IN VITRO SELECTION OF *Dendrobium* SONIA-28

PROTOCORM-LIKE BODIES AGAINST *Fusarium proliferatum*

By

RAHELEH DEHGAHI

Thesis submitted in fulfilment of the requirement for the degree of Doctor Philosophy

APRIL 2016

ACKNOWLEDGEMENT

All praise to the Almighty Allah for blessing me with sound health, mental strength and courage to conduct the research and write up of the PhD dissertation successfully. I would like to express my deepest acknowledgement to Universiti Sains Malaysia (USM) for giving me the opportunity to conduct the entire research smoothly with worthy facilities.

I am delighted here to extend sincere gratitude to my honourable supervisor Associate Professor Dr. Sreeramanan Subramaniam for his scholastic guidance in research and constructive criticism during write up. I express profound gratitude to my honourable co-supervisor, Professor Dr. Latifah Zakaria for her worthy advice during my research work and write up of the thesis.

My heartfelt thanks to my entire lab mates, friends, colleagues and well wishers who directly or indirectly inspired me every time.

Last but not the least I owe my loving thanks to my parents Javaher Sajadpour and Hojat Dehgahi, husband Alireza Junias and my two children, Niki and Amirreza Junias for their supports and understanding throughout my whole studies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYMS AND ABBREVIATION

ACRONYMS	ABBREVIATION
A_{663}	Light absorption at 663 nm
A_{645}	Light absorption at 645 nm
BAP	6-Benzylaminopurine
BSA	Bovine serum albumin
bp	Basepairs
CF	Culture filtrate
cm	Centimeter
$CaCO_3$	Calcium carbonate
DNA	Deoxyribonucleic acid
dTNP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
EMS	Ethyl methanesulfonate
FA	Fusaric acid
FW	Fresh weight
Gy	Gray
g	Gram
g/L	Gram per litre
H	Hour
kbp	Kilobasepairs
KCl	Potassium hydrochloride
M	Molarity
min	Minute
mg/L	Milligram per litre
mg/ml	Milligram per millilitre
mg/g	Milligrams per grams
$MgCl_2$	Magnesium Chloride
ml	Millilitre
mM	Millimolar
mm	Millimeter
m^{-2}	Per square meter

MS	Murashige and Skoog
nm	Nanometre
OD	Optical densities
Pa	Pascal
PCR	Polymerase Chain Reaction
PGR	Plant growth regulator
pH	Potential Hydrogen
PDA	potato dextrose agar
PVP	polyvinylpyrrolidone
RAPD	Randomly amplified polymorphic deoxyribonucleic acid
Rpm	Rotation per minute
S	Seconds
s^{-1}	Per second
SEM	Scanning electron microscope
SI	Similarity index
sp	Species
spores/mL	spores per millilitre
TBA	Tertiary butyl alcohol
TBE	Tri/Borate/EDTA Buffer
TEM	Transmission electron microscope
Tm	Melting temperature
TRIS-HCL	Tris(hydroxymethyl)aminomethane hydrochloride
v/v	Volume over volume
W	Watt
w/v	Weight over volume
µl	Microlitre
µm	Micrometer
µmol	Micromole
α	Alpha
β	Beta
γ	Gamma
$\mu mol /m^2s$	micro-moles per square meter per second
°C	Degree Celcious

PEMILIHAN *IN VITRO* JASAD SEPERTI PROTOKOM *Dendrobium* SONIA-28 TERHADAP *Fusarium proliferatum*

ABSTRAK

Dendrobium sonia-28 adalah hibrid orkid yang penting dalam industri bunga di Malaysia kerana berkeupayaan berbunga berulang kali dan berbunga padat yang kini sedang menghadapi masalah pengeluaran yang serius akibat penyakit disebabkan kulat, terutamanya oleh *Fusarium proliferatum*. Untuk mengatasi masalah ini, salah satu strategi yang sedang diusahakan adalah dengan penghasilan orkid mutan baru. Mutagenesis secara *in vitro* dengan cara kultur turasan *Fusarium proliferatum* (CF), asid fusarik (FA) dan sinaran gama boleh digunakan untuk menghasilkan mutan yang lebih baik dari segi ekonomi. Dalam kajian ini, pemilihan jasad seperti protokom (JSP) yang bertoleransi terhadap *F. proliferatum* telah dijalankan dengan menilai kesan kepekatan CF (5-20%) dan FA (0.05-0.2 mM) dan pelbagai dos sinaran gama (10-200 Gy). Hasil kajian menunjukkan bahawa kadar hidup dan berat JSP berkadar songsang terhadap inokulasi dan sinaran dos. Selain itu, kadar kematian dan kekurangan berat JSP meningkat di kalangan JSP yang lebih kecil selepas rawatan CF dan FA. Keputusan menunjukkan bahawa ujian sensitiviti radio (LD_{50}) bagi JSP lebih kurang pada 43 Gy. Kajian biokimia menunjukkan bahawa pengurangan yang ketara jumlah protein larut dan kandungan klorofil JSP yang dirawat bergantung kepada masa pendedahan dan kepekatan rawatan. Sebaliknya, terdapat peningkatan aktiviti peroxidase dalam JSP yang dirawat dengan peningkatan masa pendedahan dan kepekatan rawatan. Dos penyinaran yang rendah mempunyai kesan rangsangan terhadap jumlah protein larut dan kandungan klorofil. Analisis histologi, mikroskop elektron imbasan (SEM) dan mikroskop elektron transmisi (TEM) mengesahkan terdapatnya permukaan yang rosak dan kerosakan sel

