

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Orchid

Orchids are one of the largest families of flowers worldwide, Orchidaceae. Estimation indicated the presence of 20,000 to more than 35,000 species in over 750 genera. The distribution of orchids is quite high and they are found on all major continents of the earth except Antarctica. The highest number of orchid species is found in the tropical zone and the diversity and abundant of orchids decrease with increasing distance from the equator (Brundrett *et al.* 2001). 88 % of orchids are epiphytes or air plants (Figure 2.1A), growing on trees and the others are terrestrial, growing on soil (Figure 2.1B). Some are even parasitic and a few saprophytic, living on decaying matter.

Orchids belong to monocot, having only one cotyledon. The orchid flower consists of 3 petals and 3 sepals (Figure 2.1). The petals are colorful part of the flower. One of these petals is typically quite different than other by forming the distinctive lip or labellum that common to most orchid species and use to attract pollinators. The sepals are situated under and between the petals. The sepals can be quite colorful or green depend on the species. However, the sepals are sometimes fused together to appear as one or the two petals are very tiny and cannot be easily observed. The orchid ovary is an inferior ovary and found beneath the flower while

most plants have an ovary that is found surrounded by the floral structure in the central part of the flower (Banks, 2003).

Orchids have considerable fascination for scientists and amateurs alike due to the great diversity of them, the extraordinary form and beauty of their flowers, and sometimes bizarre methods of pollination, close relationship with insect pollinators (Smith and Read, 2008). Their flowers are very important for the economical system of many countries. They were grown commercially for the cutting flowers. Many orchid products are believed to have medicinal properties and are used as aphrodisiacs, treatment for sores, emetics and vermifuges. Moreover, one adaptive mechanism is that roots of many orchids, especially tropical species have a multiple epidermis of dead cells, called velamen, which protects the roots from excessive drying and aids in water uptake (Arditti and Krikorian, 1996; Smith and Read, 2008).



## 2.2 Terrestrial orchid

Terrestrial orchids (Figure 2.1B), or those orchids which grow in soil, make up approximately 12 % of the total number of species of orchids (Brundrett *et al.* 2001). There are two main types of terrestrial orchids, those that have tubers and those that have pseudobulbs. The terrestrial orchids are perennial in habit and die back during cold or dry seasons and grow rapidly and flower during spring and summer or when rainy season occurs (Smith and Read, 2008; Brundrett *et al.* 2001). The most fascinating thing is their dependence upon fungi to live. Many studies reported many orchids have a symbiotic relationship with various fungi in their environment, but this relationship is much more essential for terrestrial orchids than epiphytic one. It has been estimated that when a wild terrestrial orchid is dug up and transplanted from natural source, most of them will die or become to unhealthy plants because the new environment lack the correct fungi to keep it alive. In addition, the orchids depend upon the fungi which also live on roots of specific nearby trees and shrubs in a microenvironment that is of value to all the plants. Thus, when they are moved from their habitat, they will lose ability to use their fungi (Withner, 1974; Rasmussen, 1995; Smith and Read, 2008). This is the main problem for propagation and conservation of the wild terrestrial orchids.

## 2.3 Orchid seed

Orchid seeds are very small, dust-like, (Figure 2.2) and contain little food reserve. Seeds are generally less than 2 mm in length and 1 mm in width and dispersed easily by wind (Brundrett *et al.* 2001). In nature, most orchid seeds

cannot germinate or are able to start germinating but will not grow unless they are infected with mycorrhizal fungi which supplies young plants with all sugars and nutrients (Brundrett *et al.* 1996; 2003).

There are two methods, asymbiotic and symbiotic method, of orchid seed germination for orchid propagation and conservation.

Asymbiotic seed germination has become the favored method for orchid propagation and production especially tropical epiphyte orchids, using synthetic medium for orchid seeds and orchid seedling orchid culture. However, this approach with the terrestrial orchids has not been successful and only a few species have been germinated asymbiotically or can germinate in asymbiotic media, *in vitro*, but cannot survive in a greenhouse (Arditti *et al.* 1981; 1990; Smith and Read, 2008).

In symbiotic seed germination is used to grow seeds with the suitable fungi. The seeds are infected with the fungi and start to grow. The advantage of this approach is that the media used is very simple, consisting of only powdered oats with some yeast extract. The resulting mycorrhizal plants are stronger and more resistant to pathogens, such as fungal diseases, than asymbiotic plants (Brundrett *et al.* 2001).

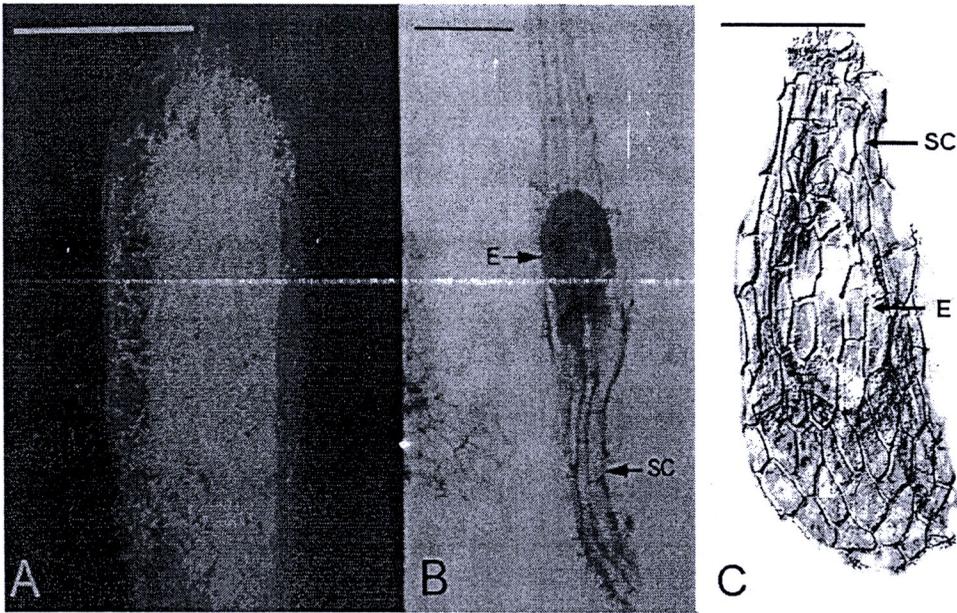


Figure 2.2 Orchid capsule and seed, the mature capsule of *Calanthe tricarina* (A), scale bar = 15 mm, seed of *Calanthe tricarina* (B), scale bar = 0.15 mm and seed of *Liparis loeselii* (C), scale bar = 0.20 mm. Note; SC = seed coat, E = embryo (Rasmussen, 1995)

In addition, many reports supported the advantage of symbiotic seed germination. Stewart and Zettler, (2002) showed that the seeds of three species of *Habenaria repens*, *H. quinquiseta* and *H. macroceratitis*, germinated within 21 days of inoculation. The seedlings infected with *Epulorhiza* sp., isolated from *Spiranthes brevibris* were transferred to peat in a greenhouse had the highest survival (88.9%). Yoder *et al.* (2000) reported that the water content of fungus-infected seeds of monkey face orchid, *Platanthera integrilabria*, was higher than uninfected seeds and the seed of *P. integrilabria* took one month to germinate in the presence of fungi while three months to germinate in the absence of fungi.

