citrus x *meyeri* atau lemon Meyer merupakan hibrid antara lemon asli dan oren manis (*Citrus limon* x *Citrus sinensis*) yang berasal dari China. Pemakanan buah lemon Meyer tidak hanya kerana rasanya yang manis, tetapi juga kerana kandungan vitamin C yang tinggi serta kompaun bioaktif yang menjadi agen antioksidan dan anti keradangan. Teknik konvensional semasa seperti cantuman dan keratan batang, yang digunakan untuk propagasi lemon Meyer di Malaysia didapati kurang berkesan untuk pengeluaran stok pokok bagi tujuan komersial. Kajian ini dijalankan bertujuan untuk menghasilkan protokol *in vitro* untuk propagasi besaran bagi lemon Meyer melalui induksi pucuk berganda dan untuk menilai pembentukan embriogenesis somatik. Pensterilan permukaan telah dijalankan ke atas eksplan pucuk dan biji benih dengan agitasi dalam 70% (v/v) etanol dan larutan peluntur komersial (Clorox®) dengan kepekatan yang berbeza. Pucuk asenik telah diinokulasi ke dalam media Murashige and Skoog (MS) separa bersama sitokinin yang berbeza (BAP, Kinetin, Zeatin and Thidiazuron) serta kombinasi BAP dengan auksin, NAA dan IAA. Induksi akar telah dijalankan ke dalam media MS separa dan WPM yang ditambah dengan auksin yang berlainan, IAA dan IBA. Pucuk yang berakar baik telah diaklimatasi dalam campuran tanah kebun hitam, tanah merah dibakar dan tanah biochar (2:1:1). Analisis polimorfisma telah dilaksanakan antara pucuk yang ditanam dan daripada subkultur yang berbeza menggunakan penanda ISSR dan SCoT. Pencahayaan, pertambahan rawatan 2,4-D tunggal dan kombinasi 0.5 mg/L 2,4-D dan sitokinin (BAP, Kinetin and TDZ) telah dinilai bagi pembentukan kalus embriogenik menggunakan segmen epikotil daripada benih yang bercambah secara

in vitro. Pencahayaan dan pertambahan sitokinin yang berlainan (BAP, Kinetin and TDZ) turut dikenalpasti dalam induksi embrio somatik. Analisis histologi dan pengimbasan mikroskop elektron telah dilaksanakan untuk memerhatikan peringkat perkembangan embrio. Kadar steril yang tertinggi bagi eksplan pucuk dan biji benih diperolehi daripada masing-masing solusi Clorox® 18% dan 3% (v/v). Purata nombor pucuk yang tertinggi didapati dalam media MS separa ditambah dengan 1.0 mg/L BAP serta kombinasi 2.0 mg/L BAP bersama 1.5 mg/L IAA masing-masing pada nilai 3.29 ± 0.221 and 3.64 ± 0.541. 100% pembentukan akar telah dikenalpasti dalam media WPM ditambah dengan 0.5 mg/L IAA. 82% kadar pokok hidup telah dicapai daripada campuran tanah di atas selepas 12 minggu aklimatasi. Tiada polimorfisma telah dikenalpasti antara pucuk yang ditanam dan daripada subkultur yang berlainan menggunakan penanda ISSR dan SCoT berdasarkan 100% monomorfisma. Induksi kalus embriogenik yang maksimum dan rapuh telah dikenalpasti di dalam keadaan gelap bersama rawatan 0.5 mg/L 2,4-D menggunakan segmen epikotil. Pembentukan embrio somatik menggunakan kalus embriogenik memerlukan keadaan bercahaya dan pertambahan 0.5 mg/L TDZ bagi menghasilkan purata nombor embrio somatik yang tertinggi pada nilai 7.31 ± 0.962. Analisis histologi dan pengimbasan mikroskop elektron telah mengesahkan pertumbuhan dan peringkat perkembangan embrio yang berbeza dalam rawatan TDZ. Kajian ini telah berjaya menghasilkan protokol *in vitro* bagi lemon Meyer menggunakan eksplan pucuk dan segmen epikotil, mengenal pasti kestabilan genetik untuk pucuk yang dimikropropagasi dan melaporkan peringkat perkembangan embrio yang berlainan.

MICROPROPAGATION OF MEYER LEMON (*Citrus* x *meyeri*)

