# *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF *DENDROBIUM* SONIA-28

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# *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF *DENDROBIUM* SONIA-28

**by**

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| A | Absorbance |
| ANOVA | Analysis of variance |
| BAP | 6-Benzylaaminopurine |
| bp | Base pairs |
| CaMV | Cauliflower Mosaic Virus |
| cm | Centimetre |
| Co | Company |
| cv | Cultivar |
| *CymMV* | *Cymbidium* Mosaic Virus |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleotide triphosphate |
| DTT | Dithiothreitol |
| EDTA | Ethylenediaminetetraacetic acid |
| *et al.* | *et alia* |
| EtBr | Ethidium bromide |

| | |
|---|---|
| Fig | Figure |
| g | Gram |
| $g.L^{-1}$ | Gram per litre |
| L | Litre |
| LB | Luria Bertani |
| Lindl | Lindley |
| M | Molar |
| *M* | Mean |
| mg | Milligram |
| $mg.L^{-1}$ | Milligram per litre |
| $mg.mL^{-1}$ | Milligram per millilitre |
| $min^{-1}$ | Per minute |
| mL | Millimetre |
| mM | Milimolar |
| mm | Millimetre |
| MS | Murashige and Skoog |
| NAA | Naphthalene acetic acid |

| | |
|---|---|
| nm | Nanometre |
| ng | Nanogram |
| *nptII* | Neomycin phosphotransferase II |
| OD | Optical density |
| PCR | Polymerase chain reaction |
| PLB | Protocorm-like body |
| PLBs | Protocorm-like bodies |
| psi | Pound per square inch |
| rpm | Revolution per minute |
| SEM | Scanning electron microscope |
| *SD* | Standard deviation |
| sp | Species |
| *Taq* | *Thermos aquaticus* |
| TBA | Tertiary butyl alcohol |
| TBE | Trishydroxymethylaminomethane-borate-ethylenediaminetetraacetic acid |
| T-DNA | Transfer deoxyribonucleic acid |

| | |
|---|---|
| TM | Trademark |
| Tris HCL | Trishydroxymethylaminomethane-hydrochloride |
| U | Unit |
| UK | United Kingdom |
| USA | United States of America |
| UV | Ultraviolet |
| V | Volt |
| w/v | Weight over volume |
| × | Times |
| % | Percentage/percent |
| $^{o}$C | Degree Celsius |
| / | Division/or |
| + | Plus |
| - | Minus |
| ± | Plus or minus |
| = | Equal to |

| | |
|---|---|
| µ | Micro |
| µg | Microgram |
| $\mu g.L^{-1}$ | Microgram per litre |
| µL | Microlitre |
| µm | Micrometre |
| µM | Micromolar |

**TRANSFORMASI MELALUI PERANTARAAN *AGROBAKTERIUM* BAGI *DENDROBIUM* SONIA-28**

## ABSTRAK

Propagasi yang berkesan bagi jasad seperti protokom (JSP) hibrid orkid *Dendrobium* sonia-28 diperolehi melalui teknik mikropropagasi. Proliferasi tertinggi bagi JSP dalam masa 21 hari telah diperhatikan dalam media separa pepejal berbanding kultur media cecair. Media dengan setengah kekuatan Murashige dan Skoog (MS) menghasilkan peratus kadar pertumbuhan JSP tertinggi berbanding media dengan kekuatan penuh ataupun dua kali ganda MS dalam kultur separa pepejal dan cecair. Dalam rawatan sukrosa, peratusan kadar pertumbuhan JSP tertinggi adalah dalam 10 $g.L^{-1}$ sukrosa. Dalam keadaan tanpa kehadiran sukrosa (0 $g.L^{-1}$) atau berlebihan sukrosa (30 $g.L^{-1}$) terbukti memudaratkan pertumbuhan dan perkembangan JSP. Peratus kadar pertumbuhan JSP adalah tertinggi (14.45%) dalam media tanpa penambahan hormon eksogen 6-Benzilamino purina (BAP) atau asetik asid naftalena (NAA). Namun begitu, penambahan BAP dan NAA dalam kombinasi BAP (4.44 atau 8.88 µM) dan NAA (8.88 µM) menghasilkan peningkatan pertumbuhan JSP kepada 14% berbanding apabila ditambah secara berasingan. Perkembangan JSP diperhatikan pada peringkat selular menggunakan analisis histologi. JSP sekunder terbentuk secara terus daripada sel-sel meristematik yang aktif membahagi pada lapisan epidermis tisu utama tanpa memerlukan pembentukan tisu perantaraan. Berdasarkan pemerhatian imbasan mikroskop elekron (IME), struktur permukaan JSP didapati sesuai untuk memudahkan jangkitan *Agrobakterium* semasa proses transformasi. Interaksi antara *Agrobakterium* dan tisu tumbuhan

