CHAPTER 1

INTRODUCTION

1.1. Research Rationale

Cercospora Fresen. (1863) *sensu lato* (*s. lat.*) or cercosporoid fungi are the most important group of fungi in agricultural field. The fungi are destructive plant pathogens and major agent of crops losses throughout the world. This group is nearly universally pathogenic, occurring on a wide range of hosts in almost all major families of dicotyledonous, most monocotyledonous families, some gymnosperms and ferns (Pollack, 1987). Cercosporoid fungi are commonly associated with leaf spots, but can also cause necrotic lesions on flowers, fruits, bracts, seeds and pedicels of numerous hosts in most climatic regions (Agrios, 2005). Furthermore, other than important pathogens of major agricultural crops such as cereals, vegetables, ornamentals, forest trees, grasses, etc., the cercosporoid fungi are also known to be hyperparasites to other plant pathogenic fungi (Shin and Kim, 2001), and are employed as biocontrol agents of alien weeds (Morris and Crous, 1994).

Crous and Braun (2003) determined four genera: *Cercospora*, *Pseudocercospora* Speg., *Passalora* Fr. and *Stenella* Syd. as true cercosporoid fungi. Numerous species of the true cercosporoid fungi have been reported from Thailand. Sontirat *et al.* (1980) enumerated 21 species of *Cercospora*. Giatgong (1980) listed 47 identified and 13 unidentified species of the genus *Cercospora* in *The Host Index of Plant Diseases in Thailand*, and Petcharat and Kanjanamaneesathian (1989) reported 49 species from various hosts. However, their reports were mainly based on the

generic concepts introduced by Chupp (1954). Further reports of new species, new records, and additions to the distribution of several cercosporoid fungi in Thailand were also published by Ellis (1976), Manoch *et al.* (1986), Pons and Sutton (1988), Barreto and Evans (1994), Crous (1998), Crous and Braun (2003), Lumyong *et al.* (2003), Braun *et al.* (2006) and Hunter *et al.* (2006).

Since crop losses caused by fungal diseases pose a serious threat to global food security, it became apparent that the threat to agriculture from the deliberate release of pathogens, such as the cercosporoid fungi, should not be underestimated. However, the information of those phytopathogenic fungi in Thailand are quite limited and, therefore, still causing many difficulties for mycologist, plant pathologist, quarantine, and other scientific societies in Thailand to identify until species level. Almost no information, regarding this group of fungi and its distribution to the host plants specific in Thailand, is available. Mostly, the publication are scattered and unspecialized to the fungi but their focus on the host, such as eucalyptus (Crous, 1998). Therefore, survey and research on diversity of this group of fungi, its distribution to the host plants, and molecular analysis of its evolution are urgently needed.

1.2. The Current Understanding of Cercosporoid Fungi

The *Cercospora* species are commonly pathogenic on plant parts, causing either distinct necrotic spots or an effuse fruiting layer without definite spots on leaves, pedicles, stems, fruits, and bracts; they are never wholly saprophytic, although often accompanying or following other fungi; they also never cause soft root (Chupp, 1954). The following information described and elucidated the characteristics of the

cercosporoid fungi, including important morphology characteristics, molecular phylogenetic relationship within this group and other related taxa, and also ecological aspects such as pathogenesis and resistance to systemic fungicides.

1.2.1. Morphology Characteristics of Cercosporoid Fungi

Chupp (1954) monographed the genus *Cercospora s. lat.*, which is one of the largest genera of Hyphomycetes with more than 3000 names. Deighton with his serial publications (1967, 1971, 1973, 1974, 1976, 1979 and 1983), Pons and Sutton (1988), Braun (1993), Braun and Melnik (1997) and other authors divided *Cercospora s. lat.* into numerous smaller genera based on morphological characteristics. Combination of morphology and molecular analysis was also carried out by Crous *et al.* (2000, 2001). From the previous intensive studies on this group of fungi, Crous and Braun (2003) published the compilation of the names in *Cercospora* and *Passalora*, and re-defined the morphological characteristics of *Cercospora s. lat.* based on morphology and molecular analysis results. The following description and illustration are the common items used to identify the cercosporoid fungi.

A. Symptoms on the Host Plants

Symptoms caused by cercosporoid fungi are variable. Leaf spots may be absent or present in every degree of distinctiveness from a faint discoloration on both leaf surfaces to definitely defined and conspicuous leaf spots with colored borders, eye-spot diseases or vein-limited lesions (figures 1a-e). Often an effuse caespituli (or fruit bodies) are visible on the lower leaf surface when no leaf spots are visible. The fungi may be so minute that a hand lens is required to detect it. The leaf may curl, dry

and drop from the plant when the disease reaches a certain stage of severity. Almost complete defoliation can be caused by the more virulent species.

