EFFECTS OF FUSARIC ACID TREATED AND GAMMA IRRADIATED PROTOCORM-LIKE BODIES (PLBs) OF *Dendrobium* HYBRID AGAINST *Fusarium proliferatum* AND *Fusarium oxysporum*

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by

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^{60}Co	Cobalt-60
ABA	Abscisic acid
AF	Asid fusarik
AIA	Indolacetic acid
ANOVA	Analysis of variance
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
BAP	6-benzylaminopurine
bp	Base pair
CAT	Catalase
Co	Cobalt
Cu	Copper
cv.	Culture variety
DAMD	Direct amplification of minisatellite DNA
DNA	Deoxyribonucleic acid
dNTP	Nucleoside triphosphates containing deoxyribose
DON	Deoxynivalenol
f.sp	*formae speciales*
FA	Fusaric acid
FAA	Formaldehyde – acetic acid – ethanol
FHB	*Fusarium* head blight
Gy	Gray
ISSR-PCR	Inter simple sequence repeat-polymerase chain reaction
JSP	Jasad seperti protokom
K	Thousand times
kb	Kilo base pair
Krad	Kilorad
LD_{50}	Half lethal dose
LSN	Lapisan sel nipis

lTCL	Longitudinal TCL
MeV	Megaelectron-Volt
O	Singlet oxygen
O_2^-	Superoxide anion
OH	Hydroxyl radicals
PAL	Phenylalanine ammonia-lyase
PCR	Polymerase chain reaction
PGR	Plant growth regulator
PLBs	Protocorm-like bodies
PTAS	Penanda terus amplifikasi satelit mini
RAPD	Random amplified polymorphic DNA
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolution per minute
SEM	Scanning electron microscope
SI	Similarity index
SPAR	Single primer amplification reaction
spores/ml	Spores per millilitre
SPSS	Statistical package for the social sciences
TBA	Tertiary-butyl alcohol
TBE	Tris-Borate-EDTA
TCL	Thin cell layer
Tm	Melting temperature
tTCL	Transverse TCL
U	Unit
VCG	Vegetative compatibility group
viz	*Videlicet*
VNTR	Variable number of tandem repeats
VP	Variable pressure
w/v	Weight over volume
Zn	Zinc

KESAN-KESAN JASAD SEPERTI PROTOKOM HIBRID *Dendrobium* YANG TERDEDAH KEPADA ASID FUSARIK DAN RADIASI GAMMA TERHADAP *Fusarium proliferatum* DAN *Fusarium oxysporum*

ABSTRAK

Dendrobium adalah bunga keratan yang popular dalam bidang florikultur dan perubatan tetapi cenderung menghadapi kerugian hasil akibat penyakit yang disebabkan oleh kulat. Tujuan penyelidikan ini adalah menggunakan rawatan mikotoksin, asid fusarik (AF) dan radiasi gamma ke atas hibrid *Dendrobium* (*Dendrobium* Waipahu Beauty × *Dendrobium* Burana White Big Flower) untuk menghasilkan kultivar yang mempunyai daya tahan terhadap *Fusarium proliferatum* dan *F. oxysporum*. AF dengan kepekatan 0.05, 0.10, 0.15 dan 0.20 mM telah ditambah ke dalam media separuh kekuatan Murashige and Skoog (MS) dan diinokulasi pada lapisan sel nipis (LSN) jasad seperti protokom (JSP) yang berusia empat minggu selama lapan minggu. Selain itu, dos radiasi gamma sebanyak 10, 20, 30, 40, 50, 60, 70, 80, 90 dan 100 Gy telah dipancar pada LSN JSP yang berusia empat minggu dan telah dipindahkan ke media MS yang baharu dengan serta-merta. LSN JSP yang dipancar dengan radiasi gamma diperhati selama empat minggu. Hasil kajian menunjukkan bahawa kepekatan AF sebanyak 0.10 mM menyebabkan kadar kemandirian JSP dan pertumbuhan tunas yang paling tinggi tetapi kadar kemandirian JSP dan pertumbuhan tunas berkurang apabila JSP diuji dengan kepekatan AF yang lebih tinggi. Didapati bahawa radiasi gamma pada 20 dan 30 Gy merupakan dos yang optima bagi kadar kemandirian JSP dan pertumbuhan tunas, namun dos yang lebih tinggi menyebabkan kematian JSP. Didapati juga bahawa dos radiasi gamma yang rendah merangsang pertumbuhan tunas dan *lethal dose* (LD_{50}) JSP adalah 63

Gy. Analisis histologi dan imbasan mikroskop elektron menunjukkan kerosakan sel dan penutupan permukaan stomata yang jelas pada JSP yang diuji dengan AF dan radiasi gamma. Walau bagaimanapun, kerosakan sel JSP lebih ketara pada kadar kepekatan AF dan dos radiasi gamma yang tinggi. Penanda terus amplifikasi satelit mini (PTAS) menunjukkan polimorfisme pada jalur DNA JSP yang telah dirawat berbanding kepada JSP kawalan. Analisa ini dilakukan sebagai kaedah awalan untuk memastikan polimorfisme telah berlaku pada LSN JSP yang dirawat dengan AF dan radiasi gamma. Selanjutnya, keputusan bioesei jambatan-daun menunjukkan bahawa kepekatan AF 0.05 mM dan dos radiasi gamma 20 hingga 30 Gy menghasilkan JSP yang mempunyai daya tahan yang paling tinggi terhadap kedua-dua spesies kulat. Oleh itu, penyelidikan ini merupakan kajian awalan saringan kadar kepekatan AF dan dos gamma yang optima berdasarkan tindakbalas LSN JSP terhadap agen mutasi tersebut.