Sangthong, (2002) showed that 678 fungal isolates that divided into 20 groups were isolated from orchid roots collected from eight locations in upper northern Thailand. Sixteen isolated fungi were inoculated to *Dendrobium scagrilinge*, an epiphytic orchid. Two *Xylaria* isolates showed significant increase of pseudobulb and leaf number while *Rhizoctonia* isolates and *Xylaria* isolates which were inoculated to orchid plantlets revealed significant increase in survival rate, root number and height. The dark colored of *Fusarium* isolates (red, pink and purple) also gave higher survival percentage than light color ones (yellow and white). Stewart and Kane, (2006) isolated six fungal mycobionts from flowering plant of *Habenaria macroceratis*, a sub-tropical terrestrial orchid. The symbiotic seed germination percent was highest (65.7%) and protocorm development was most advanced (stage 2, see Figure 3 and Table 1) when seeds were cultured with *Epulorhiza* sp. Hmac-310. Seed germination and protocorm developmental stages of terrestrial orchid were described by Miyoshi and Mii, (1995), Zettler and Hofer, (1998), Stewart and Zettler, (2002) and Stewart and Kane (2006) (Table 2.1 and Figure 2.3). In another study, seed of one terrestrial orchid, *Spathoglottis plicata*, inoculated with *Epulorhiza repens* and *Rhizoctonia globularis* resulted in the initiation of leaves 60 days after seed sowing. While, in the absence of fungi orchid seeds did not develop into seedlings and mortality gradually occurred (Athipunyakom *et al.* 2004b).

Table 2.1 Seed germination and protocorm developmental stages use for determination of growth and development of terrestrial orchid *in vitro* (Stewart and Kane, 2006)

Stage	Description
0	No germination, viable embryo
1	Swelled embryo, production of rhizoid(s)
2	Continued embryo enlargement, rupture of testa
3	Appearance of protomeristem
4	Emergence of first leaf
5	Elongation of first leaf

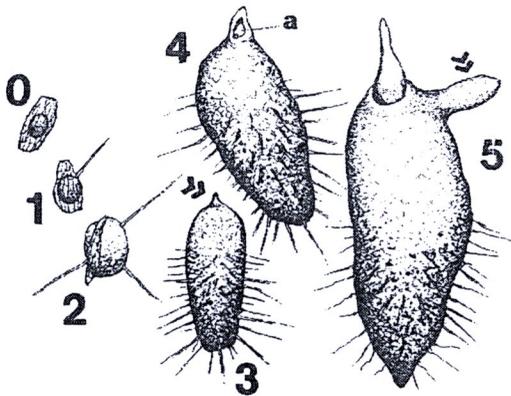


Figure 2.3 Growth stage used to determine germination and development of orchid seed *in vitro*, 0; no germination viable seed, 1; swelled embryo, production of rhizoid(s), 2; rupture of the testa by enlarged embryo, 3 appearance of promeristem, denoted by arrow, 4; appearance of first true leaf (a) and 5; elongation of true leaf and formation of branch root, denoted by arrow (Zettler and Hofer. 1998)



## 2.4 Orchid in Thailand

In Thailand orchids are found in all parts and in all different habitats ranging from evergreen hill forest (2,565 m from sea level) in Northern Thailand down to sea level (Nanakorn and Indharamusika, 1998). There are quite a large number of wild orchids, about 170 genera and 1,230 species of which 150 species are considered endemic to the country (Nanakorn and Indharamusika, 1998; Santisuk *et al.* 2006). Of these, 80% are epiphytic and most of the rest are terrestrial orchids. Some of the important epiphytic genera are *Dendrobium* spp., *Bulbophyllum* spp., *Eria* spp., *Coelogyne* spp., *Cymbidium* spp. and *Vanda* spp. and terrestrial genera are *Habenaria* spp., *Liparis* spp., *Cymbidium* spp. and *Phaphiopedilum* spp. (Nanakorn and Indharamusika, 1998), more species of terrestrial orchid that were recorded and reported in Thailand are presented in Table 2.2. In addition, northern Thailand where Queen Sirikit Botanic Garden located is rich in diversity of plant communities and is the center of plant distribution of South East Asia. The number of the native orchids in this region is estimated to be up to 600 species (Nanakorn and Indharamusika, 1998). However, most of the forest in the region are secondary or degraded and have high level of human impact in many parts due to slash and burn farming. Therefore, the studies of many factors of orchid growth for conservation in Thailand also requested.



Table 2.2 Some terrestrial orchid species in Thailand (Nanakorn and Indharamusika, 1998; Vaddhanaphuti, 2005)

Some terrestrial orchid species in Thailand	
<i>Acanthephippium sylhetense</i> Lindl.	<i>Malaxis orbicularis</i> Tang & Wang.
<i>Anoectochilus burmanicus</i> Rolfe.	<i>Nervilia aragoana</i> Gaud. *
<i>Anthogonium gracile</i> Lindl.	<i>Nervilia calcicola</i> Kerr.
<i>Arundina graminifolia</i> Lindl. (Don) Hochr.	<i>Nervilia plicata</i> (Adr.) Schltr.
<i>Brachycorythis helferi</i> Summerh.	<i>Nervilia prainiana</i> (King & Pantl.) Seid.
<i>Brachycorythis henryi</i> Summerh.	<i>Paphiopedilum bellatulum</i> Pfitz.
<i>Corymborkis veratrifolia</i> Bl.	<i>Paphiopedilum callosum</i> Pfitz.
<i>Cymbidium ensifolium</i> Sw.	<i>Paphiopedilum concolor</i> Pfitz.*
<i>Cymbidium martersii</i> Griff. & Lindl.	<i>Paphiopedilum exul</i> Pfitz.*
<i>Cymbidium sinense</i> Wild.	<i>Paphiopedilum godefroyae</i> Pfitz.*
<i>Doritis pulcherrima</i> Lindl.	<i>Paphiopedilum niveum</i> Pfitz.*
<i>Eulophia andamanensis</i> Rchb. f.	<i>Paphiopedilum parishii</i> Rchb. f.*
<i>Eulophia graminea</i> Rchb. f.	<i>Pecteilis sagarikii</i> Seid.*
<i>Eulophia macrobulbon</i> Hk. f.	<i>Pecteilis susannae</i> (L.) Rafin.
<i>Eulophia nuda</i> Lindl.	<i>Phaius indochinensis</i> Seild. & Ormerod.
<i>Goodyera procera</i> Hk.	<i>Phaius mishmensis</i> Rchb. f.
<i>Habenalia cholorina</i> Par.& Rchb. f.	<i>Phaius tankervilleae</i> (Ait.) Bl.
<i>Habenalia dentata</i> Schltr.	<i>Spathoglottis affinis</i> De Vries.*
<i>Habenalia lindleyana</i> Steud.	<i>Spathoglottis eburnea</i> Gagnep.
<i>Habenalia lucida</i> Wall. Ex. Lindl.	<i>Spathoglottis plicata</i> Bl.*

Table 2.2 (continued)

## Some terrestrial orchid species in Thailand

<i>Habenalia rhodocheila</i> Hance.	<i>Spathoglottis pubescens</i> Lindl.
<i>Habenalia rostellifera</i> Rchb. f.	<i>Taina hookeriana</i> King & Pantl.
<i>Habenalia siamensis</i> Schltr.	<i>Taina latifolia</i> (Lindl.) Rchb. f.
<i>Liparis sutepensis</i> Rolfe ex Downie.	<i>Taina viridifusca</i> Benth. & Hk. f.
<i>Malaxis latifolia</i> J. E. Sm.	<i>Thunia alba</i> Rchb. f.

