IDENTIFICATION AND MODE OF SYMBIOSIS FOR MYCORRHIZAL FUNGI AND EPIPHYTIC ORCHID, *Dendrobium crumenatum*

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by

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LIST OF UNITS, SYMBOLS AND ABBREVIATIONS

cm	Centimeter
mL	Milliliter
mm	Millimeter
µg	Microgram
µL	Microliter
µm	Micrometer
˚C	Degree Celsius
AG	Anastomosis Group
AM	Arbuscular mycorrhizae
CMA	Corn Meal Agar
DAPI	4',6-diamidino-2-phenylindole
EM	Ectomycorrhizae
ErM	Ericoid mycorrhizae
FAA	Formalin-acetic acid
FF	Fusion Frequency

OM	Orchid mycorrhizae
OMA	Oatmeal Agar
PDA	Potato Dextrose Agar
PSA	Potato Sucrose Agar
Psi	Pressure per square inch
TBA	Tertiary Butyl Alcohol
WA	Water Agar

PENGECAMAN DAN CARA SIMBIOSIS KULAT MIKORIZA DAN ORKID EPIFIT, *Dendrobium crumenatum*

ABSTRAK

Dendrobium crumenatum adalah salah satu keluarga Orchidaceae, menghasilkan bunga putih, wangi dengan warna kekuningan di bahagian tengah yang kebiasaannya dipanggil sebagai orkid merpati. Orkid ini biasanya dijumpai di Malaysia hidup secara semula jadi di atas pokok di hutan tropika. Kebanyakan orkid bergantung kepada kulat mikoriza untuk tumbuh dan terus hidup. Oleh itu, objektif utama kajian ini adalah untuk mengkaji hubungan kulat mikoriza pada akar orkid epifit (*Dendrobium crumenatum*) dan kulit kayu pokok Pukul Lima (*Samanea saman*). Hubungan kulat mikoriza pada akar orkid epifit *D. crumenatum* dan kulit kayu pokok Pukul Lima (*S. saman*) boleh ditentukan dengan memerhatikan kehadiran peloton pada akar orchid epifit. Peloton diperhatikan dengan menggunakan dua kaedah keratan; potongan akar menggunakan tangan dan mikrotom. Sejumlah 251 pencilan kulat telah berjaya diperoleh daripada akar orkid epifit dan kulit kayu pokok Pukul Lima dengan dan tanpa pertumbuhan orkid. Hanya 17 pencilan telah dikenal pasti sebagai kulat *Rhizoctonia* bak kulat iaitu empat belas diperolehi daripada akar orkid epifit dan tiga diperolehi daripada kulit kayu pokok Pukul Lima dengan pertumbuhan orkid. Tiada *Rhizoctonia* bak kulat yang berjaya diperolehi daripada akar epifit orkid mahupun kulit kayu pokok Pukul Lima tanpa pertumbuhan orkid. Sebanyak 17 pencilan kulat *Rhizoctonia* bak kulat telah dikelaskan kepada empat kumpulan iaitu Kumpulan I, II, III, dan IV berdasarkan ciri morfologi dan analisis anastomosis. Bilangan nukleus yang terdapat dalam sel-sel muda

menunjukkan bahawa keempat-empat kumpulan kulat *Rhizoctonia* bak kulat adalah binucleate. Selain itu, analisis anatomosis pada *Rhizoctonia* bak kulat menunjukkan mereka yang berjaya bercantum berkaitan dalam menentukan kumpulan yang ditentukan oleh ciri morfologi.

IDENTIFICATION AND MODE OF SYMBIOSIS FOR MYCORRHIZAL FUNGI AND EPIPHYTIC ORCHID, *Dendrobium crumenatum*

ABSTRACT

Dendrobium crumenatum is one of the families of Orchidaceae, produces white, fragrant flower with a yellow tinted throat that commonly called as pegion orchid. This orchid is the most common in Malaysia found naturally on trees in the tropical forests. Most of the orchids were depend on orchid mycorrhizal fungi to establish and survive. Thus, the main objective of this study is to understand the relationship of the mycorrhizal fungi in epiphytic orchid roots of *Dendrobium crumenatum* and bark of Rain Tree (*Samanea saman*). The relationship of the mycorrhizal fungi in epiphytic orchid roots of (*D. crumenatum*) and bark of Rain Tree (*S. saman*) can be determined by observing the presence of peloton in the root of epiphytic orchid. The presence of peloton in the roots was observed by using two method of sectioning; hand section and microtome sectioning. A total 251 isolates of fungi were successfully isolated from roots of epiphytic orchid and bark of Rain Tree with and without growing orchid. Only 17 isolates was identified as *Rhizoctonia*-like fungi, in which fourteen isolates from root of epiphytic orchid and three isolates from bark of Rain Tree with growing orchid. *Rhizoctonia*-like was isolated neither from root of epiphytic orchid, nor bark of Rain Tree without growing orchid. The 17 isolates of *Rhizoctonia*-like were grouped into four groups that is Group I, II, III, and IV based on their morphological characteristic and anastomosis analysis. Number of nuclei present in immature cells showed that all the four groups of *Rhizoctonia*-like fungi were binucleate. Besides that, anatomosis analysis

of *Rhizoctonia*-like fungi also showed that their successful hyphal fusion corresponded with the group determined by morphological characteristic.

CHAPTER 1

INTRODUCTION

Orchids are members of Orchidaceae, one of the largest families in the flowering plants. Among angiosperms, orchids are unique in their thread pattern, especially in floral structures and organ identity (Yifeng Xu *et al.,* 2006). Orchids are economically important in some country. Vanilla is a popular flavor used in food and drink, the tissues of *Gastrodia* are important in making natural medicine, and orchids also one of huge horticultural market worth 100 million dollars each year in the US alone (Griesbach, 2002). Ecologically, orchid plants are divided into three categories based on their habitats that is terrestrial orchids (grow on the grown), epiphytic orchids (grown on the others plant), and lithophytic (grow on the rock).