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ABSTRACT

*Dendrobium*s are popular cut flowers in the floriculture and medicinal fields but are prone to yield loss due to diseases caused by fungi. The aim of this research was to utilise the mycotoxin, fusaric acid (FA) and gamma irradiation treatment on *Dendrobium* hybrid (*Dendrobium* Waipahu Beauty × *Dendrobium* Burana White Big Flower) to produce cultivars that are resistant towards *Fusarium proliferatum* and *F. oxysporum*. FA of concentrations 0.05, 0.10, 0.15 and 0.20 mM were transferred to sterilised half-strength Murashige and Skoog (MS) medium and inoculated with four weeks old thin cell layer (TCL) of protocorm-like bodies (PLBs) for eight weeks. On the other hand, various doses of gamma irradiation (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 Gy) were radiated on four weeks old TCL of PLBs and were immediately transferred to fresh MS medium. The radiated PLBs were observed for four weeks. It was deduced that PLBs treated with 0.10 mM of FA resulted in highest survival rate and shoot regeneration but the survival and regeneration rate began to decline as the concentrations of FA were increased. Gamma irradiation doses of 20 to 30 Gy were optimum for survivability and regeneration of treated PLBs and low doses of gamma irradiation stimulated shoot production in treated PLBs. However, the lethality rate increased in TCL of PLBs radiated with higher gamma doses. Results also indicated that the radio sensitivity (LD_{50}) of the PLBs was approximately at 63 Gy. Besides, histology and scanning

electron microscopy (SEM) observation showed prominent cell damage and stomatal closure in PLBs treated with FA and gamma irradiation. The damage was more severe as the concentration of FA and doses of gamma irradiation increased. Furthermore, direct amplification of minisatellite DNA (DAMD) markers showed polymorphism in the treated PLBs compared to the control PLBs. This analysis was done as a preliminary study to prove that polymorphism had occurred in the DNA of the FA and gamma radiation treated TCL of PLBs. In the leaf bridge bioassay, plantlets treated with 0.05 mM of FA and 20 to 30 Gy of gamma irradiation showed most resistance towards both fungal species. Therefore, this research is a preliminary screening study where the optimum concentration of FA and doses of gamma irradiation were selected based on the reaction of treated TCL of PLBs towards these mutagens.

CHAPTER ONE

INTRODUCTION

Orchids are the most diverse botanical family in the world (Jukofsky, 2002). They are ubiquitous and grow on almost every continent and vary widely in size and form. It is proven that there are more species of orchids in the world than the number of birds or mammal species (National Parks Board, 2016). Additionally, there are also approximately 60,000 known types of orchid hybrids which cannot be found in the wild because they have been created by orchid growers (Sibin and Gangaprasad, 2012).

Due to the minute seeds and lack of food storage in the seeds, orchids faced difficulties to propagate in the wild. Therefore, the horticulture industry relies mainly on artificial propagation, though there is also a great demand for wild rare species (Royal Botanic Gardens, Kew, 2016). Recent examples include Asian slipper orchids, where 99 % of the species is under the threat of extinction because these orchids are collected to fulfill horticultural demand. In addition, many orchid species are also commercially traded as ingredients in daily products obtained from supermarkets, health stores and pharmacies. For example, anthocyanins such as Orchicyanin I, Orchicyanin II and Cyanin are incorporated in make up cleansers and deodorants (Royal Botanic Gardens, Kew, 2016).

This increased demand for orchids is due to their beneficial uses. They are widely commercialised and the price of orchids can reach thousands of dollars. For instance, the Shenzhen Nongke orchid which was developed in the laboratory by agricultural research corporation Shenzhen Nongke Group costs $202,000 per plant (Breyer, 2014). Orchids are also mainly used as a medicinal plant (Singh and

Duggal, 2009). For example, moscatilin derived from the stems of *Dendrobium loddigesii* is used for treatment of stomach and lung cancer (Bulpitt, 2005).

High demands for the orchids also caused overexploitation of this plant. Therefore, for conservation purposes, *in vitro* techniques are used. Several tissue culture techniques have been developed for orchids, including the culture of flower stalks with axillary buds, meristems, flower stalks explants and internodal segments of flower stalks for faster and higher production of the plant (Arditti and Ernst, 1993). In recent years, thin cell layer (TCL) is used as another option of explant for micropropagation of orchids. TCL is a thin layer of cells which is totipotent and able to produce more number of explants compared to the whole protocorm-like bodies (PLBs) (Teixeira da Silva, 2013).

However, Fay (1992) stated that when working with plants of conservation importance *in vitro*, somaclonal variation is sometimes inevitable. Somaclonal variation is defined as genetic variation observed among plant progenies regenerated from somatic cells cultured *in vitro*. It is usual to try and maintain genetic integrity in tissue culture because all plants regenerated from somatic cells should be clones but somaclonal variation is a very common phenomenon in micropropagation. In addition to the basic genetic implications of this phenomenon, the variation has proven advantageous in breeding programs of various crop plants (Fay, 1992). According to Kumawat et al. (2017), in somaclonal variation, some new alleles or mutation which was not available in germplasm may be isolated and these variations occur in rather high frequency.