ABSTRACT

Citrus x *meyeri* or Meyer lemon is the hybrid of true lemon and sweet orange (*Citrus limon* x *Citrus sinensis*) and native to China. The ingestion of Meyer lemon fruits is not only for its sweet flavour, but also for its high amounts of vitamin C and bioactive compounds that serve as antioxidant and anti-inflammatory agents. Current conventional techniques such as grafting and cutting used to propagate Meyer lemon in Malaysia were found to be less efficient for the production of plant stocks for commercial purposes. This study was aimed to establish *in vitro* protocol to mass propagate Meyer lemon through multiple shoot induction and to evaluate somatic embryogenesis formation. Surface sterilization was conducted on shoot tip and seed explants with agitation in 70% (v/v) of ethanol and different concentrations of Clorox® solution. Axenic shoot tips were inoculated in ½ strength Murashige and Skoog (MS) media with different cytokinin (BAP, Kinetin, Zeatin and Thidiazuron) and combinations of BAP with auxin, NAA and IAA. Root induction was performed in ½ strength MS and WPM media with different auxin, IAA and IBA. The well rooted shoots were acclimatized in black garden, red burnt and biochar soil mixture (2:1:1). Polymorphism analysis was carried out between cultivated and different subcultures of shoots using ISSR and SCoT markers. Light illumination, supplementation of single 2,4-D and combinations 0.5 mg/L 2,4-D with cytokinin (BAP, Kinetin and TDZ) were assessed for embryogenic callus formation using epicotyl segments from *in vitro* germinated seedlings. Similarly, light illumination and different cytokinin (BAP, Kinetin and TDZ) supplementation were also investigated for somatic embryos induction. Histological and scanning electron

microscopy analyses were conducted to observe developmental stages of embryos. The highest sterile shoot tip and seed explants were obtained in 18% and 3% (v/v) of Clorox® solution, respectively. The highest average shoot number was acquired in ½ strength MS media supplemented 1.0 mg/L BAP and combination of 2.0 mg/L BAP with 1.5 mg/L IAA at the values 3.29 ± 0.221 and 3.64 ± 0.541, respectively. 100% of root formation was acquired in WPM media with 0.5 mg/L IAA. After 12 weeks of acclimatization, about 82% of the survival rate was achieved. No polymorphism was evaluated between cultivated and different subcultures of shoots using ISSR and SCoT markers based on 100% of monomorphism. Friable and maximum induction of embryogenic callus were evaluated under dark condition with 0.5 mg/L 2,4-D treatment using epicotyl segments. Somatic embryos formation using embryogenic callus favoured light condition and 0.5 mg/L TDZ supplementation to produce the highest number of somatic embryos with 7.31 ± 0.962. Histological and scanning electron microscopy (SEM) analyses confirmed the formation and different developmental stages of embryos in TDZ treatments. This study has successfully established *in vitro* protocol of Meyer lemon using shoot tip and epicotyl segments, identified the genetic stability of micropropagated shoots and reported the different developmental stages of embryos.

CHAPTER 1

INTRODUCTION

Citrus plants have been recognized as one of the sought after fruit crops around the world. A broad range of *Citrus* fruits have been globally expanded for the past 30 years where more than 100 countries are extensively cultivating them (Mukhtar et al., 2005; Pérez-Tornero et al., 2010). Lemons as the third most important *Citrus* crop have been widely recognized to have high level of consumption per capita due to their refreshing flavour and nutritional values (Mellisho et al., 2011; Gironés-Vilaplana et al., 2012).

In Malaysia, various green coloured *Citrus* have been grown in large scale for local and world import industry which including pomelo, lime, kaffir lime and calamansi lime (Jantan et al., 1996; Othman et al., 2016; Hasbullah et al., 2018). However, yellow coloured *Citrus* genotypes viz. lemon has yet to be locally cultivated. *Citrus* x *meyeri* or commonly termed as Meyer lemon, is a lemon hybrid between true lemon (*C. limon*) and sweet orange (*C. sinensis*) which belongs to Rutaceae family (Miyake et al., 2012). Meyer lemon is morphologically comparable to oranges where the fruits are large with orangey-yellow or deep yellow at maturity (Lim, 2012). Nevertheless, the traits of Meyer lemon which significantly contrast to all lemons are the higher juice content due to thin rind, remarkable sweet tasted of juice and pulp and also less acidic (Uckoo et al., 2015).

However, plant propagation rate of commercial *Citrus* genotypes can be impinged by inefficiency of conventional techniques (Carimi, 2005; Goswami et al., 2013). Therefore, micropropagation via *in vitro* technique poses as a boon substitution for the conventional plant breeding methods through intensification of multiplication rate of desired genotypes for commercial purposes (Hussain et al., 2016). This alternative approach is an imperative technology that maintained aseptic cultures of cells, tissues and organs under physical and chemical *in vitro* conditions (Thorpe, 2007). It is extensively carried out for large-scale plant production where a rapid, mass production of high quality and healthy plant clones is performed within limited spaces and continuously throughout the year, independently from seasonal and location variation (Guerra and Dal Vesco., 2010, Hussain et al., 2012; Daniel et al., 2018; Panigrahi et al., 2018).

Until now, two morphogenic pathways have been extensively reported for mass propagation of more than 1000 different plant species included shoot organogenesis (SO) and somatic embryogenesis (SE) (Omar et al., 2016; Dobrowolska et al., 2017; Daniel et al., 2018). According to Thorpe (2007), the formation of axillary shoots is remarkably competent to yield genetically true-to-type regenerants, whilst somatic embryogenesis is likely to induce mass number of new plantlets albeit only limited plant species has potential for this organogenic pathway. Diverse genotypes of *Citrus* have been subjected to this *in vitro* approach, via direct shoot organogenesis (Singh et al., 1994; Perez-Molphe-Balch and Ochoa-Alejo, 1997; Bordon et al., 2000; Almeida et al., 2002; Rattanpal et al., 2011) and somatic embryogenesis (Ling and Iwamasa, 1997; Carimi et al., 1999; Carimi and De Pasquale, 2003; El Sawy et al., 2014; Amin and Shekafandeh, 2015).