organel menutup liang stomata JSP yang telah disuntik dan disinarkan. Tambahan pula, kloroplas yang herot mengesahkan kecekapan pigmen klorofil yang kurang. Pengurangan pertumbuhan anak pokok adalah lebih besar pada kepekatan dengan rawatan tertinggi. Anak pokok yang didedahkan dengan dos radiasi gamma yang rendah mempunyai perkembangan pucuk, akar dan dedaun yang lebih baik. Penanda RAPD menunjukkan corak jalur yang berbeza untuk setiap dos rawatan dan jalur khusus untuk anak pokok terpilih dan anak pokok kawalan. Keputusan bioasai jambatan-daun menunjukkan daun yang diperolehi daripada JSP yang dirawat dengan kepekatan yang lebih tinggi, menunjukkan gejala penyakit yang kurang selepas diinokulasi. Oleh itu, terdapat pertalian di antara *Dendrobium* sonia-28 secara *in vivo* dan *in vitro* terhadap rintangan penyakit. Justeru itu, pemilihan *in vitro* CF, FA dan radiasi gamma boleh menjadi kaedah yang berkesan untuk mendapatkan klon soma *Dendrobium* sonia-28. Selain itu, sinaran gamma yang diberikan pada dos rendah hingga dos sederhana boleh menghasilkan mutan JSP dengan ciri-ciri unggul. Keupayaan *Dendrobium* sonia-28 untuk terus hidup selepas jangkitan dan penyinaran membuka lembaran baru untuk pembangunan masa depan orkid transgenik yang rintang kulat.

IN VITRO* SELECTION OF *Dendrobium* SONIA-28 PROTOCORM-LIKE BODIES AGAINST *Fusarium proliferatum

ABSTRACT

Dendrobium sonia-28 is an important orchid hybrid in Malaysian flower industry for its flowering recurrence and dense inflorescences which currently facing serious production problems due to fungal diseases, especially caused by *Fusarium proliferatum*. To overcome this impediment, one of the strategies being pursued is by the production of new orchid mutants. *In vitro* mutagenesis by means of *Fusarium proliferatum* culture filtrate (CF), fusaric acid (FA) and gamma irradiation can be used to produce economically improved mutants. In this study, selection of *Fusarium proliferatum*-tolerant protocorm-like bodies (PLBs) was carried out by assessing the effects of different concentrations of CF (5-20%) and FA (0.05-0.2 mM) and various doses of gamma irradiation (10-200 Gy). Results showed that PLBs survival rate and weight were inversely related to the inoculation and irradiation doses. Additionally, PLBs death and weight reducing increased among smaller PLBs after CF and FA treatments. Results indicated that the radio sensitivity test (LD_{50}) for the PLBs was approximately at 43 Gy. Biochemical studies indicated that there was significant reduction in total soluble protein and chlorophyll contents in the treated PLBs depending upon the time of exposure and concentrations. Conversely, there were increased in peroxidase activity in the treated PLBs with increase in time of exposure and concentration of treatments. Low doses of irradiation have a stimulating effect on total soluble protein and chlorophyll contents. Histological, scanning electron microscope (SEM) and transmission electron microscopy (TEM) analyses confirmed severe surface and cell organelles damage and stomatal closure in inoculated and irradiated PLBs. Moreover, distorted chloroplasts confirmed reducing the efficiency of chlorophyll pigments. Reductions in plantlet growth were much greater at the highest concentrations of treatments. Plantlets infected with low doses of gamma radiation had better development of shoot, root and the foliage. RAPD markers showed different banding patterns for each doses of treatments and specific bands for selected and control plantlets. Leaf-bridge bioassay results revealed that leaflets obtained from PLBs challenged with higher concentrations of treatments showed less disease symptoms after being inoculated. Therefore, there is a relationship between *in vivo* and *in vitro* resistance of *Dendrobium* sonia-28 to infection. Hence, *in vitro* selection by CF, FA and gamma radiation could be an efficient method for obtaining *Dendrobium* sonia-28 somaclones. Moreover, gamma irradiation administered at low to moderate doses may generate PLB mutants with superior characteristics. The ability of the *Dendrobium* sonia-28 to survive after infection and irradiation opens new avenues for future development of fungal resistant transgenic orchids.

CHAPTER ONE

INTRODUCTION

The flowering plant family of Orchidaceae is large in size as well as being economically significant to the international floriculture industry, primarily as cut flowers and potted plants (Arditti, 1992; Khosravi et al., 2009; Poobathy et al., 2013a). In tropical Asia, a total of 6,800 orchid species were discovered in which over 1,000 wild species are located in Malaysia alone (Yang and Chua, 1990; Cribb et al., 2003; Antony et al., 2010).

A number of orchid species are becoming extinct as naturally growing plants are being harvested at random and habitats of these plants are greatly disturbed. Since traditional methods used for cultivation of orchids have proven to be quite difficult, it is extremely important to develop efficient and authentic techniques for conservation of orchids in the form of germplasm conservation (Hirano et al., 2005, 2009). Furthermore, endangered plant species and genetic resources may be effectively conserved using *in vitro* methods (Engelmann, 2011).

The genus *Dendrobium* belongs to one of the three largest families of Orchidaceae (Leitch et al, 2010). *Dendrobium* species are widely employed in horticultural, agricultural and medicinal practices (Chattopadhyay et al., 2012). *Dendrobium* sonia-28 is a commercially valuable *Dendrobium* orchid popular as a cut flower and ornamental plant because of its frequent flowering and large number of flowers for each inflorescence (Martin and Madassery, 2006; Ching et al., 2012).