Dendrobium is one of ornamental forest epiphytic orchid that have pharmaceutical value. Many traditional Chinese medicines are produced from *Dendrobium* orchid. Additionally, *Dendrobium* also used in enhanced skin quality (Bensky and Gamble, 1993). *Dendrobium* is becoming rare due to human exploitation in improving the herbal medicinal value and horticulture importance. Many orchids are now considered to be at risk of extinction as an indirect or direct result of human activities.

Dendrobium crumenatum is a tropical epiphytic orchid usually found growing on trees in the fairly exposed areas such as by roadsides or in estates and plantation. It flowers several times a year and is well known about it gregarious. *Dendrobium crumenatum* growing in the same area can bloom together depending on the change of

temperature in that area. Flowering occurs nine days after heavy rain causes a sudden drop in temperature of about 5.6˚C (Holttum, 1964). It has very unique, attractive, whitish fragrant flower that looks like a pigeon. Therefore, *D. crumenatum* is also known as pigeon orchids. However, the flowers have a short shelf-life and can survive only for two days (Beaman *et al.*, 2001).

Most of the orchids are depends on orchid mycorrhizal fungi throughout or in part of their life cycle. The orchid mycorrhizae belong to basidiomycetes or rarely ascomycetes. Many orchid mycorrhizal fungi have been classified as *Rhizoctonia*-like fungi (Otero *et al.*, 2002). Besides that, orchid mycorrhizae are also needed for orchid germination (Lee, 2002). The development of orchid plants directly depends on the presence of fungal partners. This is because orchid seeds lack of nutrient reserves. Germination in the wild is only possible upon colonization by a compatible fungus that provides carbohydrates (Leake, 1994; Rasmussen, 1995). The entire family of orchid was characterized by a distinctive relationship with mycorrhizal fungi (Brundrett, 2002). The consequence of this symbiosis is the degree of specification between fungus and orchid. The symbiosis is an important factor to determine the chances of successful seedling establishment (Bidartondo and Read, 2008).

Orchid seeds can only germinate when they are infected by a symbiotic fungus (Hadley, 1982).The fungus digests the carbohydrate, breaking them down from the polysaccharide into disaccharides and monosaccharides which the protocorms can absorb and use (Arditti, 1992). This is important for orchid since the seeds are tiny and lack of in-built nutrition of bigger seeds. After germination, orchid seeds pass through a non-green ("achlorophyllous") developmental stage. They depends on symbiotic fungi

since they cannot use fats, break down starch, obtained phosphates or photosynthesis (Hijner, 1973; Arditti, 1992).

Generally, mycorrhizal fungi are host-specific, but the symbiotic relationship between orchid mycorrhizal fungi and orchid plants are mostly non-specific (Arditti, 1992). Most endomycorrhizal fungi have biotrophic relationship with their host plants, making it non-parasitic nature. On the other hands, orchid mycorrhizal fungi are one of endomycorrhizal fungi that involve *Rhizoctonia* species that can be cultured on artificial media.

Rhizoctonia-like fungi are known as widely distributed pathogen, saprophytes, and mycorrhizal fungi of orchids. *Rhizoctonia*-like fungi are characterized by right-angle branching, a constriction at the branch point and a septum in the branch hyphae near its point of origin (Sneh *et al*., 1991). Number of nuclei in young cell is one of the characteristic in classification of *Rhizoctonia*-like fungi. Additionally, anastomosis analysis was also one of the ways to classify these fungi.

Understanding the mycorrhizal symbiosis is very important in determining orchid distribution and diversity. Availability of the fungal symbionts can play an important role in determining orchid distribution and diversity. Futhermore, most studies on orchid mycorrhizal interactions have concentrated on terrestrial orchids, whereas the majority of orchid species are epiphyte.

Epiphytic orchids often occur abundantly in a favorable habitat. The low water availability and low nutrient supply make epiphytic orchids dependent on mycorrhizal fungi. Cortical cells root that colonized by fungi display typical structures formed by hyphal coils known as pelotons (Smith and Read, 1996). Most of the researcher found that fungal colonization was restricted to roots that attached to the substrate. Even

though germination and protocorm development for epiphytic orchids in nature have not been studied, mycorrhizal fungi that are able to colonize the bark of living tree have to be studied to know the relationship between the fungi in substrate and orchid.

Dendrobium crumenatum species usually exist as an epiphyte on trees such as Rain Tree and Angsana Tree. Thus this study was undertaken to characterize the *Rhizoctonia*-like isolates from root of epiphytic orchid (*Dendrobium crumenatum*) and bark of Rain Tree (*Samanea saman*) using morphological and anastomosis analysis to understand the relationship between mycorrhizal fungi in epiphytic orchid root and bark of Rain Tree.

Therefore, the specific objectives of this thesis were:

1) To determine the relationship between mycorhizal fungi in epiphytic orchid root of *D. crumenatum* and bark of Rain Tree (*S.saman*).
2) To observe the presence of peloton in epiphytic orchid roots by using hand section and microtome sectioning method.
3) To group the *Rhizoctonia*-like fungi based on morphological characteristic and anastomosis analysis.
4) To determine the capability of anastomosis of *Rhizoctonia*-like fungi among the isolates of epiphytic orchid root (*D. crumenatum*) and bark of Rain Tree (*S. saman*).

CHAPTER 2

LITERATURE RIVIEW

2.1 Orchid

2.1.1 Classification of orchid

The orchidaceae is the largest plant family in the world with estimates of more than 25000 species (Jones, 2006). The orchid family is classified as a member of the monocot order Asparagales (Chase *et al.*, 2003). Orchids are among the most beautiful flower in the world. Their attractive colors and complex forms have amazed people throughout the ages. Indeed, they have been regarded as a noble in the world of flowers (Yong and Chua, 1990). The orchidaceae is one of the most highly and uniquely modified of all angiosperm families. Nonetheless, it still undergoes rapid diversification and speciation (Chase, 2001).

The orchid family is different compared to other plant families. Orchid flowers come in various sizes, shapes and color. Even the orchid families are different among themselves. Orchids can be found at every parts of the world except the major deserts and artic circles. Orchids have three main growth habits growing on the ground are known as terrestrial orchid, on the other plant are known as epiphytic, and on the rock are known as lithophytic (Dressler, 1990).