Currently, Meyer lemon plants available in Malaysia are very limited for commercial field establishment due to slow and less efficient of conventional methods (grafting and cutting) used by local growers albeit it has been found to thrive in the local soils. There are no studies to date on *in vitro* technique specifically for Meyer lemon. This study aims to establish an efficient *in vitro* protocol for Meyer lemon where the effects of culture media composition and plant growth regulators (PGRs) on multiple shoot induction and somatic embryogenesis were evaluated. The current study also highlights the genetic stability and structural observations of regenerated structures in Meyer lemon for further validation.

1.1 Objectives

The objectives of this study are:

1. To induce multiple shoot induction of Meyer lemon using shoot tip explants through single and combination treatments of plant growth regulators
2. To induce roots of regenerated shoots and acclimatize Meyer lemon plantlets for field adaptation
3. To evaluate and identify polymorphism between cultivated and regenerated shoots using ISSR and SCoT molecular markers
4. To induce somatic embryogenesis using epicotyl segments of Meyer lemon and to evaluate induced embryos via microscopy analysis

CHAPTER 2

LITERATURE REVIEW

2.1 *Citrus* x *meyeri* (Meyer lemon)

2.1.1 Origin and History

Most *Citrus* species are indigenous to Eastern Asia, primarily Southeast Asia regions (Goswami et al., 2013). Nevertheless, there are still plenty of *Citrus* plants have yet to be discovered and still can be located in their wild habitats. According to Uckoo et al. (2015), lemons have been categorized as one of the most sought after *Citrus* species despite of numerous new varieties or cultivars are consistently developed over the years. The recent statistics found that almost 14 million tons of lemons are extensively cultivated worldwide whereby India and Mexico are claimed as the top producers (Love and Paull, 2013).

With reference to historical perspectives, Meyer lemon was documented native to Beijing, China and has been consumed continuously around the globe for many years (Miyake et al., 2012). The fruits of Meyer lemon were initially introduced into the U.S market in 1908 by Frank N. Meyer, who was assigned for a fruit-hunting trip in China to discover new, wild plant species. Subsequently, the authority has decided to honour him and named this newly ascertained lemon species specifically after him (Lim, 2012).

However, the Meyer lemon currently available are widely known as the 'Improved' Meyer lemon developed by University of California in 1950. They have been released for public consumptions in 1975 as a defeat version of original Meyer lemon that was highly-susceptible to the most virulent and extensively outspread

Citrus disease known as the *Citrus* Tristeza Virus (CTV) (Love and Paull 2013). From then onwards, Meyer lemon has significantly gained tremendous demand from the fruit markets and is hugely favoured by the public consumers around the world as it possesses numbers of exceptional traits that has set it apart from the typical lemon fruits (Miyake et al., 2012).

2.1.2 Taxonomy and Morphology

Until the present day, *Citrus* plants have been reported for approximately 150 genera and 15,000 species in total (Hussain et al., 2016). However, the taxanomy and systematics of genus *Citrus* are comparatively complex as the overall number of *Citrus* species is very much debatable (Love and Paull, 2013). According to Chiancone and Germana (2012) *Citrus* has been categorized as one of the most vastly produced fruit crops worldwide as it consists of various and notable commercialized cultivars such as oranges (*Citrus sinensis* L. Osbeck), tangerines (*C. unshiu* Marc., *C. nobilis* Lour., *C. deliciosa* Ten., *C. reticulata* Blanco and their respective hybrids), lemons (*C. limon* L. Burm.f.), limes (*C. aurantifolia* Christm. Swing.) and grapefruits (*C. paradise* Macf.).

The trees of Meyer lemon are moderately robust with open spreading, hardy and relatively small to medium sized where the height reaching up to approximately 1.5 to 3 metres (Hodgson, 1967; Badgett, 2016). Meyer lemon is known as symmetrical tree with large, elliptic-ovate and glossy green leaves, whilst the flowers of this lemon hybrid are hermaphrodite, white, fragrant and borne in terminal fascicles. Morphologically, the fruits of Meyer lemon are large, subglobose to globose-ovoid shaped, 6.5 to 7.5 cm in average diameter and have seven segments of

pulp (Lim, 2012). Christman (2007) had reported that the surface of Meyer lemon fruit is visibly smooth that lacks the lemon nipple compared to the common lemon. Apart from that, Miyake et al. (2012) also emphasized that several other qualities of Meyer lemon that distinguish from all regular lemons include the lower levels of citric acid content and high quality of essential oils. Nonetheless, the grafted plants of Meyer lemon require at least two years while the seed grown plants need four to seven years for maturity prior to fruit bearing despite proper care (Badgett, 2016).