These advantages of somaclonal variation can be put to use to overcome the problem of diseases and pests in orchids. Although *Dendrobium* orchids have

increasing importance in floriculture, they are prone to disease caused by *F. proliferatum* and *F. oxysporum* (Swett and Uchida, 2015). These soil-borne fungi have a cosmopolitan distribution. Previous studies have reported that it is very difficult to eradicate these fungi because they are resistant to most fungicide (Pujol et al, 1997). The typical symptoms of infection on plants are discolouration which indicates rotting of tissues. Besides, infected stem of *Dendrobium* orchid showed yellowish discolouration with water soaked appearance and very friable roots (Latiffah et al., 2009a).

Hence, a valuable approach for improving the productivity of *Dendrobium* hybrid, *D5* (*Dendrobium* Waipahu Beauty × *Dendrobium* Burana White Big Flower) in this research 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). Fusaric acid which is a mycotoxin produced by most *Fusarium* spp. can be used as a selecting agent for *in vitro* selection of resistant orchids (Mahlanza et al., 2013). It has low to moderate toxicity and can cause alteration in the morphology and growth in the infected plants (Wang et al., 2014).

In addition, exposing plant genetic material such as callus to mutagens enhances the chance for isolating unique genetic material (Dhanavel et al., 2012). One of such physical mutagen is gamma ray irradiation (Soeranto et al., 2001). Induced mutation using gamma ray irradiation is commonly used to develop new species in plant breeding (Oladosu et al., 2015). It can rapidly create the variability of inherited traits in crops, both quantitatively and qualitatively (Muduli and Misra, 2008). Post induced mutation has been effectively utilised in developing new and

valuable alteration in plant characteristics that have contributed to increased yield potential or disease resistance (Gaswanto et al., 2016).

The alteration can be confirmed with morphological, cytological and molecular analysis. One of the means of molecular analysis is the direct amplification of minisatellite region DNA (DAMD) analyses. This analysis is easy to conduct and shows maximum polymorphism in DNA (Bhattacharyya et al., 2016).

In order to check the treated plantlets for resistance or tolerance towards these fungi, a leaf-bridge bioassay is conducted. Successful bioassays performed in controlled conditions have shown the efficacy of using these strategies to develop disease resistant plants (Shekhawat et al., 2014).

1.1 Objectives

The objectives of this research were:

i. To perform *in vitro* selection of disease resistant *Dendrobium* hybrid orchid protocorm-like bodies (PLBs) using different treatments of fusaric acid (FA) and gamma irradiation on the TCL of PLBs,
ii. To investigate the effects of FA and gamma irradiation on morphology, histology, and direct amplification of minisatellite region DNA (DAMD) analyses of treated PLBs,
iii. To test the survived plantlets from the FA and gamma irradiation treatment in a leaf-bridge bioassay to determine resistant, tolerant and susceptible plantlets when inoculated with *F. proliferatum* and *F. oxysporum*.

CHAPTER TWO

LITERATURE REVIEW

2.1 Orchid

The term orchid was coined by Theophrastus. In Greek, 'orkhis' means testicles and orchid was named so because the anatomy of the plant resembles testicles (Stewart and Griffith, 1995). Orchidaceae family comprises of more than 25,000 species worldwide (Chase, 2005; Wang et al., 2009). It is the largest and most diverse group of angiosperms (Jukofsky, 2002; Singh and Duggal, 2009). According to Chase et al. (2003), it is rapidly becoming one of the most researched families of angiosperms. This is due to its increased popularity around the globe for their beauty and variety (Jukofsky, 2002).

Orchids are predominantly found in the wet tropics all around the world. They are absent only from the Polar Regions and the driest of deserts (Chase, 2005). They are extremely adaptable, and can grow in almost all climates except for frigid and arid extremes. They are both pan tropical, and endemic. Most orchid species grow in tropical forests, but some can also be found in semi-desert regions, near the seashore and in the tundra. Majority of orchid species can be found in Asia, southern Central America, northwest South America, and countries that lie along the Andes Mountains (Jukofsky, 2002). Islam et al. (2015) stated that orchids can live in the soil, on rocks and even underground. However, most orchids are epiphytes, where they grow on other plants and trees. Orchids tend to obtain their nutrients from the air, rain or moisture in the soil.

Orchids are cultivated for their beautiful flowers (Chugh et al., 2009; Singh and Duggal, 2009). According to Jukofsky (2002), they greatly vary between species

in terms of size, weight and colour. There are orchids as small as a coin and others may weigh up to one ton with petals as long as 76 cm, and sprays of small flowers 3 to 4 m long. Orchid blossoms also appear in a variety of colours. In general, both hybrids and wild orchids have the following features (Jukofsky, 2002). They have bilaterally symmetrical flowers, sticky masses of pollen grains called pollinia and minute seeds containing undeveloped embryos with no nutritive materials. These seeds are only able to germinate with the presence of a symbiotic fungus under natural conditions (Seaton et al., 2010). Also, the floral arrangement of all orchid species is the same. Each orchid flower has six parts where the outer three flower parts are green sepals, and the inner three flower parts are beautifully coloured petals (Jukofsky, 2002).

It was found that the captivating flowers have variable colours, shapes and sizes. It blooms up to perfection and withstand long distances. Therefore, orchids are one of the top ten cut flowers in the international market. In the past decade, several new cultivars of orchids were released and an increased demand for orchid cut flowers was observed. Many of them have floricultural importance owing to their beautiful flowers and ability to persist for a long time. Recently, many of the commercially important orchids are artificially grown for its high price in the national and international markets (Islam et al., 2015).