dinilai berdasarkan motiliti dan kecekapan cangkuman *Agrobakterium*. Tindak balas motiliti *Agrobakterium* adalah tertinggi (1.5 unit) kepada JSP bersaiz besar (3-4 mm) tanpa mengira keadaan kecederaan tisu. Kecederaan pada tisu tumbuhan didapati tidak relevan untuk mencetuskan tindak balas motiliti *Agrobakterium* terhadap JSP. Walau bagaimanapun, kecederaan pada tisu JSP didapati signifikan dalam meningkatkan kapasiti cangkuman (75%) *Agrobakterium* pada tisu tumbuhan. JSP yang tidak ditransformasikan telah dirawat dengan geneticin, kanamycin dan neomycin untuk menentukan kepekatan perencatan minimum antibiotik sebagai ejen pemilihan. Geneticin ketara cekap dalam menghalang pertumbuhan JSP pada kepekatan 250 $mg.L^{-1}$, manakala kanamycin dan neomycin terbukti kurang berkesan sebagai ejen pemilihan akibat peratusan yang tinggi bagi JSP yang terselamat dalam kepekatan 300 $mg.L^{-1}$. Transformasi perantaraan *Agrobakterium* telah dijalankan terhadap JSP menggunakan *Agrobakterium tumefasiens* jenis LBA 4404 yang mengandungi plasmid pW1BI atau pW2KY. Plasmid pW1BI mengandungi gen *wwin1* manakala plasmid pW2KY mengandungi gen *wwin2*. Kedua-dua plasmid juga mengandungi gen *nptII* yang memberi rintangan kepada antibiotik aminoglikosida. Transformasi gen-gen *wwin1* dan *wwin2* menghasilkan 30.2% dan 36.7% pertumbuhan JSP ketika pada pemilihan pertama dalam geniticin. Selepas pemilihan selama dua bulan, transformasi gen *wwin1* dan *wwin2* menghasilkan 5.4% dan 7.6% JSP yang mampu merentang rawatan geneticin pada kepekatan 250 $mg.L^{-1}$. Analisis pada peringkat molekular menggunakan tindak balas rantai polimerase mengesahkan integrasi gen-gen *wwin1* dan *wwin2* dalam JSP transgenik yang berjaya dijana semula dengan saiz produk 300bp. Integrasi gen *nptII* juga berjaya dikesan dalam JSP transgenik yang dijana semula pada saiz 464bp. Kesimpulannya, protokol yang dibentangkan dalam kajian ini adalah sesuai untuk transformasi stabil

gen wheatwin ke dalam orkid hibrid *Dendrobium* sonia-28 sebagai langkah bagi meningkatkan rintangan orkid terhadap jangkitan kulat.

## *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF *DENDROBIUM* SONIA-28

## ABSTRACT

Effective propagation of protocorm-like bodies (PLBs) of *Dendrobium* sonia-28 was achieved by using micropropagation technique. The highest PLBs proliferation within 21 days was observed in semi-solid media over liquid culture. Half strength of Murashige and Skoog (MS) media produced the highest PLBs growth rate percentage compared to full and double strength MS media in semi-solid and liquid cultures. In sucrose treatment, the highest PLBs growth rate percentage was in 10 $g.L^{-1}$ sucrose. In the conditions of absence of sucrose (0 $g.L^{-1}$) or excessive of sucrose (30 $g.L^{-1}$) had proven detrimental for the PLBs growth and development. PLBs growth rate percentage was the highest (14.45%) in media devoid of exogenous 6-Benzylaminopurine (BAP) or Naphthalene acetic acid (NAA). However, the addition of BAP and NAA in combinations of BAP (4.44 or 8.88 µM) and NAA (8.88 µM) resulted in increased PLBs growth to 14% compared to when added separately. The development of PLBs was monitored at cellular level based on histology analysis. Secondary PLBs were directly formed from the actively dividing meristematic cells of the epidermal layer of primary tissue without any intermediate tissue development. Based on scanning electron microscope (SEM) observation, the surface structure of PLBs was found suitable for facilitating *Agrobacterium* infection during transformation process. The interaction of *Agrobacterium* to plant tissue was evaluated based on *Agrobacterium* motility and attachment competency. *Agrobacterium* motility respond was the highest (1.5 unit) toward large size (3-4

mm) PLBs regardless of wounding conditions. Wounding of plant tissue was found to be irrelevant to induce *Agrobacterium* motility response to the PLBs tissue. However, wounding in PLBs tissue was found to be significant in the increased of *Agrobacterium* attachment capacity (75%). Non-transformed PLBs were challenged with geneticin, kanamycin and neomycin to determine the minimal inhibitory concentrations of the antibiotics as selection agents. Geneticin significantly inhibited PLBs growth at 250 $mg.L^{-1}$, whereas kanamycin and neomycin proved to be poor due to high percentage of PLBs survived at 300 $mg.L^{-1}$. *Agrobacterium* mediated-transformation was carried out on PLBs using strain LBA 4404 harbouring either pW1BI or pW2KY plasmid. The pW1BI plasmid contained *wwin1* gene whereas pW2KY plasmid contained *wwin2* gene. Both plasmids also contained *nptII* gene conferring resistance to aminoglycoside antibiotics. Transformation of *wwin1* and *wwin2* genes produced 30.2% and 36.7% growing PLBs during the first geneticin selection. After two months of selection, the transformation of *wwin1* and *wwin2* genes revealed 5.4% and 7.6% of PLBs expressing resistance against the geneticin treatment at 250 $mg.L^{-1}$. Molecular analysis using polymerase chain reaction (PCR) confirmed the integration of *wwin1* and *wwin2* genes in the regenerated transformed PLBs with the size of amplification product at 300bp. The integration of *nptII* gene was also detected in regenerated transgenic PLBs at the size of 464bp. In conclusion, the protocol presented in this study is suitable for stable transformation of wheatwin genes into *Dendrobium* sonia-28 orchid hybrid as a step to improve orchid resistance against fungal infection.

# CHAPTER 1

# INTRODUCTION

The Orchidaceae family is known to be one of the largest family of flowering plants. At least 35,000 orchid species has been recorded worldwide with the most variety found on tropical mountains (Dressler, 1993). Malaysia have 3887 beautiful wild orchids, with total of 2000 species found at Mount Kinabalu of Sabah, and about 1000 species scattered all over Sarawak (Rusea and Khalil, 2008). Orchids are appreciated by many due to their availability in various habitats, high diversity and beneficial properties or simply due to their beautiful morphologies. In the book *Fundamental of Orchid Biology,* Professor Joseph Arditti compiled an inclusive history of the cultural significance of orchids around the world (Arditti, 1992).

In modern days, orchids have one of the highest trading values in the flower industry. In 2009, 77% out of US$ 80 million of Thai floriculture products were contributed by orchid export (Supnithi *et al.,* 2011). In 2010, Taiwan exported more than 14 million *Phalaenopsis* orchid plants valued at approximately US$ 82.55 million to the United States (Australia-Taiwan Orchid Media Releases, 2011). Orchids are widely consumed by many cultures around the world as food, spices, flavouring, beverages, drugs, medicine, arts, and in religion and superstition (Arditti, 1992). It is a highly popular choice in weddings and birthdays, and for Valentine's Day, Mother's Day, New Year and days to honour the dead.