In nature, orchids are associated with fungal symbionts (endomycorrhizal basidiomycetes) at least during seed germination and seedling establishment. The greatest diversity of orchid species is found in the forest that received sufficient of moisture throughout the year. However, the species compositions in the habitat are

influenced by both biotic and abiotic factors. In general, epiphytic orchids exceed terrestrial orchids, both in number of species and in number of individuals. Epiphytic orchid also occupy a wider diversity of habitats. They originated from subtropical or tropical regions, and also known to grow on the bark surface of woody plants. In some cases, the epiphytes normally inhabit the twig and trunk of woody plants in heavy rain areas in the tropical regions (Atlas and Bartha, 1993). They absorb moisture from the air rather than from surface of the bark.

Despite the wide diversity, especially their floral morphologies, these plants have specialized unifying characteristics which may reflect their isolated taxonomic position. Seed morphology and mycorrhizal fungi habit are almost certainly interdependent. Almost all of the seed of orchid species, often referred as 'dust seeds' are extremely small (0.3 - 14µg/seed). The seed consist of minute undifferentiated embryos that absence of an endosperm and have little storage (Burgeff, 1936; Arditti and Ghani, 2000). The lack of storage to support early seedling development makes all orchids in nature depends on the nutrients supply by mycorrhizal fungi. The fungi, which facilitate the process known as ' symbiotic germination' produce intracellular coils (called peloton) in the embryos of developing seedlings and in the rhizomes or roots of adult plants. Symbiotic germination and subsequent development of orchid embryos formed a swollen protocorm can only be achieved if the colonizing fungus has access to a soluble or insoluble source of carbohydrate which it translocate to the plant.

2.1.2 *Dendrobium*

Dendrobium is a large genus of tropical orchid and was established by Olof Swartz in 1799. Today the species were contains about 1,200 species. *Dendrobium* not only forming one of the biggest of the orchid groups, but also the most useful flower producers in the orchid world. Both the plants and the blooms differ significantly in shape and size, while the colors of the blooms range from white through flushes to yellow, rose and to the deepest possible crimson-purple. Many types of *Dendrobium* produced blooms, which normally last for 6 to 8 weeks. Some of *Dendrobium* leaves are quite deciduous which has thin leaves, while the others are evergreen types, with normally tough and leathery leaves.

Dendrobium is an ornamental forest epiphytic orchid of the temperate and subtropical regions that have their own pharmaceutical value. Many traditional Chinese medicines were produce from *Dendrobium* orchid. Sun dried of *Dendrobium* is mainly used to replenish body fluid. The boiled water from *Dendrobium* stem is commonly used as a tonic to moisten the stomach and lung. It is very good in for treating condition such as dry mouth, stomach pain, mouth sores and sun stroke. Additionally, this orchid also used to enhance skin quality (Bensky and Gamble, 1993).

2.1.3 *Dendrobium crumenatum*

Dendrobium crumenatum is one of tropical epiphytic orchid normally found growing on trees in fairly exposed areas such as roadsides or in estates and plantations. It blooms several times a year depends on change of temperature. *Dendrobium crumenatum* are well known of their gregarious flowering that is the

orchid that growing in the same area can flower simultaneously. It has a very unique attractive whitish fragrant flower that looks like a pigeon. Therefore, *D. crumenatum* is also known as pigeon orchids. Flowering occurs nine days after temperature drop around 5.6 ºC caused by a heavy rain (Holttum, 1964). However, the flowers have very short shelf-life and last only for two days (Beaman *et al.*, 2001).

The name of the genus is the combination of the Greek terms "dendron" is equivalent of tree and "bios" is equivalent of life. While, the name of species comes from the Latin "crumena" is equivalent as bag. The meaning of "purse or pouch" is referring to the spur of the flower (mentum). There are many common names for this orchid such as dove orchid, sparrow orchid (English), mu shi hu (Chinese), anggerik merpati and pokok merpati (Malaysia). All this different names are referring to the same species of orchid that is *Dendrobium crumenatum.*

Dendrobium crumenatum is an ideal plant for cultivation because it is easy to grow and to bloom regularly. It produces large number of sweetly scented flowers several times a year. It can quickly grow into a specimen plant, with pseudo bulbs up to 1m long, which yield a multitude of aerial growths and flowers. *Dendrobium crumenatum* will produced seedpod after pollinated by insect such as bee. The seedpod will form one week after the pollination. In wild, seed of *D. crumenatum* germinate with the help of a fungal symbiont (Hadley, 1982). Orchid seeds were pass through a non-green developmental stage where they cannot use fats, break down starch, obtain phosphates or photosynthesise. Therefore orchid will rely on symbiotic fungi to survive (Hijner and Arditti, 1973; Arditti, 1992).