However, Islam et al. (2015) reported that orchids are commonly known for their economic importance as cut flowers, but less for their medical value. Some plants like *Dendrobium crumenatum*, *Eulophia campestris*, *Orchis latifolia*, *Vanda roxburghii* and *Vanda tessellata* have been documented for their medicinal value. Some orchids have been reported to contain phytochemicals, namely alkaloids, triterpenoids, flavonoids and stilbenoids. Examples of its medicinal uses are as anti-

bacterial, anti-cancer, anti-inflammatory, demulcent, anti-hemorrhagic, and vulnerary. It is also useful in treating diabetes, diarrhea, dysentery, paralysis, impotence and malnutrition (Singh and Duggal, 2009).

In a study by Chase (2005), it was pointed out that the orchid's highly modified flowers are usually their main attraction, but the best and only truly universal character for the family is their unusual early developmental stage, the protocorm. This is the point at which orchids form a symbiotic relationship with mycorrhizae. It also compensates for its lack of a true water-retentive root system by working with these fungi during some portion of their life cycle. During this period, the fungi grow partly inside orchid roots, helping the plant to absorb water and minerals. On the other hand, the orchid produces some nutrients during photosynthesis that help the fungi to survive (Jukofsky, 2002; Cozzolino et al., 2003).

Orchid mycorrhiza has an impact on plant fitness from germination through seedling stage and throughout life. The diversity of fungi in mycorrhizal relationship with orchids involves saprotrophs, ectomycorrhizal, parasitic fungi and five major basidiomycete groups which are Heterobasidiomycetes, Hericianae, Hymenocaetanae, Thelephoranae, and Agaricanae (Rasmussen, 2002).

2.2 *Dendrobium* orchid

The genus *Dendrobium* comprises of approximately 1450 species and they are one of the largest genera in Orchidaceae. Although most *Dendrobium* species have an epiphytic growth habit, some are also found growing on rocks and cliffs and terrestrial in grasslands (Puchooa, 2004). This genus is distributed throughout

tropical Asia and the Pacific region from Sri Lanka to Japan in the North, Tahiti in the East, and New Zealand in the South (Pridgeon et al., 2014).

Chattopadhyay et al. (2012) highlighted that the *Dendrobium* genus is mainly categorised into horticultural, agricultural, medicinal or dual purpose species depending upon their utility *per se*. It occupies a leading position in ornamental orchid cut flower industry due to its high number of flowers per inflorescence and recurrent flowering (Martin and Madassery, 2006). Some characteristics of *Dendrobium* such as its long flowering life, and the genus's wide spectrum of shapes, colours, and sizes are also reasons of *Dendrobium* expanding popularity (Kuehnle, 2007).

According to Puchooa (2004), *Dendrobium* has a wide range colours. Mostly, hybrids are usually lavender, white, golden-yellow, or combinations of these colours. In some cases, unusual species and hybrids can be bluish, ivory coloured, brilliant orange or scarlet, or have interesting markings on the leaves and petals. In addition, many of the evergreen *Dendrobiums* are odourless. Though, some deciduous species such as *superbum*, *pierardii* and *parishii* have fragrance of fresh citrus scent or smell of raspberries.

It was reported that in Malaysia, orchid contributes 40 % of total production of cut flowers and the most popular are *Dendrobium* (Latiffah et al., 2009a). Unlike other groups of plants, orchids hybridise widely in nature. For the last 150 years they have been widely cross bred to produce more than 110,000 hybrids. Furthermore, 3,000 new orchid hybrids are being registered annually (The Royal Horticultural Society, 2016). They are produced in order to incorporate new colour options for ornamental plants (Cardoso, 2012).

Moreover, *Dendrobium* also has many medicinal properties. In the Chinese community, *Dendrobium* is known as medicinal plants. The main effective ingredients are polysaccharides and alkaloids which have pharmacological effects on gastritis infection, cancer, and anti-aging (Chen et al., 2013). Bulpitt (2005) highlighted that several *Dendrobium* species are used to promote peristalsis and gastric secretions, replenish body fluid, reduce fever, increase white blood cells, and reduce fidgets.

In China, *Dendrobium* is also used in treatment of stomach, oesophageal and lung cancer (Bulpitt, 2005). This medicinally important species have particular qualities of tonic, disease resistance and anti-depressant. For example, *Dendrobium macraei* contains an alkaloid; jebantine which is used as tonic. Besides, *Dendrobium moschatum* has two phenanthrenes, rotundatin and moscatin which are reported to be useful in inhibition of aggregation of platelets induced by arachidonic acid and collagen (Singh and Duggal, 2009; Chattopadhyay et al., 2012). In addition, two phenanthrenes, 4,7-dihydroxy-2-methoxy-9,10-dihydrophenanthrene and denbinobin were isolated from the aerial part of *Dendrobium nobile* Lindl. These compounds possessed strong anti-mutagenic potential and were found to be anti-carcinogenic against lung carcinoma, ovary adenocarcinoma and promyelocytic leukemia (Lee et al., 1995).

The orchid of interest in this study is the *Dendrobium* hybrid, *D5*. It is a cross between *D.* Waipahu Beauty and *D.* Burana White Big Flower (Plate 2.1). The florescence has white and purple coloured petals. This hybrid is popular because of their floriferous inflorescense, bright colour flowers and durability with long shelf life.

Plate 2.1: The orchid hybrid *D5* (*D.* Waipahu Beauty × *D.* Burana White Big Flower). Scale bar: 1 cm

2.3 Orchid diseases and the pathogens

Orchids are prone to many disease incidences especially in nurseries. Examples of virus induced diseases are *Cymbidium* mosaic virus (CYMV) and *Odontoglossum* ringspot virus (ORSV) (Soto-Valladares et al., 2012), whereas diseases induced by fungi comprises soft rot caused by *Erwinia carotovora* and petal blight caused by *Alternaria alternata* (Duff, 1993).