Note; all recorded terrestrial orchid species were found in Chiang Mai, except those species are labeled with \*.

## 2.5 Mycorrhiza

Mycorrhiza is a mutualistic symbiosis of a plant root, a fungus and the soil. In these associations the fungal groups are *Basidiomycetes*, *Ascomycetes* and *Zygomycetes*, and most plants are vascular plants (Brundrett *et al.* 1996, 2003). Evidence from fossil records show that roots evolved alongside fungal partners which may have been crucial in helping plants evolve to colonize the land, hundreds of million of years ago. Through the mycorrhizal associations, the fungal partner receives photosynthetically derived carbon compounds from the host plant while the fungus transfers essential minerals such as nitrogen and phosphorus from decaying organic matter to the host plant (Molina and Trappe, 1984; Puplett, 2006).

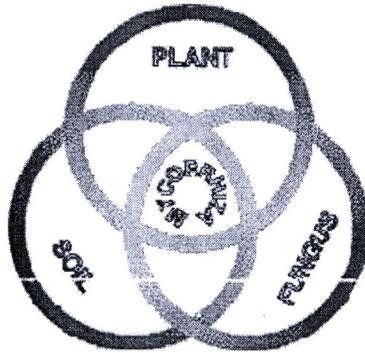


Figure 2.4 Mycorrhizal associations; the interaction of plant roots, soil (nutrients in soil) and fungi (Brundrett *et al.* 1996)

The mycorrhizal association types (Brundrett *et al.* 1996), there are seven different types of mycorrhizal associations have been recognized, involving different groups of fungi and host plants and distinct morphology patterns (Table 2.3). The different forms of mycorrhizal association types are presented by Figure 2.5.

The seven different types of mycorrhizal associations;

1. Vesicular-arbuscular mycorrhizas or arbuscular mycorrhizas (VAM or AM). The most common of mycorrhizal associations, occurring around 90% of all vascular plants. The fungi belong to the *Zygomycetes* and produced arbuscules, hyphae and vesicles within root cortex cells. Fungal spores are formed in soil or roots. These associations are defined by the presence of arbuscules.

2. Ectomycorrhizas (ECM). The fungi are *Basidiomycetes*, *Ascomycetes* and *Zygomycetes* which form a mantle around the roots and a Hartig net between root cells in the epidermis or cortex.

3. Orchid mycorrhizas. The most fungi are *Basidiomycetes* which form coils of hyphae within roots of plant in the family Orchidaceae.

4. Ericoid mycorrhizas. The most fungi are *Ascomycetes* which form hyphal coils in outer cells of the narrow hair roots in the plant order Ericales.

5. Ectendo-, (6) Arbutoid and (7) Monotropoid mycorrhizal associations. These associations are similar to ECM associations, but have specialized anatomical features (Table 2.3).

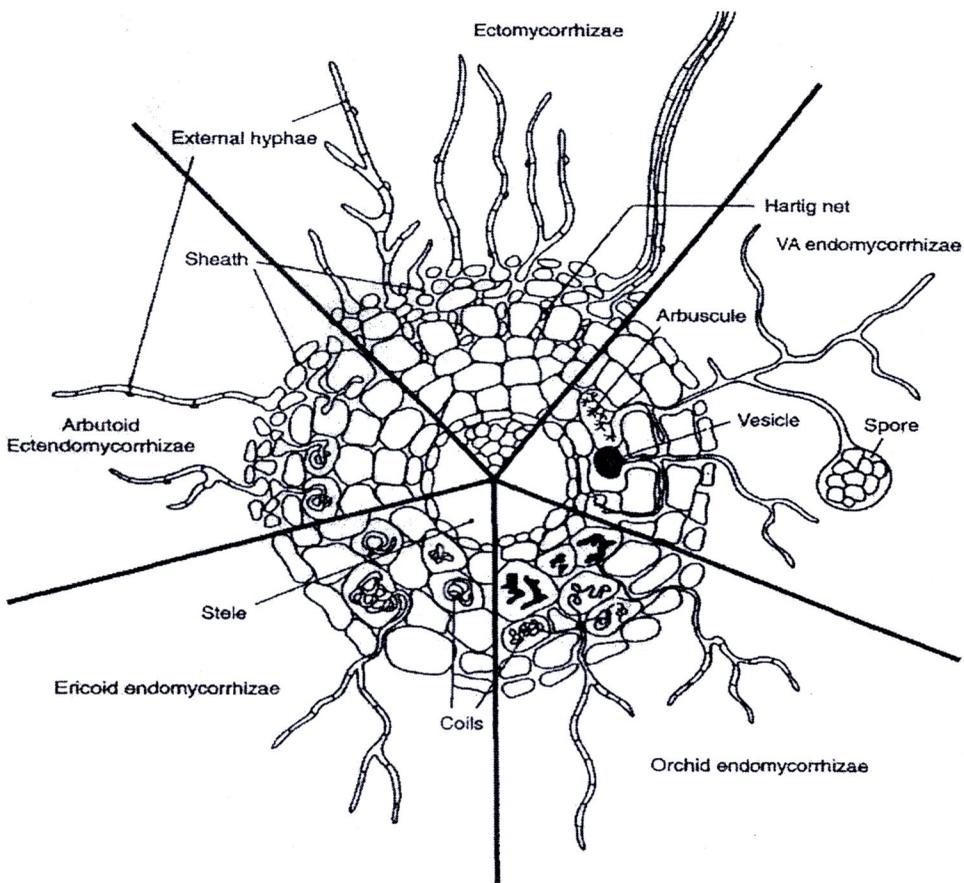


Figure 2.5 A schematic overview of the different forms of mycorrhizas (Selosse and Tacon, 1998)

Table 2.3 Types of mycorrhizal associations (Brundrett *et al.* 1996)

	AM	ECM	Extendo- mycorrhizas	Arbutoid- mycorrhizas	Monotropoid- mycorrhizas	Ericoid- mycorrhizas	Orchid- mycorrhizas
<b>Root structures</b>							
Septate hyphae	(+) -	+ -	+ -	+	+	+	+
Hyphae in cells	+	(+) -	+	+	+	+	+
Hyphae coils	+ -	-	+	+	-	+	+
Arbuscules	+	-	-	-	-	-	-
Mantle sheaths	-	+ (-)	+ (-)	+	+	-	-
Hartig net	-	+	+	+	+	-	-
Vesicles	+ -	-	-	-	-	-	-
<b>Host plants</b>							
	Vascular plants	Vascular plants	Gymnosperm and Angiosperm	Ericales	Monotropaceae	Ericales	Orchidaceae
<b>Fungi</b>	<i>Zygomycetes</i>	Most <i>Basidiomycetes</i> , but some <i>Ascomycetes</i> and <i>Zygomycetes</i>			<i>Asco-</i> , ( <i>Basidio-</i> )	<i>Basidiomycetes</i>	

Note: - = absent, + = present, (-) = sometimes absent, (+) = sometimes present, + - = present or absent, *Basidio-* = *Basidiomycetes*,

*Asco-* = *Ascomycetes*



The advantages of mycorrhizas, Plants receive more nutrients from soil by mycorrhizal associations. Some fungi are able to break down substances which plant can not use unaided, such as cellulose, thus making more nutrients available to the plant. The numerous hyphae extending from the plant's root will increase the area of uptake of nutrients and water. Furthermore, the mycorrhizal fungi can protect plants from drought, predators (nematodes, insects), pathogens and other stresses and increase rate of plant growth (Molina and Trappe; 1984, Puplett, 2006).