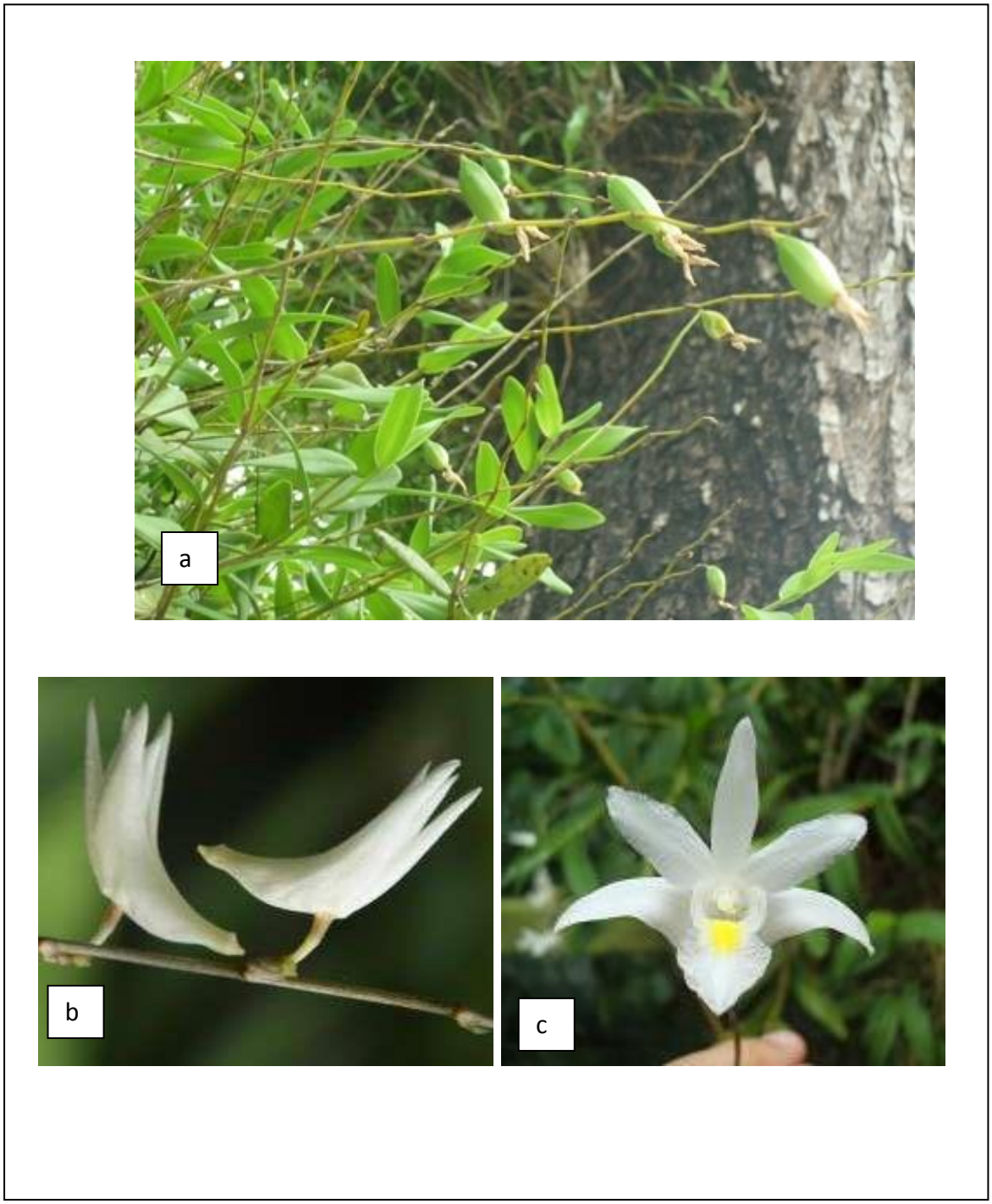

Figure 2.1: *Dendrobium crumenatum.* (a) Seedpods (b) White flower look like pegion, (c) White flower of with yellowish tinted throat

2.2 Mode of symbiosis for Mycorrhizal fungi.

Mycorrhizae is the root associating with two different organisms, the fungus (myco-) and the root (-rrhiza) that colonize the cortical tissue of roots (Garbaye, 1994). Mycorrhizal fungi are the main pathway through which most plants obtain mineral nutrients and become important in the terrestrial ecosystem functioning. In this mutualistic symbiosis, plants exchange the photosynthates to get mineral nutrients. Besides that, plants also increasing their resistance to disease, drought, and extreme temperatures (Smith and Read, 1996). The mycorrhizal fungi penetrate the root cells and change the physical structure of the root, and provide physiological benefits for the plants. In this mutualistic symbiosis, not only plant gained the profit, mycorhizal fungi also gain profit from this relationship. Mycorrhizae were not artificially made under *in vitro* conditions, but it produced only in nature.

Mycorrhizal fungi are classified into several types, according to morphological features, role in ecological ranks, or associate with plants. Generally mycorrhizal fungi are divided into endomycorrhizae and ectomycorrhizae, sometimes including endoectomcyorrhizae mainly found in the roots of azalea (Brundrett *et al.,* 1996; Susan, 1992). Currently there are five kinds of identified mycorrhizae which are, ectomycorrhizae (EM) (mainly formed in the roots of woody plant), arbuscular mycorrhizae (AM), ericoid mycorrhizae (ErM), orchid mycorrhizae (OM), and ecto-endomycorrhizae (Peterson and Farquhar, 1994).

In particular, ectomycorrhiza produced from the roots of woody plants was suggested to be an independent organism or different from the fungus or the root, such as lichens (Agerer, 1991). In most cases, the mycobiont fungus obtained the energy sources from plant, provide nutrients to (phytobiont) plant, and protect the

root from environmental stress and plant pathogens. Mycorrhizal fungi increase the solubility of mineral nutrients in the soil, making it convenient for the plant to absorb them (Hacskaylo, 1972; Bucking and Heyser, 1998; Bucking *et al.*, 1998). They are also considered to be 'ecological niche' in the rhizosphere, allocating the carbohydrate nutrients and protecting the plant roots from physical and biological hazards. Phytohormones are involved in these relationships (Alexopoulos *et al.*, 1996; Beyeler and Heyser, 1997).

Arbuscular mycorhizae (AM) is also known as endomycorrhizae, and the most common mycorrhizae in agricultural crops. AM fungi penetrate the root's of annual plants, and form vesicles or arbuscules in the root's cortical cells (Eom *et al.*, 1994; Koske and Gemma, 1989). Arbuscule is a type of haustorium which is a fungal organ that absorbs nutrients in plant root cells, whereas, vesicle are globose bodies produced by the intercalary or terminal swelling of a hyphae. Formation of arbuscules and vesicles were stimulated by plant nutrient, especially phosphates. AM fungi were identified to be species of Glomales (Zygomycotonia) inhabiting the soil, and are spread by soil insects or small animals.

Ecto-endomycorrhizae has characteristics of ectomycorrhizae, but exhibits an endomycorrhizal character by its intracellular penetration. ErM (Ericaceae, Monotropaceae) is mycorrhizae of the Ericales, which is a kind of ectomycorrhizae. There are two forms of ErM, one with mantel (Hartig net) and the other without mantle (but with invading hyphae in cortex cells), usually forming fine root system. The fungi of ErM finally degenerate together with the cortex cells.