Figure 2.1: Meyer lemon a) plants and b) fruits in Superfruit Valley in Chuping, Perlis, Malaysia

2.1.3 Uses and Composition

More than 1500 years back, *Citrus* plants have been widely utilized as traditional remedies in China as the Chinese folks strongly believed that herbs and foods are equivalent and are inseparable. The fruits of *Citrus* are widely processed into broad by-product for instance, canned fruits, juices, wines, vinegars, jams and preserves mainly due to their mass production, advances in storage and processing techniques and consistent supply for a year round (Zou et al., 2016). However in this context, Meyer lemon was initially cultivated as a decorative ornamental houseplant in China (O'hara, 2009). With reference to Lim (2012), Meyer lemon was initially grown as part of dooryard decoration among home gardeners mostly because of its attractive foliage and sporadic, fragrant flowering. Moreover, Meyer lemon plants are claimed to be less cumbersome to be maintained as not much of pruning are required and they grow extremely well in the containers which render them fairly potential as ornamental plants (Badgett, 2016).

However nowadays, edible *Citrus* varieties are extremely familiar among fruit consumers not only because of their economical values and abundant nutritional properties, but also due to their constant availability that encourages its consumption as fresh fruits and as by-products (Ali and Mirza, 2006). Some countries are blessed with thrive conditions for successful cultivation of numerous lemon varieties for their regular purchasing (Love and Paull, 2013). This is in agreement to the report by Chiancone and Germana (2012) claiming that *Citrus* are very likely related to social background of their cultivated countries mainly as part of traditions and cultures to feature *Citrus* fruits in their cuisine.

Meyer lemon is continuously gaining recognition particularly in culinary industries as the fruits are commonly included into various food, bakery and confectionery courses owing to its distinctive lemony flavour. This lemon hybrid is very much preferred as substitute to regular lemons because of its sweeter taste and less acidic characteristics (O'hara, 2009). Lemons are vastly known for their high amounts of vitamin C and are not infrequent among chefs and home cooks (Love and Paull, 2013).The fresh fruits of lemon are typically eaten raw, crisp together with other food courses, served as food decorators, toppings or dressings, or freshly squeezed in drinks such as lemonade (Uckoo et al., 2015).

Nevertheless, the high levels of dihydrocarveol and thymol volatile components from the essential oils of Meyer lemon were significant compared to other lemon varieties, thus rendering it as a prominent food flavouring agent (Miyake et al., 2012). This fact is in parallel with Chanthaphon et al. (2008) that stated the essential oils and essences obtained from *Citrus* concentrations have been tremendously utilized in food industry as flavour boosts in soft drinks, alcoholic beverages and fruit derived products. Rao and McClements (2012) also corroborated and mentioned the utilization of lemon oils as flavouring agent is not infrequent in food and beverage industries owing to their volatile components with aromatic characteristics and hence, extensively been included in various products including baked goods, confectionary, desserts, ice cream and soft beverages.

Furthermore, the employment of *Citrus* plants in cosmetic, pharmaceutical and perfumery industries is not new and is frequently utilized as one of the active ingredients or raw materials for diverse kinds of products because of their significant contents of essential oils and polyphenols (Khan and Kender, 2007; Chiancone and Germana, 2012). In this context, Rafiq et al. (2018) reasoned that the notable

presence of phytochemical compounds and dietary fibres from *Citrus* peel are responsible for nutraceutical resources, even though the peel is likely disposed into the environment as waste product.

2.1.4 Medicinal Properties

Meyer lemon fruits have been reported to possess significant amounts of flavonoids viz. hesperidin and diosmin compounds as compared to other *Citrus* genotypes (Miyake et al., 2012). Uckoo et al. (2015) supported as they have identified the significant amounts of polymethoxylated flavones (PMFs) compounds such as narirutin, hesperidin and didymin, along with limonoids and amines compounds in Meyer lemon fruits. These advantageous compounds are frequently recognized as antioxidant, anti-proliferative and anti-inflammatory agents to against oxidative stress diseases such as cardiovascular disease (Rafiq et al., 2018).

The efficiency of bioactive compounds primarily is to hinder the incidences of chronic degenerative disorders via scavenging free radicals and reducing macromolecules damages caused by reactive species such as peroxyl radicals, alkyl peroxyl radicals, superoxide hydroxyl radicals and peroxynitrite in aqueous and organic conditions (Nakajima et al., 2014). The inhibitory efficacy and protection ability of PMFs compounds on cancer cell proliferation against microvascular endothelium during oxidative stress that essentially beneficial to hinder the growth of human cancer cells (Manthey and Guthrie, 2002). Hesperidin compound is associated with antioxidant properties due to its DPPH scavenging capacity and can dose-dependently hamper the Cu^{2+} oxidation of low density lipoprotein (LDL) *in vitro* (Zou et al., 2016).

Apart from that, groups of highly oxygenated triterpenoids namely limonoids was also effective for apoptosis induction and scavenging of free radicals that shield cell membrane lipids from oxidative damage (Zou et al., 2016). Yu et al. (2005) reported limonoids are highly competent to hinder chemically-induced abnormal tissue growth in the oral cavity, forestomach, small intestine, colon and lung of evaluated laboratory animals.