## **2.6 Orchid mycorrhiza**

The relationship of orchids with fungi is relatively unique in the plant kingdom. The main group of orchid mycorrhizal fungi belongs to *Basidiomycetes* and some are *Ascomycetes* (Table 2.4). The mycorrhizal fungi typically consist of coils of hyphae, called pelotons (Figure 2.6), within cortex cells of plant roots (Brundrett *et al.* 2001). The fungal hyphae are found within the orchid root rather than in sheaths surrounding the roots thus, the association is termed endomycorrhiza rather than ectomycorrhiza. The survival of young orchid seedlings and some adult plants that lack chlorophyll are dependent on the mycorrhizal fungi (Brundrett *et al.* 2001). Due to the achlorophyllous state (non-photosynthetic) the orchid must receive an exogenous supply of carbohydrate, minerals and other metabolites by the infection of compatible mycorrhizal fungi. Therefore, the majority of orchid seeds will not even germinate until the colonization of fungal symbiont occur (Smith and Read, 2008).

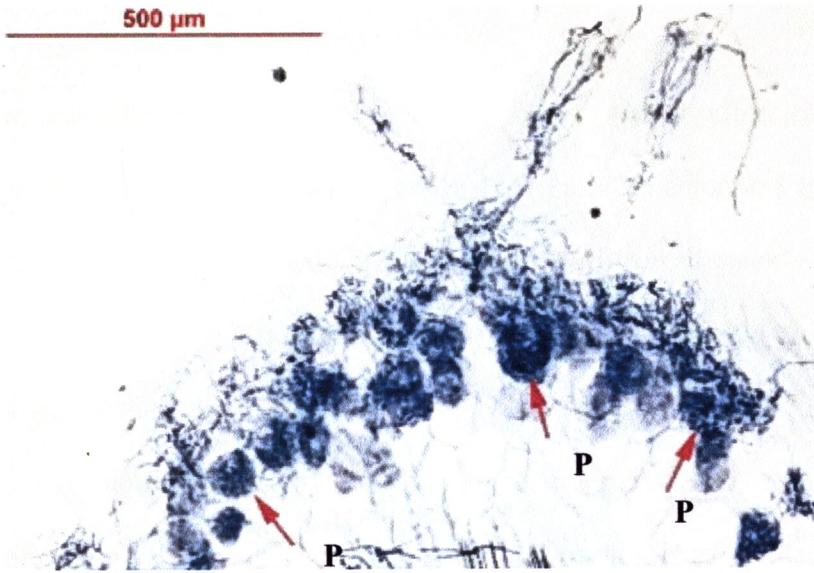


Figure 2.6 The coils of fungal hyphae, pelotons, of orchid mycorrhizal fungi within cortex cells of orchid roots, P = peloton structure, (Brundrett *et al.* 2001)

Table 2.4 The major members of orchid mycorrhizal fungi (Brundrett, 2004)

Orchid mycorrhizal fungi	
Phylum	<i>Basidiomycota</i> and some are <i>Ascomycota</i>
Families	<i>Ceratobasidiaceae</i> , <i>Tulasnellaceae</i> , <i>Sebacinaceae</i> and also many others are reported
Anamorphs	Rhizoctonia-forming: <i>Epulorhiza</i> , <i>Ceratorhiza</i> and etc, sterile mycelia and as well as <i>Fusarium</i> , etc.
Telemorphs	<i>Ceratobasidium</i> , <i>Thanatephorus</i> , <i>Sebacina</i> and etc.

## **2.7 Orchid mycorrhizal interactions**

The infection of an orchid seed by fungi occurs after early seed germination. The imbibed and swollen embryos are rapidly colonized by hyphae of suitable fungi (Smith and Read, 2008). The fungi will spread quickly from cell to cell and form coils of hyphae, pelotons, within the infected orchid embryo that consists of a few hundred cells. However, the infection is limited to suspensor cells of the embryo and epidermal hair and highly restricted compared to fungal infection of non orchid species. This suggests that orchids control the infection process and the fungal symbionts are adapted to this control (Hadley, 1982). Each intracellular peloton has a short life-span. Only a few days, it is digested and consumed by orchid cell while the surviving fungal hyphae re-colonize the orchid cells in the same time and equilibrium of both occurrences. Moreover, the orchid cell releases orcinol, a phytoalexin that causes the pelotons to collapse, to control the degree of fungal colonization, nutrients uptake and prevent parasitism by the fungi (Brundrett *et al.* 2001; Sivasithamparam *et al.* 2002; Smith and Read, 2008).

## **2.8 Isolation and identity of orchid mycorrhizal fungi**

The fungi can be isolated from roots of many adult orchids on unspecialized media and can be grown easily in pure culture. The techniques for isolation include plating fragments of surface sterilized roots on solid media and careful separation and plating of individual fungal pelotons (Smith and Read, 2008). Yamato *et al.* 2005 isolated mycorrhizal fungi by using 70% ethanol as surface sterilant for roots of orchid and crushing roots in Petri dish by using sterile glass rod to disperse the cells in root cortex. After that, pour plate technique with

media containing 0.05% sucrose as carbon source was used and the poured plates were incubated at 20 °C, dark. The fungal colonies emerging from orchid root tissues or cells were picked up and transferred to Potato dextrose agar (PDA). Zhu *et al.* (2008) isolated the orchid mycorrhizal fungi from roots of *Cremastra appendiculata* (Orchidaceae) by peeling root hair, epidermis and velamen off and the peeled roots were treated with sterile distilled water followed with 150 µg of streptomycin and penicillin for surface sterilization. The cortex cells from treated roots were suspended in sterile distilled water, the suspension of root cells was dropped on the PDA that supplemented with 100 µg/ml of streptomycin and penicillin. The cultured plates were incubated at 18 °C, dark and observed every day under dissecting microscope. The hyphae of fungi that emerged from root cells were picked up and transferred to the new media (PDA).

The main group of orchid mycorrhizal fungi has been placed in the form genus *Rhizoctonia* or Rhizoctonia-forming fungi (Moore, 1987; Anderson, 1991; Rasmussen *et al.* 1995; Weber and Webster, 2001; Sangthong, 2002; Chou and Chang, 2004; Smith and Read, 2008). The rhizoctonia-forming fungi are fungi belonging to the phylum *Basidiomycota*, class *Basidiomycetes*. All species absence yeast states and are entirely hyphal, the hyphae being divided into compartments by septa. Sexual reproduction involves the active release of basidiospore. Hyphal germination of basidiospores normally produces monokaryotic hyphae, which can anastomose with sexually compatible monokaryotic hyphae to produce a stable dikaryon (Roberts, 1999). In some genera form sclerotia that consist of modified hyphal compartments resembling chains of ellipsoid to globose cells, which become thicken walled, tinted and

agglutinated into compact hyphal masses. The sclerotia function as resistant to environmental stresses. Most, possibly all species form monilioid hyphae (Figure 2.7), chains of short swollen compartments in culture. These monilioid hyphae are precursors of sclerotium formation.