Many orchid farmers are still lagging in fulfilling the international orchid demand, as the main reason is that the orchids are still being produced through conventional breeding methods. Conventional breeding programs use sexual

hybridization (inter- and intra species) and through selection of variant or polyploids (Semiarti *et al.,* 2011). Problems such as the long growing cycle of orchids, complex procedures of conventional breeding, cross incompatibility of some orchid species, large cultivation area requirement and labour costs are the major drawbacks of this method (Manshardt, 2004). Modern micropropagation and genetic engineering methods offer orchid breeding platform to solve such problems.

Micropropagation is a procedure bypassing the sexual process in orchids for the asexual propagation of plantlets from any orchid organ, tissue or cell through the practice of plant tissue culture (as reviewed by Arditti, 2008). Micropropagation which is relatively easy to perform, shortens the length of the orchid life cycle through the modification of nutrient rich medium and the creation of similar microhabitat for the orchid to grow prosperously.

Genetic engineering is a tool for transferring specific genetic information from one species of organisms to another (Jauhar, 2006). Genetic engineering aims to modify the traits of the target plant by incorporating exogenous genes into the plant chromosomes or by silencing certain endogenous genes (Yu and Xu, 2007). It is a quick and flexible technology as the gene donor and recipient need not be of the same species or compatible for breeding and gene transfer can be done in months compared to conventional breeding (Ronald, 1997).

Orchids grown in warm and humid weather with long night period such as Malaysia and Thailand are highly susceptible to pathogen attack. Diseases caused by bacterial (eg. *Erwinia corotovora*), viral (Cymbidium Mosaic Virus: CymMV) and fungal (*Fusarium solani*) infections cause major loss of yield with visible effects such as flower and leaf discolouration, stunted growth and the death of young plant (Ichikawa and Aoki, 2000; Chang *et al.*, 2005; Sjahril *et al.*, 2006).

In 1997, Pamela Ronald and her colleagues successfully inserted the *Xa21* gene in rice, enhancing rice plants resistance to bacterial blight which in nature known to causes leaf damage and yield reduction (Ronald, 1997). The report opened up the potential of genetic engineering in the development of disease resistant plants to overcome the problem of destructive diseases. Sridevi *et al*. (2008) reported that the transformation of rice with a combination of chitinase and β-1,3-glucanase successfully conferred resistance against *Rhizoctonia solani*, which is known to cause sheath blight disease. Khan *et al*. (2006) described transgenic potatoes expressing wasabi defensin peptide as partially resistant against gray mold caused by *Botrytis cinerea*. Over the years, many transgenic plants and crops resistant to devastating diseases have been reported (Tabei *et al.*, 1998; Chan *et al.*, 2005; Noel *et al.*, 2005; Sreeramanan *et al*., 2010).

A number of transgenic orchids were also developed to express resistance against certain diseases caused by pathogen attacks. Resistance to *Erwinia caratovora*, known to cause soft rot disease, was exhibited in transgenic *Oncidium* orchid introduced with the *pflp* gene (Liao *et al.*, 2003b) and in *Phalaenopsis* introduced with the wasabi defensin gene (Sjahril *et al.*, 2006). Transgenic *Dendrobium* transformed with virus capsid protein showed high resistance to CymMV (Chang *et al.*, 2005). Transgenic *Phalaenopsis* orchid expressing CymMV coat protein which was retransformed with the *pflp* gene displayed strong tolerance to both CymMV and *Erwinia coratovora* (Chan *et al.*, 2005).

Currently, particle bombardment and *Agrobacterium tumefaciens*-mediated transformations are the two most widely used tools in orchid genetic engineering purpose (Fan, 2011; Retheesh and Bhat, 2011). *Agrobacterium tumefaciens* is a natural pathogenic soil bacterium that causes crown gall disease to the infected

susceptible plants. The infection process occurs when the bacterium transfers the T-region of its large Ti (tumor inducing) plasmid into the host plants and the genes are integrated into the plants' genome. The oncogenic function of *Agrobacterium* is removed in laboratories, thus producing a disarmed strain of the bacterium. The modification is followed by the insertion of the desired foreign DNA between the border sequences and the reduction of the large size of the native Ti plasmid. At the end of the T-DNA, located the *vir* genes, which are important components for the purpose of T-DNA transfer into host plants (Jones, 2006; Ptizschke and Hirt, 2010).

Orchids like other monocots, are not natural host for *Agrobacterium* infection therefore transformation via this system was thought difficult (Messens *et al.*, 1990). However, recent increases in number of successful *Agrobacterium*-mediated transformations were achieved in many orchids species including in *Dendrobium* sp. (Yu *et al.*, 2001), *Odontoglossum* sp. (Raffeiner *et al.*, 2009), *Cattleya* sp (Zhang *et al.*, 2010), and *Oncidium* sp. (Raffeiner *et al.*, 2009; Thiruvengadam *et al.*, 2011) thus effectively overcoming the natural barrier of *Agrobacterium*-plant interaction. *Agrobacterium*-mediated transformation system becomes favourable due to its simple technique which does not require expensive equipments and high efficiency of transformation (Veluthambi *et al.*, 2003; Barampuram and Zhang, 2011).

The convenient method of gene transfer using *Agrobacterium tumefaciens* provides a great tool to stably integrate antifungal genes into the chromosome of *Dendrobium* sonia-28. Currently, there is no record of wheatwin genes transformation mediated by *Agrobacterium tumefaciens* in this hybrid.