On the other hand, Marín and Del Río (2001) specifically attributed the anti-inflammatory efficacy to the predominant availability of diosmin compound in Meyer lemon fruits via venous capacitance and venous distensibilty reduction, and also alleviate the symptoms of severe haemorrhoids. However, the cumulative effect of PMFs compounds (hesperidin, nobiletin and tangeretin) was also responsible to encourage anti-inflammatory effect via obstructive activity against neuroinflammation where indestructible affinity to hamper iNOS and COX-2 expression in LPS and IFN-c was exhibited (Ho and Kuo, 2014; Chen et al., 2017).

Consistent consumption of Meyer lemon fruits has been linked to weight loss program mainly due to amines compounds (octopomine and synephrine) which stimulate lipolytic and thermogenic effects (Miyake et al., 2012). Strong lipolytic effect is mainly caused by cAMP-phosphodiesterase (PDE) inhibition that led to the elevation in cAMP levels and continuous stimulation to hormone-sensitive lipase (HSL), a lipolysis-triggered enzyme in human body (Dallas et al., 2008). Nonetheless, PMFs compounds also contribute to this effect through inhibition of body fat accumulation due to the increment of intracellular calcium (Ca^{2+}) in the adipocytes apoptosis which lead to weight loss maintenance and the avoidance of weight cycling (Sergeev et al., 2009; Sun et al., 2013).

Various clinical studies have suggested that physiological stress, anxiety and cortisol levels among hypertensive patients were effectively minimized through inhalation of essential oils (Hwang, 2006). Another prominent compound found in Meyer lemon essential oil namely limonene can be potentially used to treat anxiety and depression patients due to antidepressant effect on human nervous system through norepinephrine neurotransmission enhancement (Moshonas et al., 1972; Lund et al., 1982; Potdar and Kibile, 2011). A study carried out by Komori et al. (1995) has proven that *Citrus* scent was effective to restore the stress-induced immunosuppression through significant stimulation of the olfactory system and capable to normalize neuroendocrine hormone levels and immune functions better than antidepressant synthetic drugs, namely imipramine (Komori et al., 1995).

2.2 Plant Tissue Culture

Back in 1838, Schleiden and Schwann had visualized the cell potential to form into whole, complete plant under certain provided environment considering that cell is the basic structural unit for all living organisms. One decade later, Gottlieb Haberlandt, a German physiologist was the first personnel ever to culture the isolated single palisade cells from leaves on knop's salt solution containing sucrose. Although the cells was unsuccessful divided, but they were stayed alive up to one month, expanded in size and gained starch. Despite of his study was a failure, but Haberlandt is widely regarded as father of plant tissue culture as his study had given a fundamental about tissue culture technology which led to plenty and continuous tissue culture studies and researches until now (Hussain et al., 2012).

Plant tissue culture or prominently recognized as *in vitro* culture, is referring to the propagation technology of either single cell, tissue or any vegetative part of plants (explant) which inoculated into a sterile bottle culture, then is kept or maintained under entirely controlled environment and aseptic conditions. All the plant structures particularly the cells, organs, and the plants are totipotent to be morphologically or genetically cloned into full, complete genome through cell division and cell multiplication (Jha, 2005). Hussain et al. (2012) highlighted the mechanism of *in vitro* cultures whereas the plant cells or tissues are highly competent to alter infinitely their metabolisms, growth and developments that essential to regenerate the newly, whole plant.

With reference to Naik and Chand (2011), cell and plant tissue culture technology have become a major industrial influence due to their broad applications mainly for plant propagation, disease eradication, plant improvement and secondary metabolite production. This statement is supported by Hussain et al. (2012) that plant tissue culture is proven useful for secondary metabolites production from the plants which comprising medicinal and health-promoting compounds for diverse industrial manufacturing. Moreover, *in vitro* technology is extensively implemented mainly for several noteworthy advantages such as the optimal regeneration of disease-free plants as the process is performed under aseptic conditions, mass regeneration of plants clones with less human labour within limited space, maximum production of desired plants due to flexible optimization concerning numbers of key factors (nutrients media, level of plant growth regulators, light and temperature, genotype selection) within shorter duration and lastly the consistent plant production regardless of the season and weather changes (George et al., 2008). Simão et al. (2016) corroborated and mentioned that production of important phytochemicals

which restricted by seasonal variations, environmental factors and unsustainable natural harvest could be evaded as well. Thus, this is particularly effective to solve problems related to plant species of field culture (Mukhtar et al., 2005).

Generally, *in vitro* response are hugely influenced based on phytohormone perception, cell division and dedifferentiation efficacy of explants to obtain organogenetic competence, organ establishment and development (Duclercq et al., 2011). Hence, auxins and cytokinins are widely supplemented in tissue culture technology which later determined these organogenic responses. In this fate, the high amount of auxin stimulates root induction, high in cytokinins would triggered shoot induction while balance auxins-cytokinins contributes to the formation of unorganized cell mass, namely callus (Hussain et al., 2012).