Protocorm-like bodies (PLBs) can be used as a reliable source of potentially regenerable orchid tissues (Ishikawa et al., 1997; Saiprasad and Polisetty, 2003; Yin et al., 2011). *In vitro* techniques appear to be very suitable for conserving plant biodiversity as it is considered to produce a large number of clones in a relatively short time (Khoddamzadeh et al., 2013), which has provided an efficient way to rare or mass propagate commercially valuable plant germplasms especially for orchids (Hossain et al., 2013). However, there are some disadvantages such as somaclonal variations and microbial contamination under *in vitro* micropropagation conditions (Srivastava et al., 2009; Goncalves et al., 2010; Konieczny et al., 2010).

Dendrobium orchids are sensitive to pests and diseases. A considerable threat to these commercially important flowers is fungal disease. *Fusarium proliferatum* is regarded as a major pathogen of commercially cut-flower plants (Fattahi et al., 2014) including orchids (Swett and Uchida, 2015). Latiffah et al. (2009) reported that root discolourification and a yellowing of stem are strong indications of mold, chiefly associated with species from the genus *Fusarium* such as *F. proliferatum*. Thus, monitoring and management practices could be the best way to conserve orchids (Kani, 2011; Machaka-Houri et al., 2012).

In support of this contention, it has been reported that *Dendrobium* sonia-28, an important ornamental orchid in Malaysia is experiencing a reduced germination pace and risks of producing unsought progenies (Poobathy et al., 2013a). Chemical control of such pathogens is often problematic, expensive, labour and resource-intensive and can cause environmental pollution (Jayasankar et al., 2000). Moreover, conventional breeding methods for orchids present some limitations, including their long growing cycle, complicated reproductive process, and cross-incompatibility among some orchid species (Manshardt, 2004).

Accordingly, *in vitro* selection could be the best approach for pathogenic control because of benefits such as rapid testing of a broad range of individuals, easy mutant manipulation, and the presence of somaclones with higher variability in the genome (Hamid and Strange, 2000). Somaclonal variation is described as the occurrence of genetic and epigenetic changes in cell and tissue cultures (Larkin and Scowcroft, 1981; Kaeppler et al., 2000; Leva et al., 2012). Plant improvement through *in vitro* selection and somaclonal variation is a technique of *in vitro* culture for obtaining plant genotype tolerance to the abiotic or biotic stress, such as high salinity, drought and disease tolerance (Ahmed et al., 1996; Yusnita et al., 2005; Leva et al., 2012). A valuable approach for improving crop productivity in the presence of Fusarium wilt fungi is to select regenerated clones which are resistant or tolerant to fungal diseases. This can be done through mutagenic treatment or co-cultivation with pathogenic fungi (Krishna et al., 2013). The selecting agents usually employed for *in vitro* selection in disease-resistance include phytotoxin such as fusaric acid (FA) or specific fungal culture filtrate (CF) or the pathogen itself (Purohit et al., 1998; Mahlanza et al., 2013). Certain *Fusarium* species produce toxins such as FA with toxicity level ranges from low to moderate degree (Wu et al., 2008).

In vitro selection method has been used for plant disease resistance in last two decades. Over 30 plant species and selective agents from about 40 plant pathogens were examined (Švàbova and Lebeda, 2005). Some studies have also successfully used FA to select resistant planting materials (Chawla and Wenzel, 1987; Bouizgarne et al., 2006; Wu et al., 2012; Wang et al., 2014). Similarly, there were many reports on the use of culture filtrates which confirmed to be effective in selecting tolerant or resistant plants against biotic stresses (Gonzalez et al., 2006; Kumar et al., 2008; Tripathi et al., 2008; Svábová et al., 2011). Remotti et al. (1997) reported that since culture filtrates contain some phytotoxic compounds produced by pathogens, plants selected after culture filtrate inoculation may well be resistant to factors other than the main pathogen.

Plants in their natural habitat are persistently exposed to insect herbivores, fungi, viruses and bacteria. In response to attack by these organisms, plants have developed certain defence mechanisms such as induction of structural and biochemical changes (Agrios, 2005). Changes of the external environment and the inherent instability of the genetic structure in plants under natural conditions can be resulted to induced spontaneous genetic mutations. However, frequency of such mutations differs between plant species and genes and is extremely low (Drake et al., 1998). In plant breeding, induced mutation is an alternative and complementary technique for genetic modification and establishment of new genetic resources. There are a lot of physical and chemical mutagens currently used in mutation breeding (Ahloowalia and Maluszynski, 2001; Medina et al., 2005; Jain et al., 2007; Jain, 2012). X-, β- and γ-rays, neutrons and protons are some of the several energy rays that are widely used in mutation breeding.

Gamma rays are one of the most efficient sources of ionizing radiation, which induces a high frequency of mutations in plants. It has been reported that gamma rays can induce about 70 % of the world's mutant varieties (Nagatomi and Degi, 2009). Gamma rays are typically divided into two types of irradiation; chronic and acute (Nagatomi and Degi, 2009). Radiation rays can enhance mutation rate ranges more than a thousand-fold in the plants (Kovács and Keresztes, 2002). Cobalt-60 as the usual radiation source for induced-mutation, is widely used in agriculture and forestry, especially in ornamental and economically-valuable plants (Thapa, 2004; Borzouei et al., 2010). Gamma rays are an ionizing radiation that interacts with atoms or molecules to generate free radicals in cells. These free radicals can destroy important components of plant cells and differentially affect the morphology, anatomy, biochemistry and physiology of plants depending on the level of irradiation. Gamma radiation can affect plant photosynthesis, depending on the irradiation dosage (Kovacs and Keresztes, 2002; Kim et al., 2004; Wi et al., 2007). Recently, mutation breeding has been used for some important ornamental plants including orchids (Kikuchi, 2000).