One of the main orchid fungal pathogen is the *Fusarium* species (Swett and Uchida, 2015). Numerous *Fusarium* spp. have been reported to be the causal agent of root rot, dry rot, and leaf spot diseases of orchids (Leslie and Summerell, 2006). In a disease survey, root and stem rot disease of *Dendrobium* were observed in a few nurseries in Penang Island and Taiping, Perak, Malaysia. Typical symptoms on the stem and root of *Dendrobium* were discolouration with water soaked appearance and very friable (Latiffah et al., 2009a).

In earlier studies, *Fusarium* species were constantly isolated from the infected root and stem rot of the orchid plant. In pathogenicity tests on *Dendrobium* orchid, the symptoms observed were similar with symptoms on diseased plant in the nurseries. *Fusarium* species were re-isolated from inoculated plants which indicated that Koch's postulate has been fulfilled. Therefore, *Fusarium* species, namely *F. oxysporum* and *F. proliferatum* were identified as the causal agent of root and stem rots of *Dendrobium* orchid (Latiffah et al., 2009b).

2.4 *Fusarium* spp.

According to Di Pietro et al. (2003), *Fusarium* spp. are infamous to cause the vascular wilt disease in many plants. *Fusarium* spp. has more than 120 different

formae speciales (f.sp) which have been identified based on specificity to host species belonging to a wide range of plant families (Di Pietro et al., 2003). In addition, the *Fusarium* spp. causes severe loss on most vegetables, flowers, several field crops such as cotton and tobacco, plantation crops such as banana, plantain, coffee and sugarcane, and a few shade trees annually (Di Pietro et al., 2003).

Fusarium spp. are one of the most important soil borne plant pathogens which causes serious diseases in many popular ornamental plants such as carnation, chrysanthemum, lily, and orchid. Latiffah et al. (2009a) reported them as one of the major pathogen of orchids. These pathogens survive in the soils and plant debris. Spore or conidia are produced on the plant debris and susceptible plants are mostly infected by spores via rain splash and farm equipment or tools. This disease could also spread due to poor sanitation practices. Usually, infected plants are not removed from the plantations and these become the main source of inoculum (Katan, 2000).

2.5 *Fusarium oxysporum* and *Fusarium proliferatum*

The fungi of interest in this research are *F. proliferatum* and *F. oxysporum* because these fungi are the main pathogens of orchid that caused stem and root rot (Latiffah et al., 2009a). In the mentioned study, the main species isolated and identified from the root and stem rot of *Dendrobium* orchid were *F. proliferatum*, *F. oxysporum* and *F. solani.*

F. proliferatum is one of the pathogens of orchid. It can be found in a remarkably broad host range and can also grow endophytically or without causing symptoms in some hosts, such as maize, orchids and wheat (Proctor et al., 2010). Stankovic et al. (2007) observed that the microconidia of this fungus are formed in false head and in short chains with V-shaped polyphialides. The conidiogeneous cells

are both monophialides and polyphialides whereas, the macroconidia are almost straight and slightly sickle-shaped. Furthermore, there is an absence of chlamydospores. It also has dark purple pigmentation. Besides, *F. proliferatum* is a toxigenic species which produces numerous toxins, namely fumonisin B, moniliformin, fusaric acid, and fusaproliferin. A few of these toxins have phytotoxic activity (Stankovic et al., 2007).

This fungus is also considered to be a pathogen of many economically important plants (Seefelder et al., 2002). According to Logrieco et al. (2002), *F. proliferatum* is one of the species which were frequently isolated from maize pink ear rot. In Germany, this fungus widely attacks asparagus spears and garlic bulbs (Seefelder et al., 2002). Stępień et al. (2011) stated that isolates of *F. proliferatum* from a variety of hosts, such as asparagus, maize, and pineapple produces fumonisin in variable concentrations. Furthermore, Stankovic et al. (2007) confirmed *F. proliferatum* as the main pathogen of garlic and onion in Europe and that there is a potential mycotoxin accumulation risk in contaminated plants of both garlic and onion. Apart from that, Kushiro et al. (2012) concluded that *F. proliferatum* is a potent pathogen of rice and produces fumonisin B_1 in rice grains in the field.

F. oxysporum is also another pathogen of orchid. According to Latiffah et al. (2009b), *F. oxysporum* isolates can be clearly identified based on the production of oval to kidney shaped microconidia in false heads borne on short monophialides. The macroconidia are straight or slightly curved. There is also an abundance of chlamydospores. Chlamydospore enables this fungus to survive in the soil for extended periods of time and is capable of colonising crop residues and the roots of most crops. *F. oxysporum* has purple to dark purple pigmentation. The only effective control of this fungus is the use of resistant cultivars (Kantoglu et al., 2010).

According to Zhang et al. (2005), *F. oxysporum* is one of the pathogens which cause watermelon *Fusarium* wilt and melon gummy stem blight. Bottalico and Perrone (2002) deduced that this fungus is associated with head blight of small-grain cereals in Europe. They are less frequently encountered but very toxigenic on this plant. Müllenborn et al. (2008) stated that *F. oxysporum* is often related with significant yield losses due to premature senescence and reduced grain filling, and contamination of kernels with numerous mycotoxins. This fungus also contributes to *Fusarium* crown and root rot of tomato, and *Fusarium* wilt in basil (Rekah et al., 2000). In California, this fungus is an aggressive pathogen of the Pima cotton (*Gossypium barbadense*) (Kim et al., 2005a).