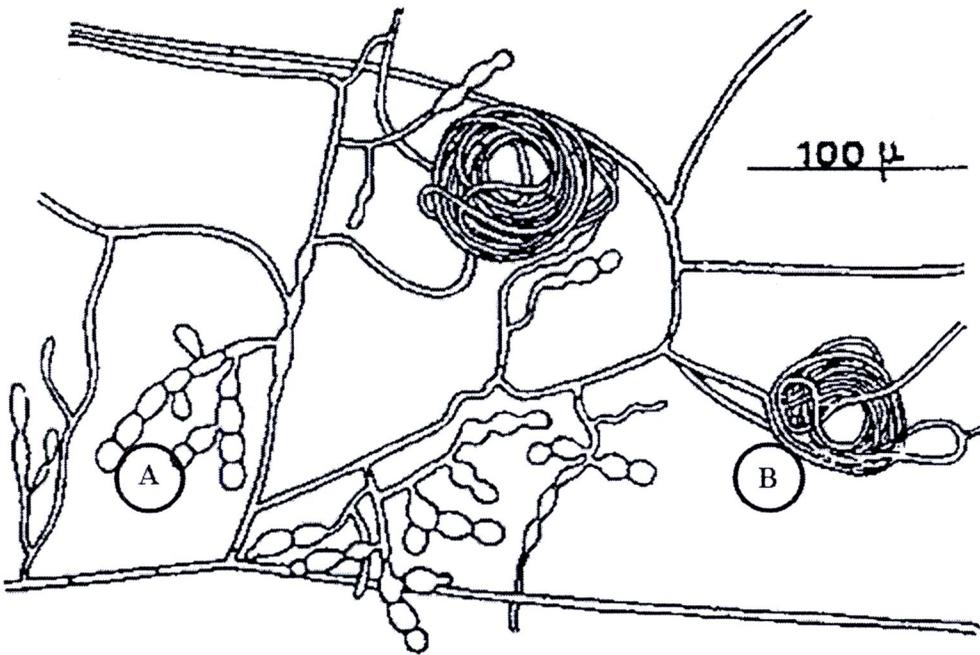


Figure 2.7 Drawing of a typical orchid mycorrhizal fungi, *Rhizoctonia repens* (*Tulasnella calospora*), isolated in pure culture. A; monilioid hyphae and B; peloton-like structure of mycelia (Smith and Read, 2008)

Most of endophytic fungi known to form orchid mycorrhizas are distinguished by their appearance in culture. The fungi which form sexual stages in culture belong to the *Basidiomycetes* (difficult and poorly repeatable produce). The orchid mycorrhizal fungi are recurrently but not exclusively of the *Ceratobasidiales*, *Exidiales* and *Tulasnellales*. The genera known to form orchid mycorrhizas are *Ceratobasidium* and *Thanatephorus* (*Ceratobasidiales*), *Oliveonia* and *Serendipita* (*Exidiales*) and *Tulasnella* (*Tulasnellales*) (Roberts, 1999; Sivasithamparam *et al.* 2002). Current classification, the Rhizoctonia-forming heterobasidiomycetes in several different orders and genera modified and simplified by Roberts, 1999 are summarized in Table 2.5.

A diversity of fungi has been found to associate with orchids. Conventional and application of molecular methods have been used for identification. Many of orchid mycorrhizal fungi have proved to be compatible with seed *in vitro* and distinguished by hyphal characters due to anamorphic mycelia of these fungi, sexual spores is not present. The mycelial characters which used such as colored, septate hyphae, branches of hyphae, formation of monilioid cells, and sclerotia. Another characteristic of many of these mycelia is that in pure culture some hyphal tips coil into peloton-like structures almost as they do within the orchid cells (Figure 2.7) (Rasmussen, 1995; Roberts, 1999; Athipunyakom *et al.* 2004a). Nowadays, the molecular approach is widely used to identify the organisms including fungi. The base sequences of nuclear rDNA especially Internal Transcribed Spacer (ITS) regions and large subunits (LSU) of nuclear rDNA (diagrams are presented in Figure 2.8) are generally used for taxonomical identification. Normally, the fungal genomic DNA is extracted by

cetyltrimethylammonium bromide (CTAB) method and using Polymerase Chain Reaction (PCR) technique to amplify the ITS sequences by using pair of primer ITS4 and ITS5 or ITS1 and ITS4. The PCR products were sequenced for identification (Bougoure *et al.* 2005; Yamato *et al.* 2005). Moreover, The mitochondrial ribosomal large subunit (LSU) DNA was used to identify *Tulasnella* isolated from a single peloton obtained from fresh roots of *Dactylorhiza majalis* and the mitochondria ribosomal large subunit DNA of fungi cultured from roots of *Neuwiedia reratrifolia* were sequenced and analyzed with sequences determined from references fungal collections and published sequences. The fungi were referred to heterobasidomycetous fungi order *Tulasnellales* and *Ceratobasidiales* (Kristiansen *et al.* 2001; 2004).

Table 2.5 Classification of the Rhizoctonia-forming heterobasidiomycetes (Roberts, 1999)

Kingdom	Class	Order	Genus	
Fungi	Basidiomycetes	Ceratobasidiales	<i>Ceratobasidium</i> (anam. <i>Ceratorhiza</i> ) (10 species)	
			<i>Thanatephorus</i> (anam. <i>Rhizoctonia</i> ) (11 species)	
			<i>Waitea</i> (anam. <i>Rhizoctonia</i> ) (one species)	
		Exidiales	<i>Ceratosebacina</i> (anam. unnamed) (3 species)	
			<i>Endoperplexa</i> (anam. <i>Opadorhiza</i> ) (3 species)	
			<i>Heteroacanthella</i> (anam. <i>Acanthellorhiza</i> ) (2 species)	
			<i>Oliveonia</i> (anam. <i>Oliveorhiza</i> ) (5 species)	
			<i>Serendipita</i> (anam. unnamed) (7 species)	
			<i>Tulasnella</i> (anam. <i>Epulorhiza</i> ) (40 species)	
			Incertae sedis; <i>Hauerslevia</i> (one species)	
		Urediniomycetes	Playgloeales	<i>Scotomyces</i> (one species)
				<i>Helicobasidium</i> (anam. <i>Thanatophylum</i> ) (2 species)