Zeng et al. (2010) indicated that stomata represent a major route for pathogen invasion and stomata closure appears to be part of a plant's immune response. Identification of plants resistant to diseases can be carried out by detecting changes in metabolites produced by plants and changes in enzyme activities once they are exposed to any stressor (Krishna et al., 2013). Different plants have evolved different complicated mechanisms for protecting themselves from the damages caused by plant pathogens. For instance, it has been demonstrated through research that antioxidative enzymes play a crucial role in conferring resistance to plants in response to biotic stresses (Mittler, 2002). Resistance to a disease involves activation of several different defence mechanisms which work in coordination to protect the plant from infection. When a plant is infected by a pathogen, higher quantities of ROS (reactive oxygen species) are produced which interact with wide range of cellular molecules including nucleic acids, proteins and lipids (Rebeiz et al., 1988; Sahoo et al., 2007).

Since these basic molecules are essentially required for structural and functional integrity of the cell, their reaction with ROS may result in irreversible harm to the cell and these damages may end up at cell death (Rebeiz et al., 1988; Sahoo et al., 2007). DNA-based markers are powerful and reliable tools for discerning genetic variation in studying evolutionary relationships (Zhang et al., 2013; Bhattacharyya and Kumaria, 2015). The RAPD analysis has been used to assess altered genetics in *Fusarium* tolerant cells (Nasir et al., 2012; Ghag et al., 2014a). *In vitro* selected variants should be finally plant-tested inoculated with the pathogen to compare the response between *in vitro* selected agents and plants inoculated with the pathogen and confirm the genetic stability of the selected traits (Jain, 2001; Flores et al., 2012).

In Malaysia, orchid industry has seen a significant increase. Furthermore, the genus of *Dendrobium* is accounted to be the main orchid cut-flower export for Malaysia. *Fusarium* species including *Fusarium proliferatum* causes yellow and black spots on root and leaves in *Dendrobium's* orchid. The controlling of the *Fusarium* infection is very difficult. The crucial economic importance to the genus *Dendrobium* holds increases the importance of conserving the valuable orchid germplasm. Thus, careful surveillance and management practices will be the best way to preserve the various different orchid genera.

1.1 Main objectives of research

The objectives of the present study are:

(i) To carry out and determine *in vitro* selection of disease resistant *Dendrobium* sonia-28 PLBs using *F. proliferatum* CF, fusaric acid and gamma irradiation,

(ii) To investigate biochemical, morphology, histology, and RAPD analyses and comparison of culture filtrate, fusaric acid and gamma irradiation treated PLBs and plantlets,

(iii) To establish leaf bridge assay technique and evaluate disease resistance of *Dendrobium* sonia-28 plantlets obtained from culture filtrate, fusaric acid and gamma irradiation treated PLBs using *Fusarium proliferatum* inoculation under *in vitro* condition.

CHAPTER TWO

LITERATURE REVIEW

2.1 Orchids: Geography, morphology and importance

The flowering plant family of Orchidaceae is very huge in size and being economically critical to the international floriculture industry, primarily as potted plants and cut flowers (Arditti, 1992; Khosravi et al., 2009). Orchidacea can be considered as one of the best recorded of all angiosperm families (Chase et al., 2015). The orchid family is one of the largest in the flowering plant kingdom, and there were around 880 genera with recent estimation ranging from 20,000 to 35,000 species in five subfamilies (Dressler, 1981; Cribb et al., 2003), with extra 800 species were being recognized and added to the orchid lists yearly (Nicoletti 2003; Bektas et al., 2013) and new orchid genera were being portrayed at a rate of around 13 per year (the average over 10 years prior to 2004) (Schuiteman, 2004; Chase et al., 2015). Over 100,000 registered commercial orchid hybrids were grown as cut flowers and potted plants (Martin and Madassery, 2006; Vendrame et al., 2007).

Both hybrids and wild orchids have the following features: bilaterally symmetrical flowers, sticky masses of pollen grains called pollinia, minute seeds containing undeveloped embryos with no nutritive materials and the ability of seeds to only germinate with the presence of a symbiotic fungus under natural conditions (Jezek, 2003; Seaton et al., 2010). At the present, numerous descriptions of new genera incorporate molecular analysis to exhibit their necessity, whereas in earlier decades, morphology has been generally accepted basis for the description of new taxa (Chase et al., 2015). Most of the orchids that are threatened and endangered are listed under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (Nikishina et al., 2007; Swarts and Dixon, 2009).

Orchids can be found in almost every region of the world, apart from marine environments and perpetually icy regions (Sharma et al., 2011). About 90% of populations of the world's orchids are found in the tropical climatic regions with Asia alone between 10,000 to 15,000 species. Mostly orchids were thermophilic, but some species can be found at the lowland, montane or submontane levels (Jezek, 2003). Besides its aesthetic value, orchids were also known as widely favoured food, beverages, spices, flavouring, medicine, drugs, arts and religions (Arditti, 1992; Arditti and Pridgeon, 2013). Although orchids are expensive, but highly in demand in the national and international markets due to their diversity in terms of size, shape, flower colour and longevity (Saiprasad et al., 2004).