Both *F. proliferatum* and *F. oxysporum* are the causal agents of a destructive disease of asparagus called *Fusarium* crown and root rot. These pathogens are transmitted by seed and colonise both vascular and epidermal tissues simultaneously (Mulè et al., 2004).

2.6 Disease control in *Fusarium* infected plants

There are various methods used by growers to control diseases in orchids. One of the methods to maintain a disease-free orchid collection is to breed healthy stock. Growers should buy only vigorous and disease-free plants (Daly et al., 2013).

In addition, Daly et al. (2013) suggested that good cultural practices should be established in the nursery as a disease control method for orchids. Benches and floors must be kept free of plant debris. All diseased plants or parts of plants must be removed from the nursery. Air flow in the breeding area should be improved in order to help dry foliage and stems to reduce bacterial diseases. Cutting and working tools must also be sterilised by dipping in alcohol or bleach (Daly et al., 2013).

Another method used to maintain disease free orchids is by using fungicides. There is a variety of fungicides available in the market. According to Müllenborn et al. (2008), chemical control is a vital method of integrated *Fusarium* head blight (FHB) control in highly infected production areas. Fungicides are applied to wheat at anthesis and as a result, reduced quantitative yield losses and mycotoxin contamination of kernels associated with FHB were observed. However, reports on the fungicide efficacy are often conflicting. Some triazole fungicides, such as metconazole and tebuconazole, were deemed effective against the FHB (Matthies and Buchenauer, 2000; Pirgozliev et al., 2002). Yet, of the two most commonly used fungicide classes (triazoles and strobilurins); strobilurins have been recorded to be associated with elevated levels of Deoxynivalenol (DON) in grain. DON is a mycotoxin produced by *F. graminearum* and *F. culmorum* which when ingested, can lead to toxicosis in human and animals (Wegulo, 2012).

Besides, chemical control of *Fusarium* is also costly, laborious and resource-intensive (Egel and Martyn, 2007). Some of them are non-biodegradable, which causes environmental pollution. It builds up in hefty concentrations in the soil and lowers its productivity in the water table causing health hazards to flora and fauna (Jayasankar et al., 2000).

2.7 Problems in orchid propagation

Besides diseases that infect the plant, orchids are also naturally difficult to propagate in the wild. The seeds of orchids are minute and contain scarce food reserves. The protocorms which are formed after the germination and growth of epiphytic orchid seeds, young seedlings and some adult plants are also not able to

produce carbon as they lack chlorophyll. Protocorms are small, spherical, food-storing underground stems (Aktar et al., 2008; Zhu et al., 2008).

Orchids also need the symbiotic relationship with the mycorrhiza to survive, which is another reason orchids do not thrive well in the wild. Teixeira da Silva et al. (2015) reported that *in vitro* culture of orchid seeds are capable of swelling without fungal influence due to minimal water uptake and optimal abiotic conditions such as light and temperature at the early stage of germination. However, in the absence of an exogenous carbohydrate supply, mycorrhizal colonisation is vital for further development of the protocorm and seedling growth. An example of mycorrhiza is the *Rhizoctonia* species (Vujanovic et al., 2000). According to Rasmussen (1995), the duration to obtain symbiotic germination may differ from one to a few months, or can even reach up to a year depending on the species and seed lots and the ability of a specific fungal isolate to prompt germination.

Orchids also propagate poorly in the wild because the seedling progenies are heterozygous and do not guarantee true-to-type plants of hybrid cultivars. According to Ferreira et al. (2006), asymbiotic seed germination and the conventional vegetative method have been frequently used by growers to propagate these plants. However, asymbiotic *in vitro* germination methods have to be developed for each species since the optimisation of tissue culture condition at each step is largely species specific (Chen et al., 2015). In addition, conventional vegetative propagation is beset with slow multiplication rate, and does not provide enough clones within short time frame.

Another major hindrance to propagation of orchids in the wild is the difficulty in seed germination, while seed development can be a long process and flowering plants are often produced only after three to five years of growth

(Malabadi et al., 2008). Therefore, *in vitro* propagation of orchids is used as an option for rapid propagation of commercially valuable orchids (Martin and Madassery, 2006).

Most species of *Dendrobium* orchids are in endangered status primarily because of anthropogenic interference in natural habitats and commercial over-exploitation. Due to this, the existence of orchid germplasms in their natural habitat is at risk (Soetopo and Purnamaningsih, 2012). Hence, the development and application of modern techniques and strategies directed towards *in vitro* propagation of orchids increases their number, provide a practical means to conserve plants in an artificial environment, and offers material for reintroduction in the wild (Teixeira da Silva et al., 2015).

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

In vitro plant regeneration is an important and essential component of plant biotechnology (Aktar et al., 2008). Ng and Saleh (2011) pointed out that propagation of orchids using micropropagation techniques has been practiced for more than a century and has resulted in the production of uniform clones in many orchid genera.

Bunn et al. (2007) stated that tissue culture technique has a great number of advantages. Thus, there is much interest for the collecting, multiplication and storage of plant germplasm. This system allows propagating plant material with high multiplication rates in an aseptic environment. Consequently, virus-free plants can be attained through meristem culture in combination with thermotherapy, thus ensuring the production of disease-free stocks and simplifying quarantine procedures for the international exchange of germplasm. Besides, the reduction of explants allows

reducing space requirements. As a result, the labour costs for the maintenance of germplasm collections is also lowered (Engelmann, 2011).