Note; anam. = anamorph

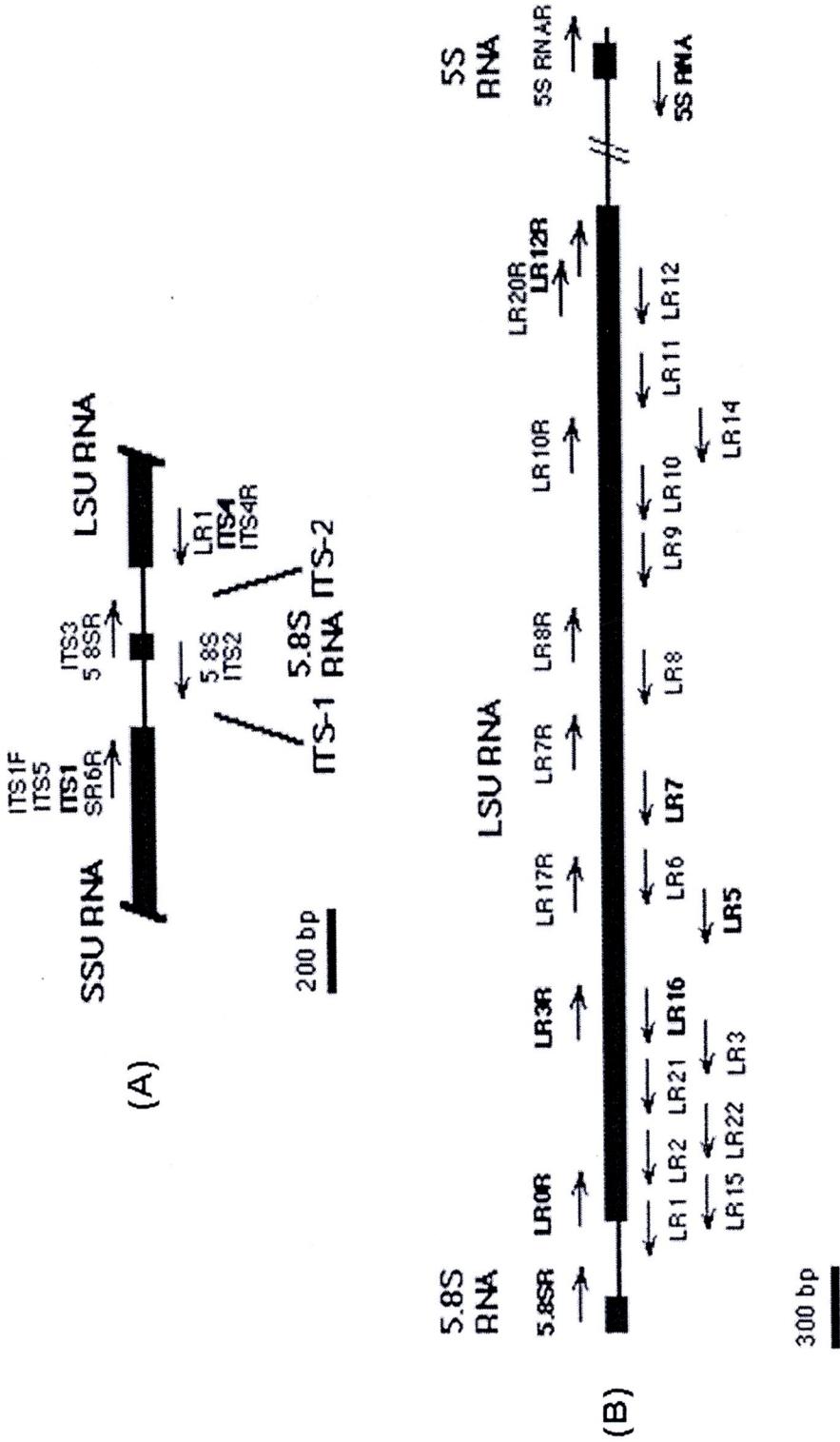


Figure 2.8 Internal transcribed spacer (ITS) regions and recognized sites of ITS primers (A) and Large subunit (LSU) RNA and recognized sites of LSU primers (B) (Duke University, 2011)

## 2.9 Roles of mycorrhizal associations

### 2.9.1 Roles in ecosystems

1. Soil hyphae have an important role in nutrient cycling by helping to prevent losses from the system, especially at times when roots are inactive (Lussenhop and Fogel, 1999).

2. Hyphae are conduits that may transport carbon from plant roots to other soil organisms involved in nutrient cycling processes. Thus, cooperating with other members of the decomposition soil food-web (Smith and Read, 2008).

3. Soil hyphae may have an important role in nutrient cycling by acquiring nutrients from saprophytic fungi (Lindahl *et al.* 1999).

4. Mycorrhizal roots and fungus fruit bodies are important as food sources and habitats for invertebrates (Fogel and Peck, 1975; Rabatin and Stinner, 1989).

5. Mycorrhizal fungus hyphae are an important food source for soil invertebrates (Ingham and Massicotte, 1994; Setälä, 1995).

6. Mycorrhizal fungi contribute to carbon storage in soil by altering the quality and quantity of soil organic matter (Rygielwicz and Andersen, 1994).

### 2.9.2 Benefits to plant

1. Increased plant nutrient provided by the fungal hyphae external to the root.

2. Increased plant nutrient supply by acquiring nutrient forms that would not normally be available to plants (Tarafdar and Marschner, 1994; Schweiger *et al.* 1995; Kahiluoto and Vestberg, 1998).

3. Some mycorrhizal fungi have the capacity to breakdown the phenolic compounds in soils which can interfere nutrient uptake (Smith and Read 2008).

4. Root colonization by mycorrhizal fungi can provide protection from parasitic fungi and nematodes (Duchesne *et al.* 1989, Grandmaison *et al.* 1993, Little and Maun, 1996, Cordier *et al.* 1998; Wehner *et al.* 2009).

5. Increased plant yield, nutrient accumulation, and/or reproductive success (Brundrett, 1996, Smith and Read, 2008).

6. Significant amounts of carbon transfer through fungus mycelia connecting different plant species. This could reduce competition between plants and contribute to the stability and diversity of ecosystems.

7. Networks of hyphae supported by dominant tree may help seedlings become established or contribute to the growth of shade under storey plants (Horton *et al.* 1999).

8. Nutrient transfer from dead to living plants may occur (Eason *et al.* 1991).

For mycorrhizal associations, there are many roles of mycorrhizal fungi that assist plant growth and fitness (Brundrett 1996; McKendrick *et al.* 2000; 2002). However, several mechanisms of mycorrhizal associations are still unclear and the study on these mechanisms is also required (Brundrett, 1991; Smith and

Read 2008). The siderophores and indo-3-acetic acid (IAA), plant growth hormone, production by mycorrhizal fungi is one of the possible mechanisms of mycorrhizal associations to help plant growth and fitness compared with non-mycorrhizal associations (Brundrett *et al.* 2001, 2003; Swarts and Dixon 2009; Wright *et al.* 2009). Moreover, there are a few studies reported the siderophores and IAA production by orchid mycorrhizal fungi. Thus, the study of these abilities of orchid mycorrhizal fungi is also required.

### **2.10 Siderophores production**

Siderophores are low molecular weight molecules, 300-2,000 daltons, water soluble and have the property to bind iron. Most of siderophores are produced by microorganisms to uptake iron in a variety of environments from terrestrial soils to the ocean surface. These compounds divided into 3 types upon a main chelating group of each siderophore. The microbial siderophores are generally classified as hydroxamates or thiohydroxamates, catecholates or phenolates and carboxylates. The functional group in hydroxamates is hydroxamic acid which is a carbonyl oxygen combined with an amino group. The catecholates ligands have adjacent hydroxyl oxygen on an aromatic ring. Most of siderophores which produced by bacteria are hydroxamates and catecholate types and some bacteria (*Rhizobium meliloti* and *Staphylococcus hyicus*) and fungi belonging to *Zygomycota* (*Mucorales*) produce carboxylates. While, the majority of filamentous fungi produce hydroxamates type (Baakza *et al.* 2004; Castaneda *et al.* 2005).