2.1.1 Orchid's market

The Asia-Pacific region has the prime share of all the world area under floriculture production (FAO, 2010). Cut flowers and many other floricultural products are key export products for many countries. These include Malaysia, where the horticultural sector has recorded phenomenal growth over the past years. In Malaysia, the floriculture industry has seen a significant increase in land under cultivation, for example, the area of land devoted to floral production from 3370 ha in 2005, has reached 7,000 ha in 2008 (Hamir et al., 2008). The export of orchids have increased from RM 12.8 million in 2003 to RM 14.3 million in 2005 (Fadelah, 2007).

Orchids are mostly traded as potted plants or cut flower in the flower industry globally (Japan Flower Trade Association, 2009; Supnithi et al., 2011). Its commercial importance, both as cut flowers and pot plants, increases globally year after year (Manners et al., 2013). Orchids share 8% of the global floriculture trade

(Martin and Madassery, 2006; Vendrame et al., 2007). Export and import trading of orchids in the world is estimated more than US $150 million dollars which 80% and 20% of this are composed of cut and potted orchids respectively.

2.2 *Dendrobium* orchid genus

Since the 18th century, more than 8000 novel *Dendrobium* hybrids and cultivars have been produced in horticulture through interspecific hybridization as been reviewed by Pongsrila et al. (2014). This genus consists of more than 1100 species distributed throughout the world, ranging from Southeast Asia to New Guinea and Australia (Puchooa, 2004). Some characters of *Dendrobium* such as its floriferous flower sprays, long flowering life, year round availability and the genus's wide spectrum of shapes, colours, and sizes are reasons of *Dendrobium* expanding popularity (Kuehnle, 2007; Fadelah, 2007; Khosravi et al., 2009).

Dendrobium is also mostly used as in medicinal products and cosmetic (Chang et al., 2010). *Dendrobium* orchid also known *Sekkoku* in Japanese or *Shih-hu* in Chinese was used in Chinese traditional medicine as tonic to improve digestion, eliminating heat and nourishing yin and promoting body-fluid production (Shiau et al., 2005; Yin and Hong, 2009). The cane of *Dendrobium* huoshanense is also used to treat ophthalmic disorder, salivary, and stomach (Hsieh et al., 2008; Yin and Hong, 2009). Dried drug of Shih-hu reach up to US$ 4000 Kg^{-1} (Shiau et al., 2005).

Most of the *Dendrobium* hybrids produce flowers that are white, golden-yellow or lavender in colour, with some having combinations of these colours (Puchooa, 2004; Kuehnle, 2006). Rare specimens may consist of bluish, ivory, brilliant orange or scarlet flowers, with exotic markings. Most of the evergreen species of *Dendrobium* do not produce fragrance while some deciduous species may produce fresh citrus-like scents, or smell of raspberries (Puchooa, 2004). Annually, *Dendrobium* usually blooms several times as well as the flower sprays make interesting cut flowers for arrangements (Puchooa, 2004; Fadelah, 2004).

Since Japan is the biggest world's importer of cut orchids, therefore Asia dominates the world trade in orchids industry. Japan imports cut flower mostly from its surrounding ASEAN countries mainly from Malaysia, Thailand, and Singapore which 90% of its imported flowers were *Dendrobium* (Japan Flower Trade Association, 2009). In the cut flower industry, *Dendrobium* has one of the top positions (Martin and Madassery, 2006; Fadelah, 2007). The genus of *Dendrobium* is accounted to be the main orchid cut-flower export for Malaysia and also for other Southeast Asian countries including Philippines and Thailand (Fadelah, 2007). *Dendrobium* is contributed 11.7% from Malaysia´s orchid exports almost for the past 10 years (Khosravi et al., 2008; Antony et al., 2010). *Dendrobium* accounts for around 80% of the total micropropagated tropical orchids usually by protocorms (Saiprasad et al., 2004; da Silva, 2013).

Small seedlings and heterozygous seedlings progenies which does not result to true-to-type plants of hybrid cultivars are accounted as problems in germination of *Dendrobium* (Martin and Madassery, 2006; Poobathy et al., 2013b). Small seed size, presence of reduced endosperm and the need of a symbiotic relationship between orchids and mycorrhizal fungi are other problems in *Dendrobium* germination (Saiprasad and Polisetty, 2003; Swarts and Dixon, 2009).

2.2.1 *Dendrobium* sonia-28

Dendrobium sonia-28 (Figure 2.1) and its other hybrid siblings are mostly popular because of their floriferous infloresscense, bright colour flowers, durability with long shelf life, free flowering characteristics and fast gowing cycle (Fadelah, 2007). *Dendrobium* sonia-28 orchid hybrid can be created by crossing two hybrids that are *Dendrobium* Caesar X *Dendrobium* Tomie Drake it is cherished for its pink-coloured blossoms as well as the quality of the cut florae (Van Rooyen Orchids Catalogue, 2007). Good air movement and strong light are essential growth conditions for the evergreen and warm-growing hybrid (Van Rooyen Orchids Catalogue, 2007).

Figure 2.1: The orchid hybrid *Dendrobium* sonia-28 (OrchidBroad.com, 2012).

2.3 Micropropagation of orchids and protocorm-like bodies (PLBs)

The choice of explant source plays a significant role in the outcome of the micropropogation (da Silva, 2013). Different explants such as shoot tips, apical buds, stem segments, root tips, leaf segments, flower buds, mature seeds, and seed- derived rhizomes have been widely used as explants to obtain regenerative plantlets in several orchids (Kuehnle, 2006; Zhao et al., 2008; Mohanty et al., 2012). *Dendrobium* generally propagated by seeds or by division of off shoots which called sexually or asexually respectively (Martin and Madassery, 2006; Qiang, 2014).