Several previous studies have employed plant tissue culture in their plant production specifically to overcome their respective issues. For instance, mass propagation of date palm *Phoenix dactylifera* L. production that has limited efficiency due to its dioecious nature (Eke et al., 2005). Apart from that, this *in vitro* technology has been described competent to eliminate diseases or infections such as pathogen elimination for *Stevia rebaudiana* Bertoni and okra (*Abelmoschus esculentus* L. monech), and also minimized the risks of bacteria, fungi and pests infestations into aquarium environment surrounding *Hemianthus callitrichoides* (Lata et al., 2013; Ng et al., 2016; Daniel et al., 2018). In addition, a genetic study by Thakur et al. (2012) also emphasized the efficacy of tissue culture protocol on *Populus deltoides* to establish genetically engineer *P. deltoides* with silviculture-beneficial traits such as insect and disease insusceptibility and herbicide tolerance via *Agrobacterium* mediated transformation.

2.3 Direct Organogenesis

Organogenesis/morphogenesis in *in vitro* technique defines as the regeneration or promotion of lacking plant cells or tissues into newly, complete form and organization to be functional for certain processes. This is commonly exhibited from inoculation of plant tissues where the explant sources namely seedling, shoot tips and nodes cultures are totipotent for the optimal shoot growth with the supplementation of appropriate phytohormones (George et al., 2008). The process of *in vitro* organogenesis has been classified into direct or indirect (Hamasaki et al., 2005).

With reference to Duclercq et al. (2011), there are three morphological stages of direct shoot regeneration which are firstly, the morphogenic competence tendency (cell dedifferentiation), followed by the induction (cell determination for specific organ initiation in response to exogenous phytohormones) and lastly the morphological differentiation (organ morphogenesis proceeding independently of exogenous phytohormones). The differentiation process during shoot morphogenesis required several biochemical processes and cellular signaling whereby growth regulators are typically mentioned as one of the key role to regulate these processes. Thus, Saha et al. (2016) were highly recommended the independent optimization of micropropagation protocols for different plant species. This is because the exogenous levels of the plant hormones are hugely affected by the amounts of endogenous ones which apparently displayed different optimal responses among vary organs, genotypes and growth stages of the plants.

This is congruent with Chitra and Padmaja (2005) study as they have proven that supplementation of BAP produced the most optimal shoot proliferation rates of mulberry (*Morus* spp.) compared to Thidiazuron treatments. Herein, they reasoned that varied response of different cytokinins are likely because of different genomes possess different uptake capacity, dissimilar rates of translocations to meristematic regions and metabolic processes which the cytokinin might be disintegrated or bonded with sugars or amino acids to produce biologically inert compounds (Chitra and Padmaja, 2005). On the other hand, several other studies of *Stevia rebaudiana* Betoni and *Eucalyptus tereticornis* were much preferred Thidiazuron and antibiotic (cefotaxime) inclusion to stimulate their most optimal shoot proliferation respectively, due to alterations of certain endogenous growth regulators had taken place (Aggarwal et al., 2010; Lata et al., 2013).

Furthermore, another enlisted factor that could hugely affect the shoot regeneration efficiency is the explant source. The maximum percentage of direct shoot organogenesis was obtained when intermediate leaves sizes (1-4 cm) of mulberry (*Morus* spp.) leaves was cultured on the abaxial side (Chitra and Padmaja, 2005), whilst the maximum shoot frequency of *Eucalyptus tereticornis* was acquired when the fifth leaf from the microshoot top (14-16 days old) used as starting materials compared to mature and younger leaves (Aggarwal et al., 2010). Apart from that, a study by Kumar et al. (2005) has identified that the reversely-positioned of the *Capsicum annuum* L. young seedlings resulted a significantly unsatisfactory results of shoot buds initiation. They inferred that this revert position possibly contribute to the suppression of apical dominance thus impede morphogenetic process.

2.4 Tissue culture of *Citrus* plants

As diverse *Citrus* species have been extensively cultured around the globe, this indicated that these plants have been considered as one of the most vital horticultural fruit cops among the public mainly due to their refreshing taste and highly beneficial for human health (Carimi and De Pasquale, 2003). Generally, the commercial *Citrus* plants are broadly propagated sexually through seed, and asexually through cutting or grafting (Ali and Mirza, 2006).

Nonetheless, Perez-Molphe-Balch and Ochoa-Alejo (1997) reported that most of *Citrus* species have difficulties in plant breeding mainly because of their reproductive nature (partial or complete sterility of pollen and ovule, gametophytic systems of self or cross incompatibility and apomixis from nucellar cells), long periods of juvenile phase and the unknown mode and nature of offspring inheritance. Therefore, the employment of plant tissue culture is capable to provide mass numbers of high qualities *Citrus* cultivars and rootstocks to the growers via speeding up the production of true-to-type and disease free *Citrus* plants (Chiancone and Germana, 2012). Moreover, this modern technique also resolved several problems encountered by certain *Citrus* genotypes such as *Citrus reticulata* cv. Khasi mandarin and *C. limon* cv. Assam lemon which are highly heterozygous and have long juvenility phase of the seed-derived plants, and the seedless 'Kaghzi Kalan' lemon that incompetent to yield sufficient production using conventional techniques in order to meet the high market demand (Singh et al., 1994; Goswami et al., 2013).