2.2.1 Classification of Orchid Mycorrhizae

Orchid Mycorrhizae (OM) is a type of endomycorrhizae, formed between plants of Orchidaceae and basidiomycetous, or rarely ascomycetous fungi. The fungi formed intracellular coils or hyphal aggregates inside the cortical cells called peloton (Peterson and Farquhar, 1994). Mycorrhizal fungi are needed for orchid seed germination. Orchid's plants also showed obligate dependence on their mycorrhizal fungi all throughout or in part of their life cycle. Protocorms of orchid will not obtain nutrients effectively and cannot grow without any interactions with mycorrhizae (Harley and Smith, 1983; Uetake *et al.,* 1992). The nurseries of *Spiranthes spiralis* grow without photosynthesis, supplied with nutrition from the mycorrhizal fungi and producing the flower shoot yearly (Harley and Smith, 1983; Uchida *et al.,* 1986). This explains why mycorrhizae were necessary for seed germination and successful growth of orchids.

Mycorrhizal relations are formed by fungal infection and establishment in plant roots. Generally, mycorrhizal fungi are host-specific, but the symbiotic relationships between orchid mycorrhizal fungi and orchid plants are mostly non-specific (Arditti, 1992). Most endomycorrhizal fungi have biotrophic relations with their host plants, resulting in no parasitic behaviour. On the other hands, orchid mycorrhizae, a type of endomycorrhizae which involved *Rhizoctonia* species, can be cultured on artificial media. Some orchid mycorrhizal fungi tend to be parasites. The balance between orchids and orchid mycorrhizal fungi in a mycorrhizal relation is very delicate, and often too much nutrition has an adverse effect on the seeds or seedlings. Therefore, the degree of mutual dependence in their general relationships is still to be determined.

2.2.2 Peloton

Peloton is a mass of entangled hyphae, having various sizes and shapes. They are ellipsoid in shape and sized 5-10 mm in diameter. Under the light microscope, thin individual hypha and/or hyphal mass can be observed in the cortical cells of orchids (Smreciu and Currah, 1989; Zettler and McInnis, 1993).The orchid plants contain pelotons in the root cortical cells, which form the mycelial mass of the infecting fungi. Previously, pelotons in the cortical cells were known as pathogenic fungi; however, they are now confirmed as symbiotic fungi. Orchid roots with pelotons are healthier than those without them (Richardson *et al.,* 1993). The pelotons usually last only for a limited period of time, and become digestive forms in the root cortical cells before degeneration.

The benefit of mycorrhizal relations over non-mycorrhizal ones to plants can be defined by the former's ability to give ecological natural niches for plants. The symbiotic relations can be detected by the formation of pelotons in orchid root tissues. This can be explained by the fact that orchid plants with pelotons show healthier life cycle, blooming every year compared to those grown from tissue culture (without pelotons) showing no continuous blooming. Except for these aspects, any other features characteristic of mycorrhizal interactions are still not be able to be understand until now.

As mentioned above, the most characterized feature in orchid mycorhizae is the peloton formation in orchid roots. Peloton is a mass of entangled hyphae, having various sizes and shapes. They are ellipsoid in shape and sized 5-10µm in diameter. Thin individual hyphae and/or hyphal mass in the cortical cells of terrestrial orchids can be observed under the light microscope (Smreciu and Currah, 1989; Zettler and McInnis, 1993). Even in pure culture, some hyphal tips coil into peloton-like

structures as in the root cells. With staining, pelotons may be categorized as small pointed, pale degenerated and heavily stained forms.

The fungus symbiotic to the orchid roots was isolated from the peloton in the cortical cells without any conidial stage throughout the life cycle, which was identified as a species of *Rhizoctonia* spp. (Arx, 1974; Currah and Zelmer, 1992; Singleton *et al*., 1992: Sneh *et al.*, 1991). The symbiotic fungus was reported to be a species of *Tulasnella* in the teleomorph state, in Basidiomycota, differing from *Thanatephorus* of the anastomosis groups (AGs) of *Rhizoctonia solani* künh. *Rhizoctonia solani* is known as a roots disease pathogen in various economic plants (Sneh *et al.*, 1991). Several studies reported that the fungus of *Rhizoctonia* symbiotic with the orchid plants which was form dolipore septa in young vegetative hyphae, make it different from the septa of Ascomycotina (Herr, 1979; Moore, 1987; 1996). The *Rhizoctonia* also become dikaryotic in the young cells and sometimes has constricted hyphae called 'monilioid hyphae'. Usually, the hyphae formed neither clamp connections nor specialized hyphae.

2.2.3 *Rhizoctonia* spp.

Rhizoctonia forming as orchid endophytes were first founded by Bernad (1909), who identified three species based primarily on sclerotial morphology and general mycelial appearance. According to Duggar (1915), *Rhizoctonia* species can be distinguished by their sclerotial and mycelia morphology. The branching patterns, constrictions of hyphae, and monilioid cells were the main mycelia morphology. Since then, numbers of mycorrhizal fungi have been referred to the genus, mainly orchid endophytes.

The genus concept of *Rhizoctonia* was established by de Candolle (1815). As reviewed by Parmeter and Whitney (1970), "the basic characters of the genus, as set forth by de Candolle, were production of uniform texture of sclerotia with hyphal threads and the association of the mycelium with roots of living plants". Unfortunately, the lack of more specific characters has led to the classification of a mixture of unrelated fungi as *Rhizoctonia* spp. (Parmeter and Whitney, 1970; Moore, 1987)

Rhizoctonia solani is the most studied species within the genus. The original description of *R. solani* (Kunh, 1858), which lacked specificity, was formulated into a revised concept of species, especially through the studies of Duggar (1915) and Parmeter *et al.,* (1967). Parmeter and Whitney (1970) stated that the current species concept of isolates of *R. solani* have the following characteristics: a) some shade of brown hyphal pigmentation, b) branching near the distal septum of cells in young vegetative hyphae, c) constriction of hyphae and formation of septa a short distance from the point of origin of hyphal branches, d) dolipore septa, e) multinucleate cells in young vegetative hyphae. Characteristic such as monilioid cells, sclerotia, hyphae greater than 5 µm in diameter, rapid growth rate and pathogenecity are normally present, but they may be lacking in some isolates.