Orchid regeneration through seed needs the infection of suitable myccorhiza fungus which usually supplies carbohydrates and nutrients to the orchid seeds (McKendrick et al., 2000; Yoder et al., 2000; Godo et al., 2010). This relationship between the fungi and orchid is called “symbiosis” where the fungus provides nutrition for orchid growth, while, the orchid provides shelter to the fungi. In nature this mycorrhizal association with an orchid seed is not common and thus a high proportion of seeds fail to survive. However, the *in vitro* technique decreases mycorrhiza dependence for seed germination and excludes disease infection and reinfection to the clonal products (Razdan, 2003, Kauth et al., 2008).

In vitro propagation of orchids has emerged as a choice for swift propagation of commercially cultivars as the conventional *in vivo* vegetative propagation presents with problems such as slow multiplication rate, high financial demand and insufficient production of clones within a short timeframe (Saiprasad and Polisetty, 2003; Martin and Madassery, 2006; Mohanty et al., 2012). *In vitro* culture has also made it possible to preserve orchids, since the advent of asymbiotic seed germination (Andronova et al., 2000; Nikishina et al., 2001; Nikishina et al., 2007; Mohanty et al., 2012). However, the maintenance of *in vitro* collections requires manual labour and causes the accumulation of somaclonal variations and phenotype-based involuntary selections, which result in the homogeneity of the orchid population (Butenko, 1999; Ivannikov, 2003; Maneerattanarungroj et al., 2007) and depletion of the gene pool (Nikishina et al., 2007; Thorpe, 2007; Sorina et al., 2013). Micropropagation is the producing of microplants via tissue culture by which is created through initiation of meristematic material such as shoot buds, stem apices and seedlings from fully-developed plants into the culture process (Debnath et al., 2006; Leva et al., 2012). Protocorm-like bodies (PLBs) resemble true protocorms (germinated orchid seed) in being round shaped (Figure 2.2) but they are derived from bud explants or shoot tips (Kuehnle, 2006; Lee et al., 2013).

Orchid tissue culture propogation has been found almost immediate commercial trading in which placed orchids within the economic reach of the average person (Arditti, 1984; Arditti, 1990; Ahmed et al., 2001; Arditti, 2010). Orchid producers have adopted the propogation technique, so that it would increase the mass and the quality of the orchids (Chugh et al., 2009; Azman et al., 2014).

Dendrobium sonia-28 is produced via an *in vitro* system based on regeneration of advanced plantlets through protocorms and PLBs (Saiprasad and Polisetty, 2003). Stages of pro-meristematic, leaf primordial and formation of the first embryonic leaves are developmental phases for *Dendrobium* sonia-28 PLBs which happened between eight to 10 days old, 13 to 15 days old and 18 to 20 days old PLBs, respectively (Saiprasad and Polisetty, 2003).

Figure 2.2: ***In vitro*** **proliferation of PLBs and plantlets from a single PLB of *Dendrobium* sonia-28 within three months of culture on semi-solid half-strength MS medium supplemented with 2% (w/v) sucrose and 0.2% (w/v) charcoal.** Bar = 1cm.

2.4 Diseases of *Dendrobium*

Survivality of orchid is correlated with the abiotic and biotic factors and their interactions for growth, development and reproduction. Particularly, in the face of climate change, abiotic elements foist significant and dreaded threats to orchid conservation (Dixon et al., 2003; Barman and Devadas, 2013). Factors such as overharvesting, climate change, habitat demolition, and decreasing pollinator populations bestow to the susceptibility of orchids to extinction (Machaka-Houri et al., 2012).

Fusarium diseases have been reported in orchids from various locations around the world which mostly in the tropics and sub-tropics (Swett and Uchida, 2015). *Dendrobiums* are susceptible to several pathogens and the infections they transmit (Samuels and Brayford, 1994: Hirooka et al., 2005; Hirooka et al., 2006; Halleen et al., 2006). *Dendrobium*´s susceptibility expands in regions with high night temperatures and humid climate. Above 50% loss of orchid is mainly because to Fusarium wilt (Wedge and Elmer, 2008). The controlling of the infection is very difficult, because the spores of *Fusarium* which are easily dispersed through irrigation water, air, and contact to infected plant or infected soil (Wedge and Elmer, 2008). Despite years of selection of resistant cultivars and conventional breeding due to the evolution of divergent lineages of virulent races of *Fusarium*, the desease is not controllable (Swarupa et al., 2014).

The crucial economic importance to the genus *Dendrobium* holds increases the importance of conserving the valuable orchid germplasm. Thus, careful surveillance and management practices will be the best way to preserve various orchid genera (Kani, 2011; Machaka-Houri et al., 2012). Furthermore, it has been reported that *Dendrobium* sonia-28, an important ornamental orchid in Malaysia is experiencing a reduced germination pace, susceptible to fungal diseases and risks of producing unsought progenies (Poobathy et al., 2013a).