The isolation of different tissues and organs cultures for most *Citrus* cultivars are attainable via plant tissue culture due to optimal plant regeneration and biotechnological development where the description of various tissue culture protocols are well reported (Tavano et al., 2009). Numerous studies regarding *in vitro* technique of *Citrus* rootstocks and explants have been published and discussed whereby most of them emphasized that the regenerative ability of the plants are hugely influenced by critical factors such as genotype, explant sources and composition of culture medium (Pérez-Tornero et al., 2010). The aforementioned statement was supported by Tavano et al. (2009) and Thakur et al. (2012) where they were specifically highlighted that the key factors contribute to an effective *in vitro* protocols was hugely affected by genotypes, explants sources, cytokinin and auxin levels and culture conditions. According to Perez-Molphe-Balch and Ochoa-Alejo (1997), these are because of different responses were observed from previous *in vitro Citrus* studies where each particular species yielded different responses to the culture conditions employed. Therefore, it is compulsory to optimize the regeneration protocol among different *Citrus* cultivars as the qualitative and quantitative differences in organogenic response were significantly remarkable (Bordon et al., 2000).

Based on previous literatures, *Citrus* explants that extensively used to encourage the maximum number of direct shoot buds include the internodal and epicotyl segments of various *Citrus* plants such as *Citrus aurantifolia*, *C. reticulata* Blanco cv. Monica, *C. aurantium* L., *C. macrophylla* Wester, *C. sinensis*, *C. limonia* L. Osbeck, *C. jambhiri* Lush (Perez-Molphe-Balch and Ochoa-Alejo, 1997; Bordon et al., 2000; Almeida et al., 2002; Rattanpal et al., 2011). An earlier study by Singh et al. (1994) also manifested that efficacy of shoot tips explants for both *C. reticulata*

cv. Khasi mandarin and *C. limon* to yield the highest frequency of direct shoot regeneration. Apart from that, high rates of cell proliferation induced via *in vitro* technique have been utilized for genetic transformation and mutagenesis studies (Pérez-Tornero et al., 2010). This is in agreement with El Sawy et al. (2014) as they emphasized that plant tissue culture is totipotent to regenerate high regeneration of tissue explants and embryogenic cells particularly for protoplast fusion and genetic transformation studies among *Citrus* species. This is mainly due to the source of genetically homogeneous plant cells and tissues that are consistently reproduced that necessary in every genetic manipulation work (Pérez-Tornero et al., 2010).

In this context, researchers have integrated biotechnological tools such as somatic hybridization and genetic transformation into *Citrus* breeding programs mainly because of their high vulnerability towards phytopathogens and pests (Perez-Molphe-Balch and Ochoa-Alejo, 1997). A number of agronomically important genes have been introduced to various *Citrus* cultivars to generate new pest and disease resistant or tolerant species and to accelerate the improvement process, selection and the release of new *Citrus* cultivars for a better *Citrus* industry worldwide (Almeida et al., 2003). Unfortunately, the study of genetic transformation of some *Citrus* species and hybrids have encountered several difficulties such as low genetic transformation efficiency, problems for rooting of the transformed plantlets and high number of escapes (Almeida et al., 2002). Therefore, an effective, highly-specific *in vitro* regeneration protocol on each *Citrus* species is vital to overcome issues for instance, the recalcitrant nature in certain species such as *Citrus limonia*, *C. volkameriana* and *C. aurantium*. This will significantly influenced gene transfer activities and also the effectiveness of genetic transformation outcomes (Tavano et al., 2009).

2.5 Somatic embryogenesis

Way back in 1958, somatic embryogenesis (SE) regeneration pathway was initially discovered by Steward and Reinert in *Daucus carrota* or wild carrot. Over the years, numerous studies regarding the development of somatic embryogenesis have been performed where somatic embryos are successfully induced from various plant species including angiosperms, gymnosperms and ferns. Herein, it was proven that somatic embryogenesis is possible via two different ways; directly where the somatic embryos are formed from the explants without callus intervention, and indirectly where the somatic embryos induction takes place through intermediate structure, namely callus (Xu et al., 2015).

George et al. (2008) have defined somatic embryogenesis as a process where the somatic cells are competent for cell differentiation or cell division into somatic embryos and capable to develop into a complete, functional whole plant because they contain all the necessary genetic information. The aforementioned statement was reasoned by an earlier study by Zimmerman (1993) that the ability of somatic cells to regenerate structurally and developmentally normal embryos indicated the genetic process for embryogenesis is completely contained within the cells.