According to Soetopo and Purnamaningsih (2012), germplasms are very important asset as raw materials in any orchid breeding program. For example, orchids, such as *Dendrobium* and *Phalaenopsis*, which contain certain endangered species, require immediate conservation. Thus, a possible choice for *ex situ* conservation in orchid is via cloning technique by tissue culture. This technique results in vegetative propagation which produces offspring in mass number and genetically similar to the parental plant (Soetopo and Purnamaningsih, 2012).

Tissue culture has certainly played an important role in multiplication of many commercially important orchids, including *Dendrobium* for many years. In orchid, this technique reduces dependence on mycorrhiza for seed germination and excludes disease infection and reinfection to the clonal products (Kauth et al., 2008). Micropropagation in orchid is done using different explants such as shoot tip, leaf, stem, flower stalk, or root segment (Martin and Madassery, 2006). Micropropagation of orchid has also been done using inflorescence axis, flower bud, rhizome segment and TCL (Rangsayatorn, 2009).

Study conducted by Lee et al. (2013) proved that shoot tips have been effectively used for the induction of shoot buds and PLBs of many orchids such as *Cymbidium* and *Dendrobium*. It is a reliable method for tissue culture of sympodial orchids like *Dendrobium, Cymbidium, Arundina, Phaius* and *Anoectochilus*. Apart from that, PLBs can also be induced directly from other explants, such as flower stalk buds, root tips and leaf segments. The indirect regeneration of PLBs from embryogenic callus culture could use both solid and liquid suspension cultures. The formation of protocorms from germinated seed and the subsequent induction of PLBs

from these explants has become a reliable method for propagating orchids (Ng and Saleh, 2011).

Unlike shoot tips, foliar explants can be easily obtained and do not require the sacrifice of the mother plant. Besides, their availability is not seasonal like inflorescence explants (Temjensangba and Deb, 2005). Effective micropropagation using leaf explants relies on many factors, such as medium nutrient composition and the growth hormones, explant orientation, part of the leaf taken, source of the leaf (*in vitro*/*in vivo*), and most importantly the age of the leaf. Furthermore, inflorescence segments also appear as effective explant for micropropagating orchids such as *Phalaenopsis* and *Dendrobium* (Chugh et al., 2009).

Apart from that, *in vitro* derived rhizomes also serve as an available source of explants for many terrestrial orchids. This is a very effective method for rapid propagation of commercially important rhizomatous orchids like *Cymbidium*. In spite of the limited morphogenetic ability of root meristem of higher plants, including orchids, the utility of root explants for micropropagation purposes is increasing due to their year round availability, low oxidation rate, and the ease with which they can be explanted (Chugh et al., 2009).

Besides, Aktar et al. (2008) observed that in tissue culture, the frequency of callus inductions and plant regeneration depends on many factors, such as genotypes, type of explants and composition of media. Nutrient composition is considered as the most important source of variation in plant tissue culture. Various culture media have been used for efficient plant regeneration in orchid previously. Among them, Murashige and Skoog (MS) medium was found to be most efficient for PLBs formation and plantlet regeneration of *Dendrobium* orchid when supplemented with

2,4-Dichlorophenoxyacetic acid (2,4-D) (Murashige and Skoog, 1962; Nasiruddin et al., 2003).

According to Ahmad et al. (2010), the successes of hybridisation technology in producing enormous number of orchid hybrids with attractive characteristics and the introduction of the *in vitro* technology to mass-propagate clonal planting materials have been a remarkable boost for the orchid growing industry. Wide range of successful cultivars with attractive combinations of bud number, spray length, flower colour and form, fragrance, vase life, seasonality, and compactness have been attained through hybridisation (Ahmad et al., 2010).

On the other hand, tissue culture has been extensively used as the standard method of germinating seeds and propagating seedlings for the industry. Thousands of plants can be cloned and grown in a relatively short duration through the meristem cloning technique (Ahmad et al., 2010). These two technologies combined have been very reliable in supporting the orchid industry. It has been easier to mass propagate and supply commercially attractive hybrids and varieties to the growers for the market (Ahmad et al., 2010).

Studies conducted by Park et al. (2000) confirmed that micropropagation through PLBs formation is favoured by commercial growers of most orchid genera because large number of PLBs can be obtained in a short period of time. The large-scale propagation of PLBs can also be attained using a bioreactor system. Also, due to the high capabilities of PLBs to regenerate into complete plantlets, they are the most common target tissue for genetic transformation studies in orchids (Sreeramanan et al., 2008).

Furthermore, Cordova II and Thammasiri (2016) stated that PLBs can also serve as plant material for cryopreservation. PLBs are well-differentiated tissues that

are sometimes considered as orchid embryos that develop with two discrete bipolar structures, *viz*. the shoot and root meristem. Hence, these structures are highly totipotent when grown on plant growth regulator (PGR)-free medium. Moreover, the PLBs that formed directly from meristem tissue have a higher genetic stability than those produced by callus (Ng and Saleh, 2011).

2.9 Thin cell layer (TCL) system

Another explant for micropropagation of orchids is the TCL. Teixeira da Silva (2013) stated that TCL is an explant which is a thin layer of cells usually measuring a few millimeters (mm) in thickness, but with variable proportions of length and diameter. A TCL can be prepared from any explant source by cutting a thin section less than 5 mm thick with a sharp blade.