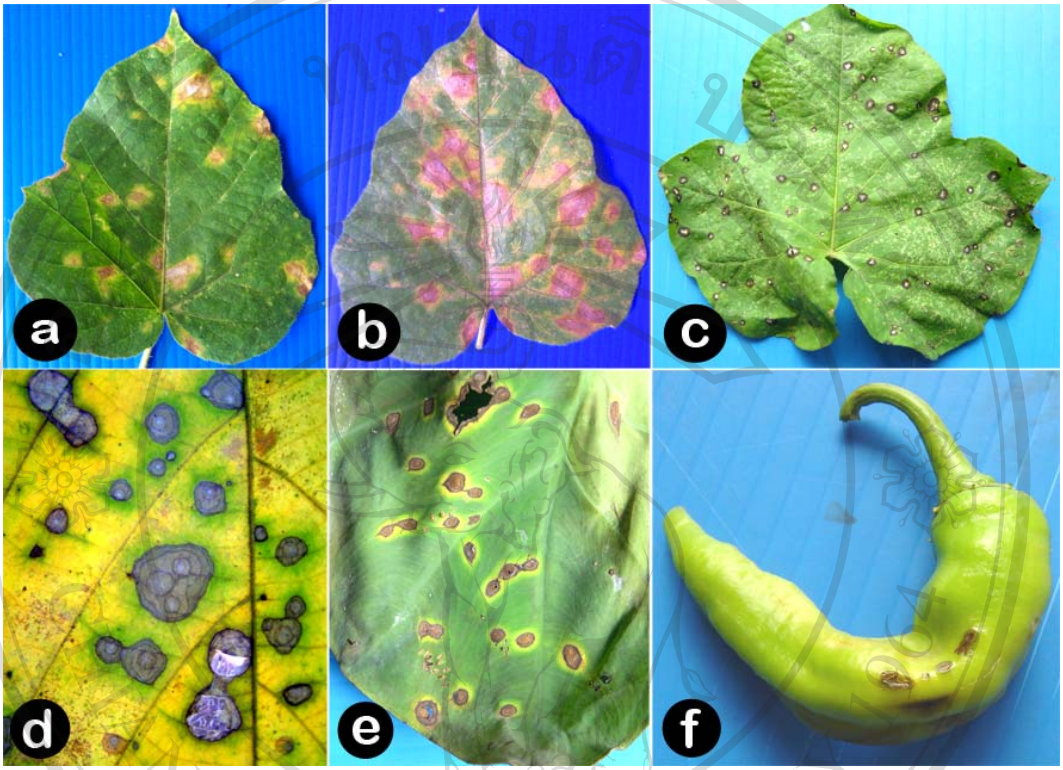

Figure 1 Various types of cercospora leaf spot symptoms on leaves and fruit.

Many cercosporoid fungi also affect the blossoms, fruits (figure1f), pods, succulent petioles, and young stems. Frequently, the dying and shrinking portion dries ears away from the living leaf tissue, leaving a shot-hole effect. One to numerous spots may turn the entire leaf yellow or brown, after which it shrivels and dies. In describing the symptoms of the individual *Cercospora* species, the shot-hole effect and defoliation are rarely mentioned. Most herbarium specimens are pressed leaves, therefore, only the leaf symptoms as they show in freshly collected or herbarium material need here be taken into account.

B. Caespituli (Fruit Bodies)

Caespituli of cercosporoid fungi, commonly is called fruit bodies, is defined as turfs of conidiophores as seen under microscope or hand lens (figure 2). The caespituli could be distributed on the upper surface (epiphyllous), lower surface (hypophyllous), both surfaces (amphigenous); evenly distributed on the spot or aggregated along the margin of the spot. The caespituli structure appearances often velvety, floccose, arachnoid, as effuse patches, punctiform (as minute black pustules), mouldy, and the colors are variable from sooty, dark, grey, olivaceous to whitish.