In contrast to zygotic embryos, somatic embryos are documented as bipolar structures and have no apparent vascular connection with the original tissues albeit morphologically both embryos are resemble to each other with typical embryogenic organs, the radicles, hypocotyls and cotyledons parts (Agisimanto et al. 2012). This is corroborated by Zimmerman (1993) that several factors that prominently distinguish somatic and zygotic embryos such as firstly, the morphogenesis dependence on polar auxin transport. In somatic embryogenesis pathway, the embryos treatments with polar auxin transport inhibitors tend to obstruct the

morphogenesis to move to the next embryo stage while, zygotic embryos are less affected with the same inhibitors. Secondly, the dormancy difference between these two pathways was identified once torpedo stage surpassed. In this regard, zygotic embryos will develop to cotyledon stage, followed by maturation stage then prepare for desiccation and dormancy. On the other hand, somatic embryos would continuous to grow and differentiate, activates the shoot and root apical meristems with no apparent dormancy state occurs (Zimmerman, 1993).

In general, the prime interest of somatic embryogenesis is mostly for plant mass propagation, germplasm conservation and exchange, and also for gene banks establishment via cryopreservation, sanitation, metabolite production and synthetic seed manufacturing (Chiancone and Germana, 2012). Nonetheless, the studies of somatic embryogenesis are not only for high regeneration efficacy, but also for the infrequent occurrence of somaclonal variation. The most prominent feature of somatic embryos is that plants regenerated from this morphogenesis pathway are predominantly normal and possess no phenotypic and genotypic variation because they are produced from single cells with stringent selection for embryogenesis of normal cells (Omar et al., 2016). Besides, the implementation of somatic embryogenesis in germplasm preservation is mainly to initiate high-efficiency of transformation systems which including the production of genetically modified plantlets from single cell and also to avoid mosaics occurrence within short period of time and lesser labour (Xu et al., 2015).

2.6 Somatic embryogenesis of *Citrus* plants

Although *Citrus* plants and their wild relatives have noticeably high genetic diversity traits, however plant improvement studies of *Citrus* via conventional methods was found to be difficult due to the presence of nucellar embryos, high degree of heterozygosity, long juvenility and sterility (Carimi, 2005). Moreover, the production of new *Citrus* cultivars is occasionally required to create new marketing opportunities and to combat threatened diseases which are possible through genetic transformation programs. Therefore, somatic embryogenesis approach is commonly performed for an efficient plant recovery as some modified genes with highly-desired traits such as hostility to *Phytophtora*, *Citrus* canker, *Citrus* greening (Huanglongbing), *Citrus* variegated chlorosis, blight and drought can be integrated with complementary parent genes without altering the important characteristics of specific cultivars or cultivars groups (Omar et al., 2016). Similarly, Kazmi et al. (2015) performed this organogenic pathway on Kinnow mandarin as an approach to develop varieties with disease, salt and drought tolerant important to sustain large-scale production of this fruit crop.

Besides, *in vitro* propagation techniques appear to be a viable and efficient to overcome difficulties associated with conventional breeding methods. According to Pati et al. (2015), this modern technique is competent to reproduce mass number of desired plants within comparatively shorter time span rather than conventional ones and also ideal to mass regenerate the disease-free new plants. In this regard, Gholami et al. (2013) specifically emphasized that numerous studies of *Citrus* species have been extensively established and documented. With reference to Carimi (2005), somatic embryogenesis is particularly preferred due to the genetic fidelity of somatic cells or tissues as those cells are originated, formed and developed directly from

maternal tissues. This is corroborated by Carimi and De Pasquale (2003) as they mentioned that most of *Citrus* genotypes are apomictic and polyembryonic in nature which responsible for the formation of genetically uniform plants mainly due to the emergence of adventitious embryos grown directly from mother plant cells. Thus, it was proven that this morphogenesis pathway encourages the production of vigorous, morphologically and genetically identical new plantlets which is indifferent from the original explants (Agisimanto et al., 2012).

Several earlier studies of *Citrus* somatic embryogenesis are well reported albeit different regeneration efficacy has been observed among these *Citrus* genotypes (Carimi and De Pasquale, 2003). One of the studies was the induction of genetically similar somatic embryos from immature seeds of six different monoembryonic *Citrus* genera through callus intervention by Ling and Iwamasa (1997). Apart from that, a study conducted by Carimi and De Pasquale (2003) yielded the maximum somatic embryo regeneration with highly-pathogenic tolerant and shorter fruit bearing duration using stigma and style of various *Citrus* flowers such as lemons, sweet oranges, mandarins and grapefruits. Similarly, El Sawy et al. (2014) obtained the highest embryogenesis percentage and maximum number of embryos using stigma explants of variegated lemon and citron. On the other hand, the maximum induction of somatic embryogenesis in Kinnow mandarin was achieved within relatively a short period of time using nucellus tissues in MS media supplemented BAP solely (Hussain et al., 2016). Furthermore, a number of *Citrus* studies also indicated that the inclusion of malt extract was proven essential for the optimal regeneration of somatic embryos of six *Citrus* genotypes (*C. deliciosa* cv. Avana, *C. limon* cv. Berna, *C. madurensis* cv. CNR P9, *C. medica* cv. Cedro, *C. tardiva* Hort cv. CNR P6 and *C. sinensis* L. cv. Ugdulena 7), *C. aurantifolia, C.*