## 1.1 Objectives of research

The objectives of this research project were:

a) To micropropagate *in vitro* PLBs of *Dendrobium* sonia-28 through the modification of the strength of culture media, level of sucrose, liquid or semi-solid media, and plant growth regulators supplementation,

b) To study the amenability of PLBs obtained from optimum culture condition for transformation and regeneration through histology and scanning electron microscopy analysis,

c) To investigate the role of chemotaxis and quantification of *Agrobacterium tumefaciens* attachment as indication of the *Agrobacterium* virulence capability,

d) To determine the minimum inhibitory concentration of geneticin, kanamycin and neomycin as potential selection agents for selection of transgenic *Dendrobium* sonia-28 PLBs,

e) To transform *Dendrobium* sonia-28 with *wwin1* and *wwin2* genes for potential fungal disease resistance via *Agrobacterium*-mediated transformation.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Orchid development for commercialisation

The Orchidaceae is a large family of flowering plants with 35,000 orchid species recorded worldwide (Dressler, 1993). Orchids are highly traded as cut flower or potted plant in the flower industry worldwide (Japan Flower Trade Association, 2009; Supnithi *et al.*, 2011). Apart from its aesthetic value, orchids also widely favoured as food, spices, flavouring, beverages, drugs, medicine, arts, and in religion and superstition (Arditti, 1992). Orchid commercial production is initially achieved via conventional breeding programme. Improvement and development of new hybrids involves the use of sexual hybridization and through selection of variant or polyploids (Semiarti *et al.*, 2011). Closely related orchid species are sexually hybridised and the progeny with the most positive trait is selected. The selected progeny will be hybridised with another orchid or with one of its parent, in a process called "back-crossing" until the desired trait is produced.

However, conventional breeding program is very inconvenient for mass production of orchids due to the long life cycle of orchid, the extended period of selection process, and the difficulty in manipulating the specific desired trait (Chai and Yu, 2007; Chai and Yu, 2008). Breeding barrier such chromosome incompatible between inter- or intraspecies orchid is a major drawback to this programme (Hsu *et al.*, 2010). The program also requires huge cultivation area, high labour cost, high material cost, and high maintenance cost (Singh *et al.*, 2007; Matsui, 2010).

Conventional breeding is also at disadvantage due to the requirement of specific cultivation of certain orchid to overcome problems such as pest, diseases, mycorrhiza interaction and environmental stresses (Mishiba *et al.,* 2008). For example, endangered orchids such as *Dendrobium hallieri* and *Dendrobium singkawagense* were difficult to cultivate due to complications in imitating the microhabitats suitable for the growth of the orchid specific myccorhiza (Siregar, 2008). Hence, micropropagation and genetic engineering procedures were extensively studied to offer orchid breeding new potential for advanced orchid development and improvement.

## 2.2 *Dendrobium* genus

*Dendrobium* is a large genus of the Orchidaceae family which has more than 1,100 species around the world and is highly valued in the ornamental flowers industry (Luo *et al*., 2008). *Dendrobium* is sympodial orchid and mostly epiphytes (Kuehnle, 2007) along with a few terrestrial (such like *Dendrobium lobii* Teijsm. and Binn. and *Dendrobium salaccense*) or epipteric (Rusea and Khalil, 2008).

The value of *Dendrobium* attributed to potted plant sales at wholesale in Hawaii increased from US $2.4 million in 1991 to US $5.6 million in 2000 (Kuehnle, 2007). *Dendrobium* contributed 11.7% from RM 40 million of Malaysia's orchid exports, annually (Khosravi *et al*., 2008). Japan imported cut flower from its surrounding ASEAN countries mainly from Thailand, Singapore and Malaysia with 90% of the imported flowers are *Dendrobium* (Japan Flower Trade Association, 2009). Floriferous flower sprays in wide range of colours, sizes and shapes are the

main attractions to *Dendrobium* high market demand as ornamental and cut flower (Kuehnle, 2007).

*Dendrobium* is also widely used as in cosmetic and medicinal products. *Dendrobium*, also known as *Shih-hu* in Chinese or *Sekkoku* in Japanese, derived a group of sesquiterpene alkaloids used in Chinese traditional medicine as tonic to improve digestion, promote body-fluid production, nourishing *yin* and eliminating heat (Shiau *et al*., 2005). The stem of *Dendrobium huoshanense* is used to treat salivary, stomach and ophthalmic disorder (Hsieh *et al*., 2008). Dried drug of *Shih-hu* reach up to US$ 4000 $Kg^{-1}$ (Shiau *et al*., 2005). In India, *Dendrobium* was used as indigenous medicine in Ayurvedic system (Singh *et al*., 2007).

### 2.2.1 *Dendrobium* sonia-28

*Dendrobium* sonia is a cross between pollen parent *Dendrobium* Tomie Drake and seed parent *Dendrobium* Caeser. The hybrid was registered in Bangkok Orchid by P. Chittraphong in 1984 (The International Orchid Register). *Dendrobium* sonia-28 (Plate 2.1) and its other hybrid siblings are famous due to their fast growing cycle, durability with long shelf life, floriferous inflorescence, bright colour flowers and free flowering characteristics (Fadelah, 2007). The sepal has pointed end and white background with bright purple at the upper half. The lateral petals are broad and are purple in colour with little white shade at the base (as reviewed by Xiang and Hong, 2003).

*Dendrobium* sonia-28 has been reported in a number of interesting researches focusing on quality improvement. Martin and Madassery (2006) reported revised media compositions to improve *in vitro* micropropagation of PLBs production,

shooting and rooting of *Dendrobium* sonia-28. The growing need to preserve the unique germplasm of *Dendrobium* sonia-28 had encouraged numerous studies to investigate the potential of *Dendrobium* sonia-28 for cryopreservation technology (Hwa *et al.*, 2009; Hooi *et al.*, 2010). A novel cytokinin oxidase, which function is to regulate cytokinin production in plant, was isolated from *Dendrobium* sonia-28. The gene is designated as *Dendrobium* soni*a Cytokinin Oxidase* (DSCKX1) and immense study on its function on orchid flowering and shelf life are being investigate (Yang *et al.*, 2002; Yang *et al.*, 2003).