The revised concept of *R. solani* (Parmeter and Whitney, 1970) placed this species on solid taxonomic ground. In the absence of a strong genus concept, fungi related to *R. solani* have been referred to as *R. solani*-like fungi, rather than *Rhizoctonia* spp. (Parmeter *et al.,* 1970). Ogoshi (1975) enhanced the specificity of the genus concept of *Rhizoctonia* by elevating the following characteristics of *R. solani* to genus level that is the young vegetative hyphae was branching near to the distal septum of cells, formed constriction of hyphae and formation a short distance

of septa from the point of origin of hyphal branches, and the presence of dolipore septa. The characteristic of *Rhizoctonia* also absence of clamp connection, conidia, rhizomorphs and sclerotia differentiated into rind and medulla. Based on this revised genus concept, species *Rhizoctonia* are differentiated by mycelia color, number of nuclei per cell in young vegetative hyphae and morphology of teleomorphs. The teleomorphs of *Rhizoctonia* spp. belong to the sub-division Basidiomycotina; class Hymenomycetes (sub –class Holobasidiomycetidae) except for *Sebacina* spp. which belong to the sub-class Phragmobasidiomycetidae.

A broader concept of *Rhizoctonia* and *Rhizoctonia-like* fungi has been summarized by Moore (1987). Using the criteria of septal pore apparatus and teleomorps, he assigned members of the *Rhizoctonia* complex to Ascomycetes, Ustomycetes, Holobasidiomycetes and Heterobasidiomycetes. The septal morphologies of these groups are distinct. Ascomycetes have transparent septa with large central pores and two or more Woronin bodies. The septa of basidiomycetes are three layered. Uscomycetes have simple septa and small pores with acute rims. Holobasidiomycetes have dolipore septal complexes with parenthesomes that are generally perforate. Heterobasidiomycetes have dolipore septal complexes with parenthesome that are imperforate or vesiculate (Moore, 1987).

Classification of *Rhizoctonia* spp. has evolved mainly from studies of isolates obtained from diseased plants. Before the 1970s, most of the agriculturally important isolates of *Rhizoctonia* were classified as *R. solani.* Based on results Duggar (1915) and Parmeter *et al.*, (1967), it became clear that *R. solani* comprised a number of taxonomic species representing various teleomorphic states.

Saprophytic species and species involved in mycorrhizal associations with orchids and other plants comprise another important and diversified component of *Rhizoctonia* spp. Intensive studies of orchid mycorrhizal fungi (Currah *et al.*, 1987; Warcup and Talbot, 1966; 1967; 1971; 1980) indicated that in addition to the above mentioned species there are a considerable number of teleomorphs with *Rhizoctonia* anamorphs that have not yet been classified to species. In contrast, a number of isolates of *Rhizoctonia* spp. is still in a developmental stage. For some anamorphs, a teleomorph has not been determined, while for other anamorphs the species has not been designed.

Identification techniques based on number of nuclei per hyphal cell, hyphal anastomosis and morphology of teleomorphs are currently most useful for investigators whose primary interests are in ecology and epidemiology of *Rhizoctonia* spp. Relatively simple and efficient methods are required to characterize the typically large collections (approximately 100-3000 cultures) of *Rhizoctonia* spp. isolated from plants or soil under specific cropping practices, rotation, soil types, etc.

The species of genus *Rhizoctonia* with monilioid mycelia were divided into two groups based on the number of nuclei in the young cells: binucleate and multinucleate (Sneh *et al.*, 1991; Moore, 1987 and 1996). The species of multinucleate *Rhizoctonia* were categorized to be plant pathogenic fungi, including *R. cerealis, R. fumigata, R. oryzae, R. oryzae-sativae*, and *R. solani*. They cause various diseases in plants. However, some isolates of *R. solani* were reported to have symbiotic relationships with the protocorms of orchid (Harvais and Hadley, 1967). This can be attributed to orchids' ability to produce many secondary products that inhibit the fungus.

2.3 Anastomosis Analysis

A system of anastomosis grouping based on hyphal fusion has been widely accepted over the last 35 years, as the basis for recognizing groups and taxa among the several fungi that constitute the form genus (Ogoshi, 1975; Sneh *et al.,* 1991).These methods have been applied to the different taxonomic entities of *Rhizoctonia,* including isolates of *R. solani,* binucleate *Rhizoctonia* species (Oghosi *et al.,* 1983a) or *Rhizoctonia* isolates with teleomorph belonging to genus *Waitea.* A hyphal anastomosis reaction represents an expression of somatic or vegetative incompatibility (Anderson, 1982) and, from a biological point of view, is part of the several mechanisms involved in compatibility and sexual recognition processes, thats allow the preservation of unique heterokaryons in fungi.

An anastomosis group is represented by a collection of genetically-related isolates, according to their capability to anastomose hyphae among them. These reactions can vary from a complete fusion between hyphae, including cell walls and cytoplasmic membranes (the common situation found in anastomosis reactions in one given isolate with itself), to a complete absence of reaction. Reactions in which cell walls, and probably cytoplasmic membranes, connect but no fusion occurs (generally followed by death in the connected and adjacent cells), are typical among members of the same anastomosis group.

The term Fusion frequency (FF) is an expression of the incidence hyphal fusion between two isolates (Carling *et al.,* 1988; Yokoyama and Ogoshi, 1988). There are three types of hyphal fusion that is perfect fusion, imperfect fusion and contact fusion (Matsumoto *et al.,* 1932). Perfect fusion includes complete fusion of cell walls and cytoplasms with continuous living cytoplasm in the fusion site. Perfect fusion is observed only among hyphae originating from a common isolates that is

self fusion (Yukoyama *et al.*, 1983; 1985). Fusion between different isolates (non-self fusion) usually results in plasmolysis of fused cells or imperfect fusion (Yokoyama and Ogoshi, 1986). Contact between hyphae without lysis of cell walls at the site of contact (contact fusion) is not considered anastomosis.