According to Tranh Than Van (1973), TCL consists of explants of a small size, such as stems, leaves, floral inflorescences, flower primordial and cotyledons excised from different plant organs, either longitudinally or transversally. Longitudinal TCL (lTCL) contain only one type of tissue, for instance, a monolayer of epidermal cells. In a transverse TCL (tTCL), a small number of cells from different tissue types, such as epidermal, cortical, cambium, perivascular and medullary tissue, as well as parenchyma cells are included (Chugh et al., 2009).

Based on the research by Nayak et al. (2002), TCL of actively growing tissues such as shoots, stem nodes and PLBs have been successfully used for plantlet regeneration in orchids (Malabadi et al., 2008). TCL explants containing only few layers of cells are able to regenerate either into root, flower bud or shoot. This culture system was proven to be more successful than other conventional *in vitro* culture methods. This is proven with regard to the total output of plantlets of orchids as well

as in other plants such as *Aranda* Deborah, *Dendrobium candidum*, *Rhynchostylis gigantea*, *Lilium* spp., *Sorghum bicolor* and *Heliconia psittacorum* (Rangsayatorn, 2009).

TCL has also been used as an explant for *in vitro* plantlet regeneration in a few other plant species such as *Panax ginseng* and *Digitaria sanguinalis* (Teixeira da Silva, 2013). TCL technology has advanced orchid tissue culture, making mass clonal propagation easier and more reproducible. This system was more efficient than other conventional *in vitro* methods with regard to the total output of plantlets in a few orchid species including *Aranda* Deborah (Lakshmanan et al., 1995a) and *Spathoglottis plicata* (Teng et al., 1997).

Teixeira da Silva (2013) proposed three protocols for TCL of PLBs formation. In Protocol 1, the PLB is subcultured as a whole PLB. The amount of PLBs regenerated is generally lower than in Protocol 2 and 3. In Protocol 2, a whole PLB is cut at the shoot apical meristem and basal part. Then, the PLB is cut symmetrically length-wise to yield two half-moon-shaped explants. When each half-moon shaped PLB explant is re-plated on the same medium, several secondary PLBs are formed near and at cut surfaces after 30 to 45 days. In protocol 3, a whole PLB is also cut at the shoot apical meristem and basal part. Then, the PLB is cut longitudinally or transversely into three to four slices. According to Teixeira da Silva (2013), protocol 3 is better compared to protocol 1 and 2 because the rate of PLB formation is higher in protocol 3.

Nayak et al. (2002) have emphasized that TCLs have advantage in terms of economy, time and material. Using TCL, more than 80,000 *Vanilla planifolia* plantlets could be produced from thin sections obtained from a single shoot tip in a year as compared to about 11,000 plantlets produced by the orthodox shoot tip

method (Lakshmanan et al., 1995a; Chugh et al., 2009; Jing et al., 2014). Besides, in *Cymbidium* Sleeping Nymph within seven days of inoculation, a slight expansion and formation of a small protuberance was observed at the periphery of the tTCL. The protuberance grew in size and developed into a PLB within 30 days of culture (Vyas et al., 2010). According to Malabadi et al. (2008), tTCL was used as an efficient propagation method for the fast multiplication of *Eria dalzelli*. The tTCL technology is very effective for small scale tissue culture industries and commercial production of plantlets.

In addition, thin sections ease the diffusion of nutrients and growth-promoting substances at the site of regeneration. It also eliminates correlative control imposed by other tissues (Lakshmanan et al., 1995b). This technique is advantageous because of the optimal induction of several centres of meristematic activity, which are generally present in the protocorms. Another advantage of using TCLs is that they need only a small amount of plant material and medium volume (Teixeira da Silva, 2003).

2.10 Somaclonal variation

According to Gozukirmizi et al. (1990), one of the major problems of many tissue cultured plants is somaclonal variation. It is the variation originating in cell and tissue cultures. The growth of plant cells *in vitro* and their regeneration into whole plants is an asexual process which involves only mitotic division of the cell. Ideally, it is expected to get clonal multiplication of genetically uniform plants. However, due to somaclonal variation, uncontrolled and random spontaneous variations occur during the culture process. The highly mutagenic conditions of tissue culture environment causes somaclonal variations at morphological,

cytological, molecular and biochemical levels by activating genetic and/ or epigenetic mechanisms (Gozukirmizi et al., 1990).

This variability expressed in micropropagated plants could be due to or related to; oxidative stress damage inflicted upon plant tissues during *in vitro* culture (Cassells and Curry, 2001). According to Wachsman (1997), oxidative stress results in elevated levels of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, hydroxyl, peroxyl and alkoxyl radicals. The enhanced production of ROS during environmental stresses can pose a threat to cells by causing peroxidation of lipids, oxidation of proteins, damage to nucleic acids, enzyme inhibition, activation of programmed cell death pathway and ultimately leading to death of the cells (Sharma et al., 2012).

Moreover, different factors affect the frequency of development of somaclones under *in vitro* conditions (Cassells and Curry, 2001). The numerous stress factors includes exposure to sterilants during sterilisation, wounding, replacing sugar to photosynthesis in the leaves, imbalances of media components such as high concentration of PGRs, the disturbed relationship between high humidity and transpiration and lighting conditions (Joyce et al., 2003; Nivas and D` Souza, 2014).

According to Jain (2001), there are some disadvantages of somaclonal variation. This variation may not occur for complex agronomic traits, and many characters change in the opposite or unwanted direction. Besides, the variations that occur are in an unpredictable nature and genetically unstable.

On the other hand, somaclonal variation also has its advantages. It is useful in crop improvement through creation of novel variants. Induced somaclonal variation is used for genetic manipulation of crops with polygenic traits. It can also be a vital tool for plant breeding via generation of new varieties. This could exhibit disease