**Plate 2.1:** **Flowers of *Dendrobium* sonia-28.** A hybrid between *Dendrobium* Tomie Drake and *Dendrobium* Ceasar. Bar = 1 cm.

## 2.3 Micropropagation for orchid development and improvement

Micropropagation through plant tissue culture provided an important breakthrough for mass propagation of many orchid species which have highly heterozygous genotype and have extremely slow sexual reproduction capability (Kanjilal *et al.,* 1999). Micropropagation is also a powerful tool for large scale propagation in short time span of plant materials and is the best method to eliminate pest infestation (Rout *et al.,* 2006)

Micropropagation begins with the collection of plant material and surface sterilized to free the plant material from fungus, bacteria or virus. Any tissue or organ of the plant can be used as starting material. For example, *Aerides crispum* was successfully regenerated from its leaf section (Sheelavanthmath *et al.,* 2005) and from shoot-tip explants of *Phalaenopsis* (Tokuhara and Mii, 2001) and in *Dendrobium fimbriatum* (Roy and Banerjee, 2003). Plant material is introduced into *in vitro* culture which is provided with nutrient rich medium suitable for orchid growth. After successful establishment of plant material *in vitro*, the plant material can be chopped into small pieces and transferred into new media for multiplication or propagation and regeneration process (Dutra *et al.,* 2008).

Japan, Taiwan, Thailand, Netherlands and many more giant orchid producers has adopted the technique to increase orchid mass and quality (Thammasiri, 1997; Griesbach, 2002; Chugh *et al*., 2009). Various orchids have been reported successfully propagated *in vitro* including *Aerides maculosum* (Murthy and Pyati, 2001), *Oncidium sp*. (Kalimuthu *et al.,* 2007), *Laelia speciosa* (Avila-Diaz *et al*., 2009), *Dendrobium sp.* (Rangsayatorn, 2009; Pornpienpakdee *et al.,* 2010), *Vanda*

*sp*. (Kishor and Devi, 2009), *Arachnis*, *Malaxis*, and *Cleisostoma* (Deb and Imchen, 2010).

### 2.3.1 The advantages of orchid micropropagation

The main advantages of micropropagation are the rapid clonal propagation of elite genotype for large scale commercial demand and as a potential germplasm conservation procedure (Kumar and Singh, 2009). Thousands of plants can be propagated from a mother tissue, such as young psedobulb, within 1-2 years time (Thammasiri, 1997; Slater *et al.,* 2003). The technique offers a solution to overcome the problem with small number of seeds in many orchid species and to prevent the rapid collection of wild orchid for commercial production (Basker and Narmatha Bai, 2006). Only minimum area of nursery is required for commercial propagation and low cost production are beneficial for farmers (Razdan, 2003a).

*Ex vitro* orchid seed require the infection of suitable myccorhiza fungus to increase the survival rates, to enhance vegetative and reproductive growth, to fasten flowering and increase flower quality, and to reduce disease infection (Chang, 2007). The *in vitro* technique eliminates mycorrhiza dependence for seed germination and prevents diseases infection and reinfection to the clonal products (Razdan, 2003b).

## 2.4 Orchid micropropagation media

Murashige and Skoog (1962), Knudson C (1946) and Vacin and Went (1949) media were used for micropropagation of orchid culture. Media modification and the addition of various formulas such as organic substrate, plant growth regulators

(PGRs), activated charcoal and carbon source are important to improve orchid development (Ferreira *et al.*, 2006; Thomas, 2008; Avila-Diaz *et al.,* 2009; Rahman *et al.,* 2009).

The Murashige and Skoog (MS) media was initially revised for rapid tobacco growth (1962). Over the years, MS media become widely used in many orchids in *vitro* propagation including *Aerides* (Murthy and Pyati, 2001), *Dendrobium* (Martin and Madassery, 2006; Aktar *et al.,* 2008), *Phalaenopsis* (Sinha *et al.*, 2010) and *Gramatophyllum* (Pemsin and Kanchanapoom, 2011). Protocorm-like bodies (PLBs) formation for a number of orchid species seems to shows better response to MS media compared to other orchid propagation media. Kishi *et al.* (1997) and Aktar *et al.* (2008) established MS medium as the best in the proliferation of PLBs of *Dendrobium* and *Vandofinetia* orchids compared to Knudson C solution, Vacin and Went medium and New *Phalaenopsis* medium. MS salt concentration plays an important factor to the proliferation rate of the PLBs (Kishi *et al,* 1997). Optimum media salt concentration is essential for *in vitro* orchid to provide sufficient nutrient require to promote metabolism and cell growth and to prevent toxic effect of media salt (Fadel *et al.,* 2010)

### 2.4.1 Exogenous plant growth regulators (PGRs)

The effect of exogenous PGRs such as Benzylaminopurine (BAP), Naphthaleneacetic acid (NAA), Thidiazuron (TDZ), Kinetin (Kin), and Indole-3-acetic acid (IAA) to orchid growth and development are well documented (Sheelavanthmath *et al.,* 2005; Zhao *et al.,* 2008).

The roles of BAP and NAA are often studied for PLBs proliferation experiment. Lee and Lee (2003) and Martin and Madassery (2006) reported efficient induction and proliferation of PLBs of *Cypripedium* formosanum and *Dendrobium* sonia on MS media supplemented with 4.44 µM BAP alone. Luo *et al.* (2008) reported that the highest frequency of PLBs induction in *Dendrobium densiflorum* was promoted by 5 $mg.L^{-1}$ BAP either alone or in combination with 0.1-0.5 $mg.L^{-1}$ NAA. PLBs formation in *Cymbidium* was also improved in media contained the combination of 2 $mg.L^{-1}$ BAP and 1 $mg.L^{-1}$ NAA (Hamada *et al.,* 2010). Fujii *et al.* (1999) reported that addition of NAA alone was found to be inhibitory to the formation of PLBs while BAP promoted production of small sized PLBs in *Cymbidium* orchid. However, NAA plays an important role to enhance the size of existing PLBs (Fujii *et al.,* 1999).