Currently, there are several accepted classifications based on the anastomosis group (AG) concept for both multinucleate *(Thanatephorus)* and binucleate *(Ceratobasidium)* taxa within the *Rhizoctonia* species complex (Carling, 1996). In the first group of taxa, 14 anastomosis groups have been described (Carling *et al.*, 1999, 2002a), including 13 different groups (named from AG 1 to AG 13) whose members are generally only capable of fusing hyphae among themselves. There is another anastomosis group named AG BI (bridging isolate) that includes isolates capable of fusing hyphae among themselves and also with members of other AG. Currently, AG BI is thought to be a member of AG 2 that could suggest a paraphyletic origin for this AG. According to recent studies (Carling, 2000), AG BI may not represent the only 'bridging isolate' group, as new isolates have been described with the same behaviour in some of the classical AGs (i.e. AG 3, AG 6, etc.). Moreover, some of these AGs described for *R. solani* isolates have been further subdivided in anastomosis subgroups (i.e. AG 1, AG 2, AG 4, AG 6 and AG 9), based on criteria different to anastomosis pairing, including pathogenicity, colony morphology, DNA complementarity, pectic zymograms, etc. (Carling, 1996).

From a biological perspective, the different anastomosis groups described in binucleate *Rhizoctonia* represent a highly heterogeneous group of fungi, due to the fact that taxonomic relationships between these isolates are difficult to associate or assign to a specific teleomorph.

CHAPTER 3

MATERIALS AND METHODS

3.1 Sample collection

Aerial root of epiphytic orchid species, *Dendrobium crumenatum* and bark of Rain Tree (*Samanea saman*) with and without growing orchid (*D. crumenatum*) were collected from five locations in the Universiti Sains Malaysia (USM) main campus. The five locations were described as area A, B, C, D, and E. The orchid root was divided into two parts that is primary and secondary root. The older part of root classified as primary root, and the new part of root classified as secondary root. Each area consisted of 5 replicates of primary orchid roots, secondary orchid roots, and bark of Rain Tree with and without growing orchid. The sample collected were packed in sealed plastic bag to prevent desiccation and transported to the laboratory in the same day. The sample analysis was processed in the same day of collection to prevent the peloton from disintegration.

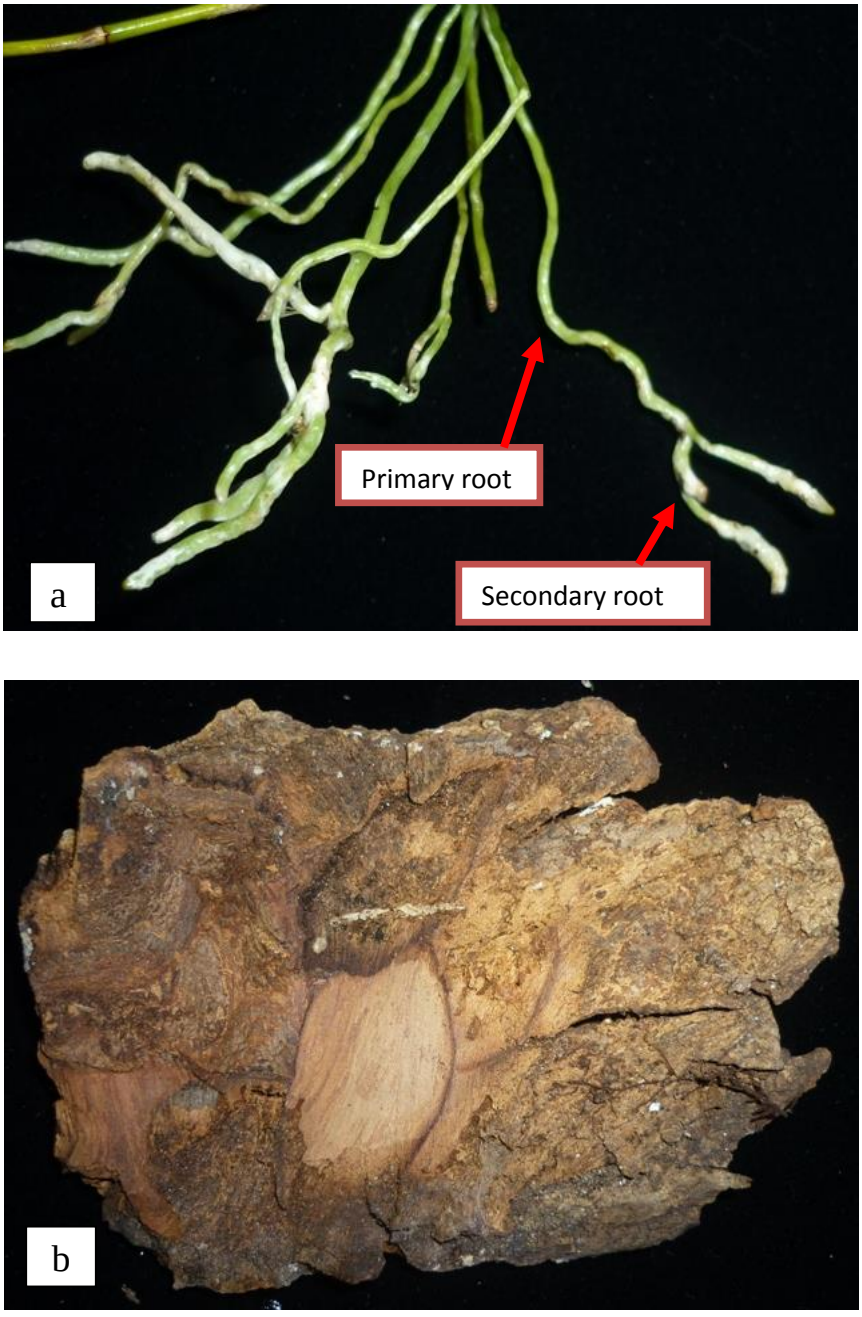

Figure 3.1: (a) Primary and Secondary orchid roots (*D. crumenatum*) and (b) Bark of Rain Tree (*S. saman*).