Iron is an essential trace element for all creatures in the world, especially for microorganisms. It is used as cofactor for electron transportations, protein synthesis, reduction of ribotide precursors of DNA, heme formation and other essential purposes (Neilands, 1995; Khamna, 2009). In the natural sources, soils, iron is found as ferric ion ( $\text{Fe}^{3+}$ ) that the microorganisms are unable to assimilate directly. Therefore, the iron chelating compounds called siderophores are produced by several microorganisms. These compounds have a high affinity for  $\text{Fe}^{3+}$  and form complex with  $\text{Fe}^{3+}$  which is able to be imported into cells by microorganisms (Figure 2.9). The producing of siderophores is one of mechanisms of biological control in term of competition by siderophores producing microorganisms uptake an essential iron from environments better than other. The types of mechanisms of biological control are shown in Table 2.6. In addition the processes of siderophores production by chemical synthesis are often slow and expensive. Thus, there are several reports that studied on siderophores production; Milagres *et al.* (1999) reported fungi (*Thermoascus aurantiacus*, *Aspergillus flavus*, *A. niger*, *A. tamari*, *Fusarium melano*, *Paecilomyces varioti*, *Cunninghamella* sp., *Penicillium spinolosum*, *P. indofitico*, *Metarhizium anisopliae* and *Rhizopus* sp.) and bacteria (*Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus*, *S. epidermidis* and *Bacillus cereus*) could produce siderophores using chrome azural S (CAS) agar plate assay for detection siderophores production. Moreover, there are some fungi belonging to *Ascomycetes*, *Zygomycetes* and *Deuteromycetes* also were reported that could produce siderophores (Baakza *et al.* 2003; Perez-Miranda *et al.* 2007). In addition,

the capacity of various mycorrhizal fungi to produce siderophores was reviewed by Haselwandter, (1995) and Verma, (1998). Data are presented in Table 2.7.

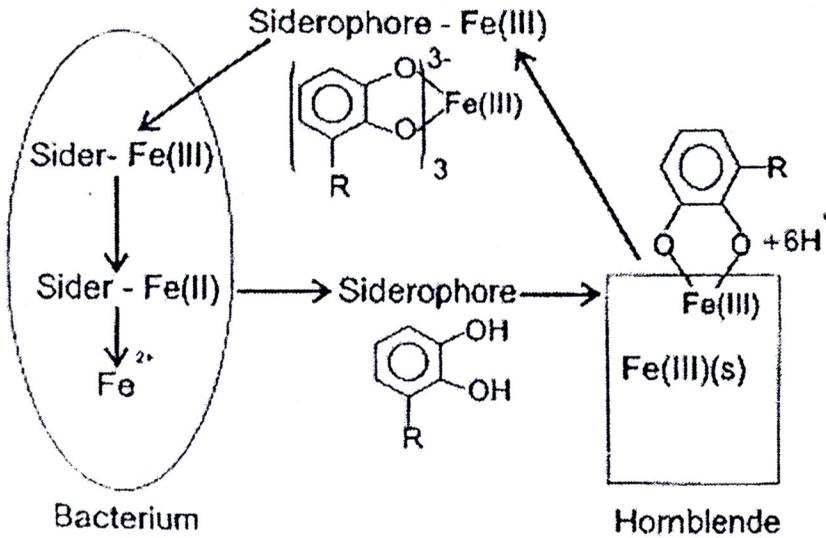


Figure 2.9 Mechanism of iron acquisition by microbial siderophore (Kalinowski *et al.* 2000)

Table 2.6 Types of interspecies antagonisms leading to biological control of plant pathogens (Pal and Gardener, 2006)

Type	Mechanism	Example
Direct antagonism	Hyperparasitism, predation	Lytic/some non lytic mycoviruses
		<i>Ampelomyces quisqualis</i>
		<i>Lysobacter enzymogenes</i>
		<i>Pasteuria penetrans</i> <i>Trichoderma virens</i>
Mixed-path antagonism	Antibiotics	2,4-diacetylphloroglucinol
		Phenazines
		Cyclic lipopeptides
	Lytic enzymes	Chitinases
		Glucanases
Proteases		
Unregulated waste products	Ammonia	
	Carbon dioxide	
	Hydrogen cyanide	
Physical, chemical interference	Blockage of soil pores	
	Germination signals consumption	
	Molecular cross-talk confused	
Indirect antagonism	Competition	Exudates/leachates consumption
		Siderophores scavenging
		Physical niche occupation
	Induction of host resistance	Contact with fungal cell walls
Detection of pathogen-associated, molecular patterns		
Phytohormine-mediated induction		

Table 2.7 Predominant siderophore types in major mycorrhizal types (Verma, 1998)

Mycorrhiza	Siderophore type	Chemical structure
Ectomycorrhiza	Hydroxamates	Unknown
Arbuscularmycorrhiza	Hydroxamates	Unknown
Ericoidmycorrhiza	Hydroxamates	Ferricrocin, fusigen
Arbutiodmycorrhiza	Unknown	Unknown
Monotropoidmycorrhiza	Unknown	Unknown
Orchid mycorrhiza	Unknown	Unknown
Ectendomycorrhiza	Hydroxamates	Ferricrocin

### 2.11 Plant growth hormone, Indole-3-acetic acid (IAA), production

Plant growth hormones are organics produced naturally in higher plants, controlling growth or physiological functions. The major types of plant growth hormones can be divided to 5 types: Auxins, Gibberellin, Cytokinin, Ethylene, and Abscisic acid. Indole-3-acetic acid (IAA) is one of the most physiologically active auxins and a major plant growth hormone found in almost all plants. Furthermore, IAA is a common product of L-tryptophan metabolism by several microorganisms including some fungi especially that associated with plant roots or rhizosphere soil and endophytic fungi (Hasan, 2002; Nasim *et al.* 2004; Sridevi and Mallaiiah, 2008). Microbial IAA biosynthesis pathways are shown in Figure 10. IAA is a derivative of indole ring compound. This compound has the ability to

improve plant growth by stimulating cell elongation, root initiation, increase seed germination, seedling growth and effect on phototropism and apical dominance (Davies, 2004; El-Tarabily, 2008).

Nowadays, IAA production from microorganisms including several fungi was studied especially for plant growth promoting bacteria and fungi. The microorganisms which produced IAA are shown in Table 2.8. In addition, some studies reported that IAA produced from several bacteria and fungi had ability to improve seed germination, root formation and plant growth. For examples, Hasan, (2002) reported that *Fusarium oxysporum* isolated from rhizosphere soil of *Glycine max* (soybean) had an ability to produce IAA and enhance the germination of seeds. Niemi *et al.* (2002) reported the IAA produced from *Pisolithus tinctorius* and *Paxillus involutus*, ECM, improved root formation and growth of Scots pine. Therefore, IAA production from plant associated fungi especially endophytic fungi, rhizosphere soil fungi and mycorrhizal fungi was studied.