### 2.4.2 Liquid and semi-solid media culture system

The micropropagation media culture system is either semi-solid or liquid. The liquid media system provides a simple procedure for plantlets inoculation without the need of systematic plantlet arrangement and the media requires constant shaking on automated rotator or roller type bioreactors (Park *et al.,* 2000; Wawrosch *et al.,* 2009). The continuous shaking of the liquid culture supply plantlets with ample oxygen for optimum respiration and equal nutrient distribution for enhanced nutrient uptakes as well as to promote the induction and proliferation of numerous axillary buds (Kanjilal *et al.,* 1999; Mehrotra *et al.,* 2007).

The semi-solid media is solidified by gelling agent such as agar and gellan gum. The semi-solid media provides matrix to support plants above the surface of the

media and in upright position (Prakash *et al.,* 2004). In some plant species, gelling agents is essential to prevent hyperhydricity which is detrimental to plant physiology typical seen on plant cultured in excess of water (Ibrahim *et al.,* 2005). Liu *et al.* (2006) suggested that culture system has a significant impact on the proliferation of PLBs of *Phalaenopsis* and *Doritaenopsis*.

### 2.4.3 Carbon source for orchid growth

Sugars such as sucrose, fructose, maltose, sorbitol and glucose are the common choice of carbon sources supply for *in vitro* orchid propagation. The optimisation of sugars addition to media plays an important role to enhance orchid growth (Murdad *et al.,* 2010). Sugars provide carbon and energy for orchid respiration (Hew and Yong, 2004) and synthesis of macromolecules and cell constituents (Yu, 1999). Sugar is also essential structural element for DNA, RNA, cell wall, protein and lipid synthesis in plant (Yu, 1999).

Sucrose has been accounted to be more superior to other sugars for *in vitro* orchid growth (Jawan *et al.,* 2010). Zha *et al.* (2007) described that sucrose is easily available to cell and directly participate in glycolytic and penthose phosphate pathways for cell growth, thus optimising the growth of PLBs of *Dendrobium huoshenense* compared to glucose and fructose. Apart from providing carbohydrate to the plant, sucrose was also found to act as osmotic role to the culture system (Biahoua and Bonneu, 1993). Paivo Neto and Otoni (2003) stressed on the role of osmotic potential contributed by carbon source to the morphogenesis development of *in vitro* plantlets. In *Helianthus annus,* osmotic pressure is a detrimental factor to induce somatic embryogenesis (Jeannin *et al.,* 1995).

## 2.5 Protocorm-like bodies (PLBs)

Protocorm like-bodies are spherical tissue masses that are unique to orchid and resemble an early stage of orchid embryo or protocorm development (Tisserat and Jones, 1999) and are formed from tissue explants and callus (Arditti, 2008). The ability of the PLBs to regenerate into a new plant makes PLBs the most suitable target explants for transformation. Direct generation of PLBs from mother tissue produces clones with genetic uniformity which is an important factor in the transformation studies (Mishiba *et al.*, 2008).

The PLBs can be induced and propagated easily on specific media for rapid and continuous supply of plant materials for large scale transformation purposes (Belarmino and Mii, 2000; Mishiba *et al.*, 2005). Furthermore, the PLBs of *Dendrobium* contain abundant coniferyl alcohol, which could induce the virulence gene in the bacterium, therefore enhance transformation efficiency (Nan *et al*., 1997). Many transformation protocols had successfully developed for genetic engineering of orchids targeting PLBs of *Phalaenopsis* (Chai *et al.*, 2002), *Cymbidium* (Chin *et al.*, 2007), *Vanda* (Shrestha, 2007), *Cattleya* (Zhang *et al.*, 2010), and *Dendrobium* (Quan *et al.*, 2010) as initial explants.

### 2.5.1 Microscopy observation of PLBs morphology through histology and scanning electron microscopy (SEM)

Histology is the first important step to the study of plant morphogenesis. A good histology examination reveals information on cellular morphogenesis changes and organisation in plant body which is essential for the design of hypothesis for

many experiments (Yeung, 1999). Histology can be used for the selection of the best culture system producing competence plant cell for genetic transformation (Creemers-Molenaar *et al.,* 1994). Apart from that, the precise location of the origin of the said competent cell can be targeted to enhance transformation efficiency or other mutagenesis technique (Mendoza *et al.,* 1993). Histology determination can also be used as an important tool for the isolation of mutants and nonchimeric cells (Kanchanpoom and Korapatchaikul, 2012).

Scanning electron microscopy (SEM) has been used as reliable analysis of tissue surface structure. Plant identification and characterisation, study of plant tissue function and mechanism, as well as investigation of plant interaction to stress and pathogens were to name a few of established practice of SEM observation (Elad *et al.,* 1983; Cochard *et al.,* 2000; Sandalio *et al.,* 2001).

## 2.6 *Dendrobium* orchid fungal diseases

*Dendrobium's* plant is susceptible to most of the fungal diseases, particularly, in region with humid climate and high night temperature. Fungal infections cause major loss of yield with visible effect on flowers and leaves discoloration, stunted growth and killing young plant. More than 50% loss of orchid is due to *Fusarium* wilt (Wedge and Elmer, 2008).

*Fusarium proliferatum* race B, was found to cause black spots disease on *Dendrobium*'s leaf result in adverse effect on the orchid valuable traits and stunted its growth (Ichikawa and Aoki, 2000). The symptoms on infected leafs is minute black speckles on the leaves appeared at an early stage and then enlarged to irregular, angular black spots of 5.0 X 2.0 mm and spread rapidly (Ichikawa and Aoki, 2000).