2.4.1 *Fusarium proliferatum*

Fusarium proliferatum is a species with increased potential for producing diverse mycotoxins and a major pest of many crops (Kushiro et al., 2012), including commercially important such as cut-flower plants (Fattahi et al., 2014). Due to *Fusarium proliferatum*, it has been found to cause black spot disease on *Dendrobium*´s leaf, result in unfavourable effect on the orchid valuable, stunted its growth and traits (Ichikawa and Takayuki, 2000). Small black speckles on the leaves are symtoms of an infected leaf which appeared at an early stage, then, they tend enlarge to irregular, angular black spots of 5.0×2.0 mm and spread rapidly (Ichikawa and Takayuki, 2000). Five *Fusarium* species have been confirmed to be pathogens of orchids which *Fusarium proliferatum* has been detailed as a foliar pathogen that causes yellow and black spots on root and leaves in orchids (Swett and Uchida, 2015). Latiffah et al. (2009) reported that *Dendrobium's* orchid root yellowish and discolourification stem mainly associated with *Fusarium proliferatum* attacks.

2.5 *Fusarium* control

Susceptibility to various fungal, bacterial and viral pathogens is the reason for the yield decreasing in many commercially important plants. Attempts to control these infections which can seriously decrease marketability rely heavily on the increased use of chemical insecticides and fungicides. Although some such as chlorothalonil, azoxystrobin, fludioxonil and Palladium worked well, the development of resistance has a severely hindered success (Wedge and Elmer, 2008; Wedge et al., 2013).

Chemical control of *Fusarium* is highly in cost, laboures and resource-intensive (Bezier et al., 2002; Egel and Martyn, 2007). Furthermore, some chemically synthesized fungicides are non-biodegradable, caused environmental pollution, build up hefty concentrations in soil, and lowering its productivity in the water table causing health hazards to flora and fauna (Jayasankar et al., 2000; Komárek et al., 2010). In the case of antifungal compounds, a better understanding of orchid-fungus interactions is relevant (Kani, 2011; Machaka-Houri et al., 2012). Chemical control methods of *Fusarium* using fungicides are practically not effectual, particularly, steam sterilization of the soil as a *Fusarium* chemical control is an expensive method (Esmaiel et al., 2012; Ghag et al., 2014a).

In plant improvement, proper management strategies and traditional breeding technologies play an essential role. The standard breeding programs have been employed to integrate flattering genes of interest from inter crossing genera and breed into the plants to influence stress tolerance. Even with that conventional breeding methods have not been successful and have failed to provide succesful results (Rai et al., 2011). The program also needs big areas of cultivation, expensive labour, material and maintenance (Matsui, 2010). Moreover, conventional breeding is also at drawback because there is a need of specific cultivation of certain orchids to overcome problems such as diseases, pest, fungal interaction and environmental stresses (Mishiba et al., 2008).

Transgenesis is another strategy that has been and is being pursued which has led to the engineering of stable transformants of orchids (Chai et al., 2007; Swarnapiria, 2009; da Silva, 2013). Genetic transformation is now a globaly used procedure for introducing genes from distant gene pools into many plant species by using this technique it has progressed stress tolerant plants and substantial efforts have been made to produce stress-tolerant plants (Borsani et al., 2003; Yamaguchi and Blumwald, 2005; Singh and Singh, 2014). But, the main problem in extension of this technique to various is the supress of transgene, consequent reducing of low transformation frequency and gene expression (Mandal et al., 1997; Rai et al., 2011). The transgenic approach is limited by the availability of the desired gene and by the lack of efficient transformation and plant regeneration protocols (Kumar et al., 2012). Therefore, the aptness to produce orchid transgenics with high germination tolerance and potentials to fungal infections still remains a main challenge. The production of such plants necessitates the discovery of potential target molecules (Kani, 2011; Machaka-Houri et al., 2012).

Selection of suitable somaclonal variants having desired characteristics is another alternative strategy to develop plants with improved characters (Esmaiel et al., 2012; Ghag et al., 2014b). Resistance is a qualitative character and consequently, through selection, it is possible to obtain more resistant variaties (Esmaiel et al., 2012).

2.6 Somaclonal variation system

The maturation of plant cells *in the vitro* and their regeneration into mature plants is an asexual process that only involves mitotic division of the cells. In this context, the happening of uncontrolled and random impetous variation when culturing plant tissue is a major problem (Leva et al., 2012; Nwauzoma and Jaja, 2013). Gao et al. (2010) and Bairu et al. (2011) stated that occurred variation in plant micropropogation is mostly undesired.

Undirected genetic variability happening in plant tissues culture may have novel agronomic traits that might not be accomplished by conventional breeding (Jain, 2001; Piagnani et al., 2008). The happening of genetic variation between plants regenerated from *in vitro* culture which has been referred to as somaclonal variation (Larkin and Scowcroft, 1981; Lestari, 2006; Nwauzoma and Jaja, 2013; Bhojwani and Dantu, 2013). Somaclonal variation may yield desirable genotypes as novel cell lines or plants of agronomic and commercial advantages (Bhojwani and Dantu, 2013). Somaclonal variation can suit a very important component of the plant breeding in which variation regenerated from somatic cells can be utilised for the introduction of new tolerance, agronomic or quality traits (Jain, 2013).

Larkin and Scowcroft in 1983 have proposed the word of somaclones' and have described 'Somaclonal variation' in sugarecane plants (soma=vegetative, clone=identical copy). Tissue culture regenerated variants have also been called calliclones, phenovariants, protoclones and subclones (Skirvin et al., 1994; Yadav et al., 2009). Somaclonal variation is not limited to the plant kingdom. There have been hundreds of reports of cell line variants among animal tissue cultures (Skirvin et al., 1994; Bairu et al., 2011).