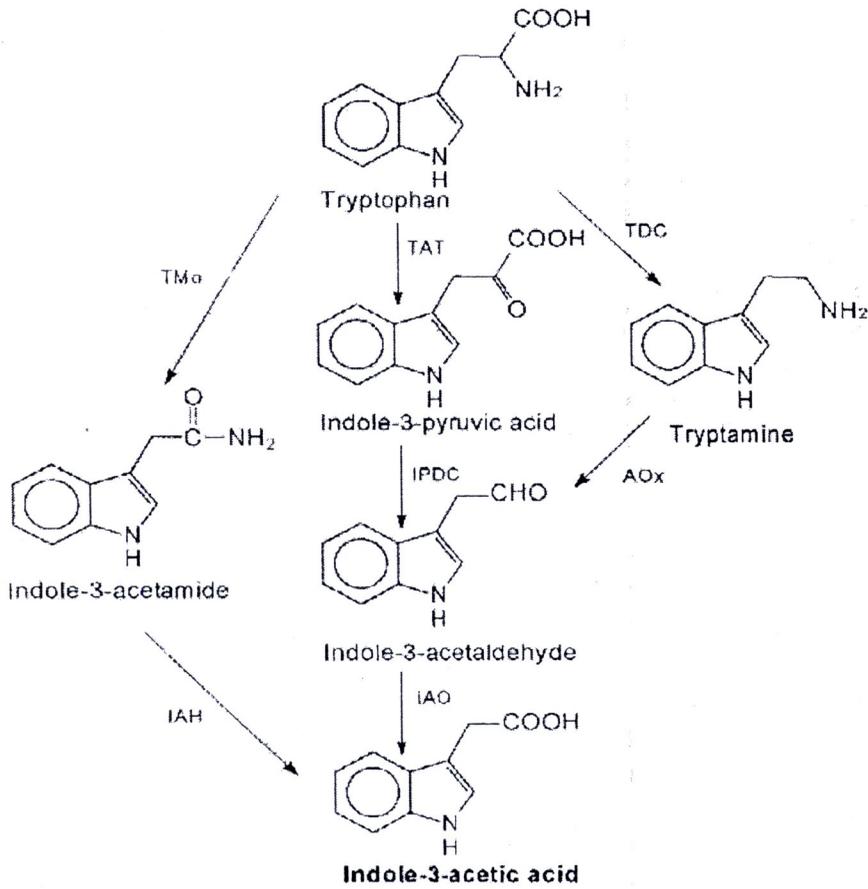


Figure 2.10 Pathways for the microbial biosynthesis of indole-3-acetic acid (IAA) from tryptophan (Davies, 2004)

Table 2.8 Several microorganisms which are able to converted L-tryptophan to Indole-3-acetic acid (IAA)

Microorganism	Source of isolation	Source
Fungi		
<i>Collectotrichum gloeosporiodes</i>	<i>Aeschynomene</i>	Maor <i>et al.</i> 2004
<i>Candida incommunis</i>	<i>Gossypium</i> sp.*rh	El-Mehalawy <i>et al.</i> 2007
<i>Eupenicillium senticosum</i>	<i>Gossypium</i> sp.*rh	El-Mehalawy <i>et al.</i> 2007
<i>Fusarium oxysporum</i>	<i>Corchorus olitorius</i> *rh	Hassan, 2002
<i>F. oxysporum</i>	<i>Sesamum indicum</i> *rh	Hassan, 2002
<i>F. oxysporum</i>	soyabean <i>Glycine max</i> *rh	Hassan, 2002
<i>Pisolithus tinctorius</i>	ECM of <i>Pinus sylvestris</i> L.	Niemi <i>et al.</i> 2002
<i>Paxillus involutus</i>	ECM of <i>Pinus sylvestris</i> L.	Niemi <i>et al.</i> 2002
Bacteria		
<i>Agrobacterium</i> sp.	<i>Pholidota articulata</i> *rh	Tsavkelova <i>et al.</i> 2006
<i>Azotobacter</i> spp. (10)	crops of wheat and cauliflower *rh	Ahmad <i>et al.</i> 2004
<i>Bacillus</i> sp.	<i>Paphiopedilum appletonianum</i> *rh	Tsavkelova <i>et al.</i> 2007
<i>Bacillus</i> sp.	<i>Paphiopedilum appletonianum</i> *rt	Tsavkelova <i>et al.</i> 2007
<i>Bacillus</i> sp.	<i>Pholidota articulata</i> *rt	Tsavkelova <i>et al.</i> 2007
<i>Bacillus</i> sp.	<i>Triticum aestivum</i> *rh	Erturk <i>et al.</i> 2008
<i>Bacillus</i> sp.	<i>Rubus occidentalis</i> *rh	Erturk <i>et al.</i> 2008
<i>B. megaterium</i>	<i>Triticum aestivum</i> *rh	Erturk <i>et al.</i> 2008
<i>B. subtilis</i>	<i>Lycopersicon esculentum</i> *rh	Erturk <i>et al.</i> 2008
<i>B. simplex</i>	<i>Rubus occidentalis</i> *rh	Erturk <i>et al.</i> 2008
<i>Burkholderia</i> sp.	<i>Paphiopedilum appletonianum</i> *rh	Tsavkelova <i>et al.</i> 2007
<i>Burkholderia</i> sp.	<i>Pholidota articulata</i> *rh	Tsavkelova <i>et al.</i> 2007
<i>Bradyrhizobium</i> sp.	<i>Arachis hypogaea</i> *rt	Deshwal <i>et al.</i> 2003

Table 2.8 (continued)

Microorganism	Source of isolation	Source
Bacteria		
<i>Chysoebacterium sp.</i>	<i>Pholidota articulata</i> *rh	Tsavkelova <i>et al.</i> 2007
<i>Comamonas acidovorans</i>	<i>Rubus occidentalis</i> *rh	Erturk <i>et al.</i> 2008
<i>Erwinia sp.</i>	<i>Pholidota articulata</i> *rh	Tsavkelova <i>et al.</i> 2007
<i>Erwinia sp.</i>	<i>Paphiopedilum appletonianum</i> *rt	Tsavkelova <i>et al.</i> 2007
<i>Flavobacterium sp.</i>	<i>Pholidota articulata</i> *rt	Tsavkelova <i>et al.</i> 2007
<i>Flavobacterium sp.</i>	<i>Pholidota articulata</i> *rh	Tsavkelova <i>et al.</i> 2007
<i>F. indologenes</i>	<i>Glycine max</i> *rh	Erturk <i>et al.</i> 2008
<i>Klebsiella sp.</i>	crops of bean, maize and rice *rh	Chaiham and Lumyong, 2011
<i>Paenibacillus polymyxa</i>	<i>Triticum aestivum</i> *rh	Tsavkelova <i>et al.</i> 2007
<i>Pantoea sp.</i>	<i>Pholidota articulata</i> *rh	Tsavkelova <i>et al.</i> 2007
<i>Paracoccus sp.</i>	<i>Pholidota articulata</i> *rh	Tsavkelova <i>et al.</i> 2007
<i>Pseudomonas spp. (11)</i>	crops of wheat and cauliflower *rh	Tsavkelova <i>et al.</i> 2007
<i>Pseudomonas sp.</i>	<i>Paphiopedilum appletonianum</i> *rh	Tsavkelova <i>et al.</i> 2007
<i>Pseudomonas sp.</i>	<i>Pholidota articulata</i> *rh	Jeon <i>et al.</i> 2003
<i>P. fluorescens</i>	soil	Sridevi and Mallaiah, 2008
<i>P. putida</i>	<i>Pterostylis vittata</i>	Tsavkelova <i>et al.</i> 2007
<i>Stenotrophomonas sp.</i>	<i>Pholidota articulata</i> *rh	Tsavkelova <i>et al.</i> 2007
Actinomycetes		
<i>Actinomadura sp.</i>	<i>Stemona tuberosa</i> *rh	Khamna, 2009
<i>Norcadia sp.</i>	<i>Stemona tuberosa</i> *rh	Khamna, 2009
<i>Nonomurea sp.</i>	<i>Curcuma mangga</i> *rh	Khamna, 2009
<i>Streptomyces sp.</i>	<i>Curcuma mangga</i> *rh	Khamna, 2009

Note; \*rh = rhizosphere soil, \*rt = root