Latifah *et al.* (2008) reported that root discolourification and stem turned yellowish indicates rotting occurance on *Dendrobium* orchid was found to be strongly associated with *Fusarium* species namely *Fusarium oxysporum, Fusarium proliferatum* and *Fusarium solani*.

*Fusarium* causing black spot or wilt is currently controlled using fungicide chlorothalonil and azoxystrobin (Wedge and Elmer, 2008). However, the management and control of infection is difficult due to spores of *Fusarium* are easily dispersed through air, water irrigation and contact to infected plant or infected soil (Wedge and Elmer, 2008).

## 2.7 Genetic engineering for orchid improvement

Genetic engineering in plant offers attractive advantages and opportunities for development of many important traits such as to confer heavy metal resistances as tool of phytoremediation (Song *et al.,* 2007), to increase higher yield production (Bhalla and Smith, 1998) and to exhibit tolerance against abiotic stress including extreme temperature and high salinity (Wang *et al.,* 2010).

For orchid genetic engineering continuous researches mainly focused on the improvement of aesthetic value, multiple flowering shoot, and shelf life longevity (Teixeira da Silva *et al.,* 2011). Yu *et al.* (2001) and Semiarti *et al.* (2007) reported success in transforming class 1 *knox* gene, DOH1 and BP/KNAT1, into *Dendrobium* and *Phalaenopsis* orchids. As a result, the transformed gene expression significantly enhanced multiple flowering shoot production in both orchids. *Dendrobium* orchid transformed with lucifearse (LUC) gene with silenced knox gene shows improved flowering and vase life compared to non-transformed orchid (Chia *et al.,* 2001). The

expression of ethylene receptor mutant *etr1-1* gene transformed into *Oncidium* and *Ododntoglossum* orchids notably prolonged their shelf life by reducing the transgenic orchids' sensitivity to exogenous ethylene (Raffeiner *et al.,* 2009).

### 2.7.1 Development of disease resistant orchids through genetic engineering

Orchid disease caused by plant pathogens is detrimental for orchid yield and quality. Currently, only transgenic orchid resistant to *Erwinia coratovora*, which known to cause soft rot disease in orchid, and Cymbidium Mosaic Virus (CymMV) were extensively explored. Orchid with enhanced resistance against *Erwinia coratovora* were established through transformation with sweet pepper ferredoxin-like protein (*pflp*) or wasabi defensin genes (Liao *et al*., 2003b; You *et al.,* 2003; Sjahril *et al.,* 2006). CymMV disease resistant orchids were developed by transforming orchid with viral *capsid protein* or *coat protein* genes of the CymMV (Borth *et al.,* 2004; Chan *et al.,* 2005; Chang *et al.,* 2005). Currently, there is limited information on transformation of orchid to confer resistance against fungal diseases.

## 2.8 *Agrobacterium tumefaciens*-mediated transformation

The two commonly used tools in orchid genetic engineering are *Agrobacterium*-mediated (Men *et al*., 2003a; Mishiba *et al.,* 2005; Raffeiner *et al.,* 2009; Retheesh and Bhat, 2011) and microprojectile bombardment (Kuehnle and Sugii, 1992; Yang *et al.,* 1999; Men *et al.,* 2003b; Tee and Maziah, 2005; Fan, 2011). *Agrobacterium*-mediated system is more preferable as it offers simple yet precise transfer and integration of gene into the target tissue with stable transformation of

many single copy insertions and large T-DNA size of up to more than 150kb (Veluthambi *et al*., 2003).

In nature, the *Agrobacterium tumefaciens* infects wounded plant and cause crown gall disease. During the infection process, the *Agrobacterium* transfered a DNA segment (T-DNA) of its tumor inducing plasmid (Ti-plasmid) into the plant and subsequently integrated with the plant genome (Nester *et al.,* 1984). The oncogenic genes of the T-DNA induced excessive production of auxin and cytokinin thus causing the crown gall formation (Riva *et al.,* 1998).

The *Agrobacterium*-mediated transformation system, the Ti-plasmid is disarmed by the deletion of the T-DNA and replaced with constructed desired gene. The newly inserted genes most often comes with plant selection marker such as *nptII* gene (Semiarti *et al.,* 2010), *hptII* genes (Subramaniam and Rathinam, 2010), *gusA* gene (Rahman *et al.*, 2011) and *gfp* gene (Julkifle *et al.*, 2010), bacterial selection marker, usually kanamycin resistance gene, and gene of interest (Matthysse, 2006; Arcos-Ortega *et al.*, 2010).

### 2.8.1 Transformation in orchid mediated by *Agrobacterium tumefaciens*

*Agrobacterium* transformation in monocotyledonous species was initially difficult due to the nature of *Agrobacterium* which do not infect monocotyledon plants. Nan *et al*. (1997) has reported that orchids are recalcitrant to the formation of crown gall tumor caused by the *Agrobacterium tumefaciens*. However, increasing number of accomplished gene transformation using this method in monocotyledonous species reported recently on rice increase the possibility of

manipulating this tool into the most efficient gene transformation protocol in orchid improvement challenge (Coca *et al.,* 2004; Sridevi *et al.,* 2008; Rahman *et al*., 2011).

Optimisation of various biotic factors, such as bacterial density, target tissues, and abiotic factors such as co-culture media composition and period of inoculation were intensely studied to improve transformation efficiency (Sreeramanan *et al.,* 2008; Subramaniam and Rathinam, 2010). Successful transformation of *gusA* and hygromycin resistance (*hyg*) genes was obtained in *Vanda* Tokyo Blue (Shrestha *et al.* 2007). A number of orchids species were successfully transformed include *Phalaenopsis* (Chai *et al.,* 2002; Mishiba *et al*., 2005), *Aerides* (Sheelavanthmath *et al.,* 2005), *Oncidium* (Liau *et al.,* 2003a), *Dendrobium* (Chai *et al.,* 2007), *Vanilla* (Malabadi and Nataraj, 2007) and *Cymbidium* (Chin *et al*., 2007).