Somaclonal variation has been caused because of alterations in chromosome number, structure and point mutations, or amplification, transposition and deletion of deoxyribonucleic acid (DNA) order (Neelakandan and Wang, 2012; Landey, 2013; Jain, 2013). Cytogenetic changes such as variation in ploidy level, structural changes, and number of chromosomes represent big alterations to the genome and they are sometimes generated during *in vitro* differentiation and proliferation (Kaeppler et al., 2000; Neelakandan and Wang, 2012; Landey, 2013). The chromosomal changes may produced a stable alteration which transferred to the progeny (Haines, 1994; Fu et al., 2013).

However, the amount of somaclonal variation depends on the plant genotype, age of plant, culture medium compounds, the time of culture and the number of subculture cycles (Duncan, 1997; Sahijram et al., 2003; Peredo et al., 2006; Bairu et al., 2011; Landey, 2013). The true rate of somaclonal variation is difficult to ascertain because of many individual genes to examine. Many somaclones were identical which suggests a common origin.

One of the vital potential benefits of somaclonal variation is the creation of additional genetic variability in co-adapted, agronomically useful cultivars, without the need to retreat to hybridization. Somaclonal variation will be useful if *in vitro* selection method is available (Brown and Thorpe, 1995; Ketema, 1997; Roychowdhury and Tah, 2013). It was supossed that somaclonal variants can be entensified during *in vitro* culture for some haracters, which includes resistance to disease pathotoxins, tolerance and herbicides to chemical stress or environmental (Bhojwani, 2012).

Somaclonal variation has a few disadvantages. For instance, somaclonal variation is not always resulted to the wanted plant lines (Niizeki and Lu, 2003; Semal, 2013). It is very necessary to screen a lot of materials as possible. Second, somaclonal variation usually results in changes in multiple traits. Finally, it is very crucial to point out that a big deal of effort is needed to screen the somaclones. Most of the time somaclonal variants are not novel or useful (i.e. aberrant phenotypes), the variation generated could be unstable or not reproducible (Duncan, 1997, Jain, 2001), although some variants show positive changes other traits could be altered in a negative way (Karp, 1994; Landey, 2013).

Somaclonal variants can be detected using a few techniques which are mainly categorized as morphological, leaf morphology, physiological/biochemical such as plant height, and abnormal pigmentation (Israeli et al., 1995; Leva et al., 2012) and molecular traits to determine somaclonal variation (Sorina et al., 2013). Somaclonal variation has led to the selection of several variants with increased resistance to pests, diseases, and herbicides (Brar and Jain, 1998; Predieri, 2001; Pandey and Mukerji, 2006; Lee, 2015).

Epigenetic variation is also known as physiological variation or developmental. It involves nonpermanent changes which may be unstable and non-heritable and potentially reversible (Kaeppler et al., 2000; Leva et al., 2012). In disparity, enduring changes are heritable and sometimes represent expression of preexisting variation in the source of plant or are an effect of induced variation (Larkin and Scowcroft, 1981; Leva et al., 2012). Epigenetic modifications are mostly found in DNA (methylation) and histones and are associated with changes in the gene expression (Kaeppler et al., 2000; Zhang and Meaney, 2010; Ahmad et al., 2010; Vanyushin and Ashapkin, 2011). In general, genetic stability is high in shoot tips than from explants that have no preformed shoot meristems, such as leaves, roots, or protoplasts (Skirvin et al., 1994; Kaur and Sandhu, 2015).

Plant advancement through somaclonal variation and *in vitro* selection are a few techniques of *in vitro* culture to procure plant genotype tolerance to the abiotic or biotic stresses (Ahmed et al., 1996; Yusnita et al., 2005; Xu et al., 2012).

2.6.1 Mutation breeding

Somaclonal variation may be one of the most advantageous sources when reliable early selection methods for the trait of interest are available (Kumar and

Arya, 2009; Gupta, 2011). Mutagenesis is a skill which is being utilized by both human beings and nature in order to upgrade the quantitative and qualitative traits in plants against diverse abiotic and biotic stresses (Maluszynski et al., 1995; Ahloowalia and Maluszynski, 2001; Wu et al., 2012; Yunus et al., 2013; Perera et al., 2015). Mutagenic agents are more helpful than harmful and without them evolution of species would have been arrested at a very primitive stage (Fishbein, 2012).

Plant water content is significant in its radiosensitivity, since most of the frequent main quarry of ionizing radiation is the water molecule (Predieri, 2001; Miguel and Marum, 2011; Draganic, 2012). Mutation affects cells and mutated cells have to grow out into group and layer of cells. Various layers have different radiosensitivity, maybe because of differential mitotic activity and organogenic properties (Broertjes and Van Harten, 2013). If more than one cell is present at the moment of mutagenic event, chimerism will be occurred. Chimeras will be transformed into the plant progenies by repeated multiplication (Yang and Schmidt, 1994; Mba, 2013). Consequently, it is mostly advantageous to dissociate chimeras by following subcultures up to M1V3–M1V4 generations (Jain et al., 1998; Mandal et al., 2000; Yunus et al., 2013). Subculture will maintain and secure the stability of mutant traits and guarantee that the chosen mutants are secure from chimeras (Yunus et al., 2013). Induced mutation needs screening of very large population, since, induced mutation lays in the low recovery frequencies (10^{-4} to 10^{-6}) of specific single gene mutants in M2 populations (Esmaiel et al., 2012). Nevertheless, somaclonal variation frequently happens at very high frequencies (up to 10% per cycle of regeneration) than radiation or chemical persuade mutation, making it a feasible alternative to mutagenesis and a preciouse tool for the plant geneticist to