Table 3.1: The samples of epiphytic orchid root and bark of Rain Tree collected from five locations in Universiti Sains Malaysia (USM).

Location	Habitat	Orchid species	Sample
Area A	*S. saman*	*D. crumenatum*	• Primary root • Secondary root • Bark (with growing orchid) • Bark (without growing orchid)
Area B	*S. saman*	*D. crumenatum*	• Primary root • Secondary root • Bark (with growing orchid) • Bark (without growing orchid)
Area C	*S. saman*	*D. crumenatum*	• Primary root • Secondary root • Bark (with growing orchid) • Bark (without growing orchid)
Area D	*S. saman*	*D. crumenatum*	• Primary root • Secondary root • Bark (with growing orchid) • Bark (without growing orchid)
Area E	*S. saman*	*D. crumenatum*	• Primary root • Secondary root • Bark (with growing orchid) • Bark (without growing orchid)

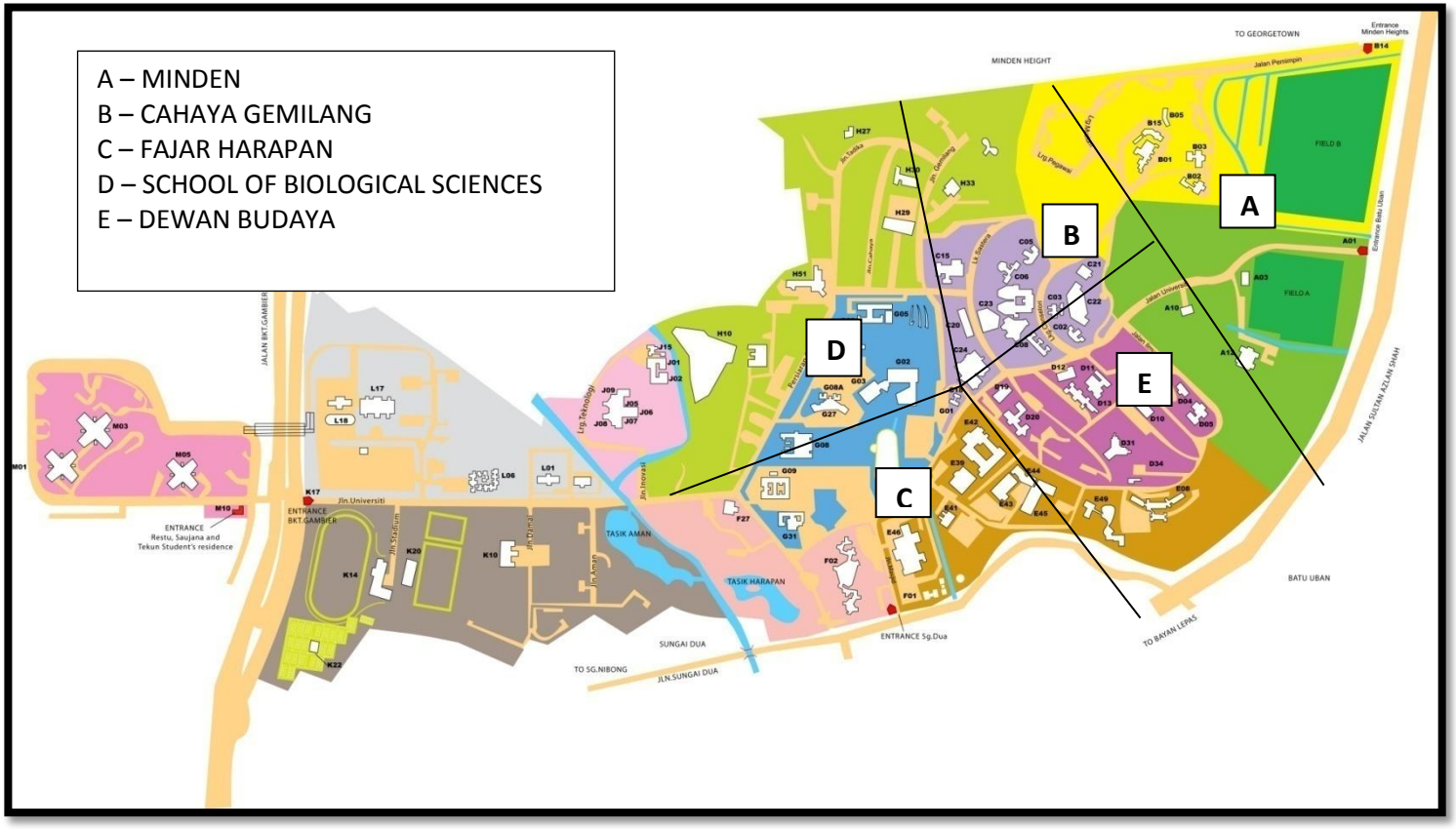

Figure 3.2: Location of sample taken from five areas in Universiti Sains Malaysia (USM)

3.2 Preparation of Culture Media

3.2.1 Potato Sucrose Agar (PSA)

To make 1L of Potato Sucrose Agar (PSA), potatoes were washed and peeled. The potatoes were cut into cube size about 1 cm × 1 cm. After that, 200 g of the potatoes were weight and boiled with 500 ml distilled water to extract broth. Then, 20 g of sucrose was added into potato broth in the beaker and stirred until dissolved. Sucrose mixed potato broth was poured into autoclave-safe Schott Glass bottle in which 17 g of agar was previously added. It was autoclaved for 20 min at 15psi, 121 ºC.

3.2.2 Potato Dextrose Agar (PDA)

To make 1L of Potato Dextrose Agar (PDA), 39g PDA powder (OXOID CM 0139) was weight and added into beaker. Then, 1L of distilled water was poured into beaker containing PDA powder and stirred. After that, it was decanted into Schott Glass bottle and autoclaved.

3.2.3 Water Agar (WA)

To make 1L of Water Agar (WA), 17 g of agar powder were weighted and added into beaker. Then, 1L of distilled water was poured into beaker containing agar powder and stirred. After that, pour the content of beaker into Schott Glass bottle and autoclaved.