## 2.9 Pathogenesis-related (PR) protein to express fungal resistance

Pathogenesis-related (PR) proteins are defined as a group of plant proteins induced upon pathological infection on plants or related situations. Van Loon and Antoniw (1982; as reviewed by Buchel and Linthorst, 1999) reported that the accumulation of PR proteins correlates with the onset of the systemic acquired resistance (SAR). At least 17 families of PRs has been classified and named as PR 1-17 (Gorjanovic, 2009).

Wheatwins are a type of chitinase of PR4 family. Four known wheatwin protein, referred to as wheatwin 1, 2, 3 and 4, were found to be highly homologous to barwin of barley (Caporale *et al.,* 2003). As the name suggested, the protein were isolated from wheat kernel, and the '-win' is referring to the cDNAs encoding wound-inducing proteins. Bertini *et al.* (2003) shown that the protein is expressed in

coleoptiles, root and leaf of wheat upon wounding and strongly induced in the presence of salicylic acid and methyl jasmonate. Investigations on wheatwin proteins proved that the proteins possess antifungal and antimicrobial activity. The antifungal activity action is regulated by the enzymatic activity of ribonucleases (Caporale *et al.,* 2004; Bertini *et al.,* 2009). Wheatwin proteins were able to inhibit the *in vitro* growth of fungal hyphae and inhibit spore germination (Caruso *et al.,* 2001). Transgenic tobacco transformed with wheatwin proteins significantly increases antimicrobial activity against *Phytophtora nicotinae* (Fiochetti *et al.,* 2008).

## 2.10 *Agrobacterium*-plant interaction

### 2.10.1 Competence of *Agrobacterium* chemotaxis to PLBs

In the process of bacterial infection, the first vital step is the attraction of the bacteria towards the target explants. Bacterial chemotaxis is an effective method to determine the interaction between bacteria and target tissue. The *Agrobacterium*-plant interaction is highly influenced by their motility. *Agrobacterium* with defected motility may lose their virulence for T-DNA transfer to plant (Deakin *et al.,* 1999). Therefore, it is crucial to determine the *Agrobacterium* behaviour and motility before attempting transformation process.

Chemotaxis studies the microorganism behaviour in response to chemical stimulus. Swarm agar plate proposed a convenient protocol for chemotaxis analysis (Perez-Hernandes, 2000). Swarm agar plate is made using skimped amount of agar which makes sloppy agar plate. Swarming behavior is bacterial mobility specific on solid surfaces. This locomotion is observed on various eubacteria such as *Proteus, Serratia* and *Escherichia* (Lengeler, 2004). Bacteria are inoculated in the center of

the plate and the tested chemical compound or plant cells are placed at a distance from the point of inoculation. The bacteria consumed nutrients from the media and slowly creating a concentration gradient as they migrate outward (Shaw, 1995; Lengeler, 2004). The distance covered by the swarming bacteria can be seen and measured to determined the tactically respond of the bacteria towards the tested chemicals or tissue exudates (Perez-Hernandes, 2000).

### 2.10.2 Competence of *Agrobacterium* attachment to PLBs

Finally to be able to initiate infection, *Agrobacterium* required to efficiently attached to the host plant. Previously wounding of plant was taught necessary to commence attachment competence of *Agrobacterium* to plant cell (Matthysse, 1981). Recent studies proved that attachment of *Agrobacterium* to plant cell is affected by many factors including bacterial motility, bacterial fibrils formation and plant surface morphology (Smit *et al*., 1992; Gorski *et al.*, 2003). Orchids, like other monocots, are not natural host to *Agrobacterium* infection. Therefore, early assessment of *Agrobacterium*-plant interaction in monocot cell is important to determine the type of tissue or wounding type suitable for transformation. *Agrobacterium tumefaciens* were observed to greatly respond to the callus of sorghum (Verma *et al.,* 2008) and the PLBs of *Phalaenopsis* orchid (Subramaniam *et al.,* 2009a).

## 2.11 Plant selection markers for orchid

Selective marker are used to enable individual transformed plant cells to grow out of a non-transformed cell mass, facilitating the generation of transgenic plants

(Xue *et al.,* 2006). An effective selective marker can greatly improve the orchid transformation system. Selection using transient expression of *gfp* (green fluorescent protein) and/or *gusA* genes offer a fast detection of gene transfer usually consuming only 3-10 days of detection after transformation (Belarmino and Mii, 2000; Liao *et al.,* 2003a). These markers are usually used for the purpose of optimising factors influencing gene transfer efficiency (Sreeramanan *et al.,* 2008; Julkifle *et al.,* 2010).

Selective markers such as *manA* and *bar* genes are also popular for their shorter selection and regeneration period of transgenic plantlets (Knapp *et al.,* 2000; Chai *et al.,* 2007; Thiruvengadam *et al.,* 2011). Plant transformed with resistance genes against pathogen can be directly selected based on the expression of the resistance against the challenged pathogen (You *et al*., 2003).

Antibiotic based selection agents such as kanamycin, hygromycin and paramomycin is often used to achieve selection for stable transformation of *nptII* or *hptII* genes (Suwanaketchanatit *et al.,* 2007; Swarn and Rajmohan, 2008). In this system, antibiotic substrate create toxic to non-transformed cell thus inhibit cell development. The expression of *nptII* and *hptII* genes in transformed cell would promote transformed cell to survive the selection periods (Tee *et al.,* 2011).

A review by Miki and McHugh (2004) revealed that 44-77% of plant transgenic studies used *nptII* gene as selectable markers. The *nptII* selection marker is benefited from the availability of various aminoglycosides antibiotics containing 3'-hydroxyl group as selection agents. The added advantages of this selection marker are the rare occurrence of endogenous *nptII* activity in many plant tissues and the flexibility to manipulate the regulation of *nptII* expression without creating expression instability (Aragao and Brasileiro, 2002).