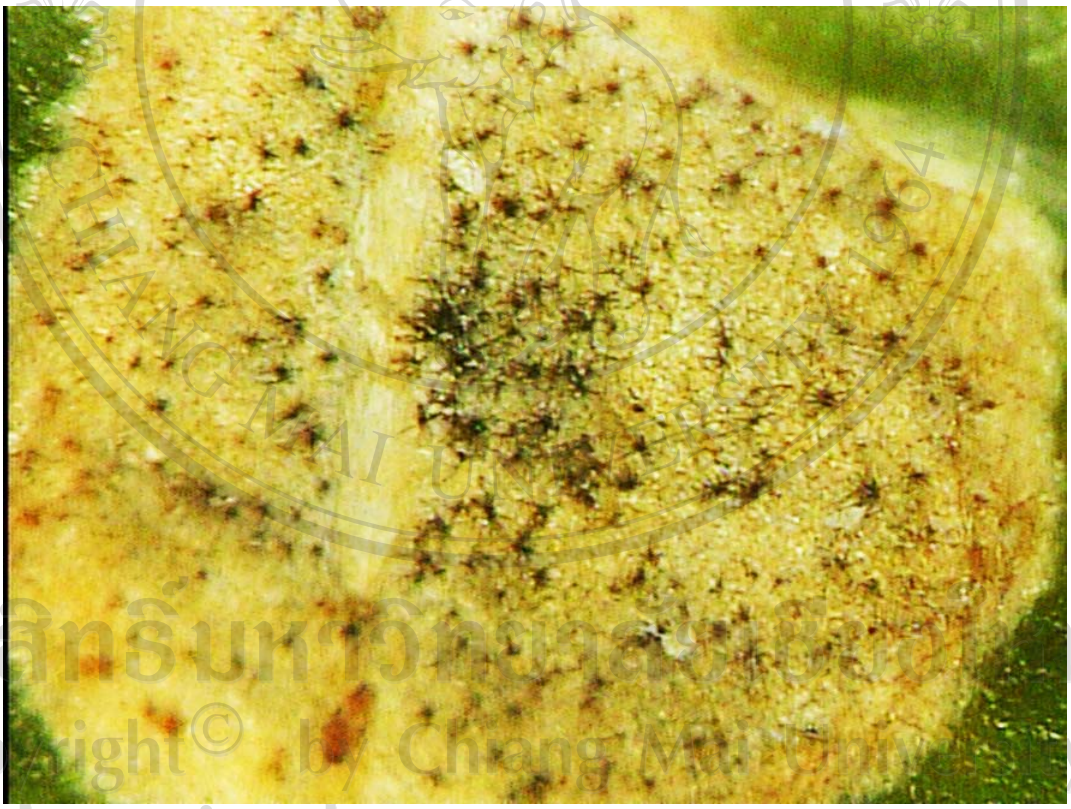

Figure 2 Appearance of caespituli (as turfs of conidiophores) of genus *Cercospora* on the leaf spot of *Coccinia grandis* (L.) Voigt.

C. Conidiophores, Conidiogenous Cells, and Conidiogenesis

A conidiophore is defined as the entire system of fertile hyphae bearing conidia, it may be either simple or branched, and includes the conidiogenous cell (s) (Ulloa and Hanlin, 1999). It can be reduced to a single fertile cell if the conidiophore and the conidiogenous cell are identical, or the conidiophore is composed of a single conidiogenous cell and a single or several supporting cells, or it consists of a system of conidiogenous cells with or without differentiated supporting structure (hyphal cells, stipe) (Gams *et al*., 1987). Some authors, for instance Hawksworth *et al.* (1983) and Pons and Sutton (1988), preferred to confine the term conidiophore to complex structures composed of two or more cells and only mention of conidiogenous cells in the other case. In the cercosporoid fungi, there are numerous species with tufts of mixed conidiophores. Some of them are continuous and one-celled, other conidiophores are septate, composed of two or more cells. Therefore, in this thesis, a wider concept of the term conidiophore is applied as one-celled conidium-bearing structures can either be called conidiogenous cell or conidiophore, depending on the particular case. Micronematous refers to conidiophores which are morphologically hardly distinguished from ordinary hypha, but macronematous conidiophores are well-differentiated (Ulloa and Hanlin, 1999). Conidiophores may be colorless (hyaline) or variously pigmented, and the pigmentation is an important taxonomic feature. Conidiophores may be formed singly, erumpent through the substratum or arising from free, creeping hyphae as lateral branchlets, or they are caespitose, i.e. arranged in loose or densely fascicles (tufts) (figures3a-d).

Conidiogenous cells can be formed as part of an undifferentiated hypha, and they also can form a unicellular conidiophore in case of conidiogenous cell and

conidiophore are identical such as several species of genus *Passalora*, or they can mostly form part of a pluricellular conidiophore. In this case, they can be either terminal, intercalary, or pleurogenous. If they are formed laterally or terminally but not in continuity with the main axis, they are therefore called discrete. The conidiogenous cells appear as various shapes such as ampulliform, lageniform, sphaerical, etc., and within a conidiogenous cell, the conidiogenous locus is the point, area or zone at which a conidium is released. It can be fixed or varying (Ulloa and Hanlin, 1999).

A conidiogenous cell may be unilocal/single locus (figs 3-b, d) or multilocal/more than two loci (figures 3a, c). The loci can be apical, lateral or circumspersed (all around the conidiogenous cell) (Hennebert and Sutton, 1994). Old conidiogenous loci are often well–discernible by their denticle–like, papilloid, thickened, darkened or refractive structure. A conidial scar, the minute structure at the end of conidiogenous cell resulted from a conidiogenesis, is a recognizable portion where the conidium has been liberated (the basal part of the conidial septum). Conidial scars may be conspicuous by thickened walls with dark coloration (figures 3c-d), by being refractive, bulging or protuberant (often papilla–shaped). The distinction between “darkened” and “refractive” is often difficult, especially in minute and hardly or only slightly thickened scars, however, both phenomena are often combined. A scar on a conidium at the point of former attachment to the conidiophore is termed hilum. Conidiogenous cells provided with conspicuous conidial scars are said to be cicatrized. Tooth-like projections supporting the young conidia are called denticles (conidiogenous cells provided with denticles are defined to be denticulate). Scars and denticles are usually formed in a sympodial succession. True denticles are

more or less subcylindric to tapered, mostly formed laterally or terminally on more or less straight, sometimes swollen.

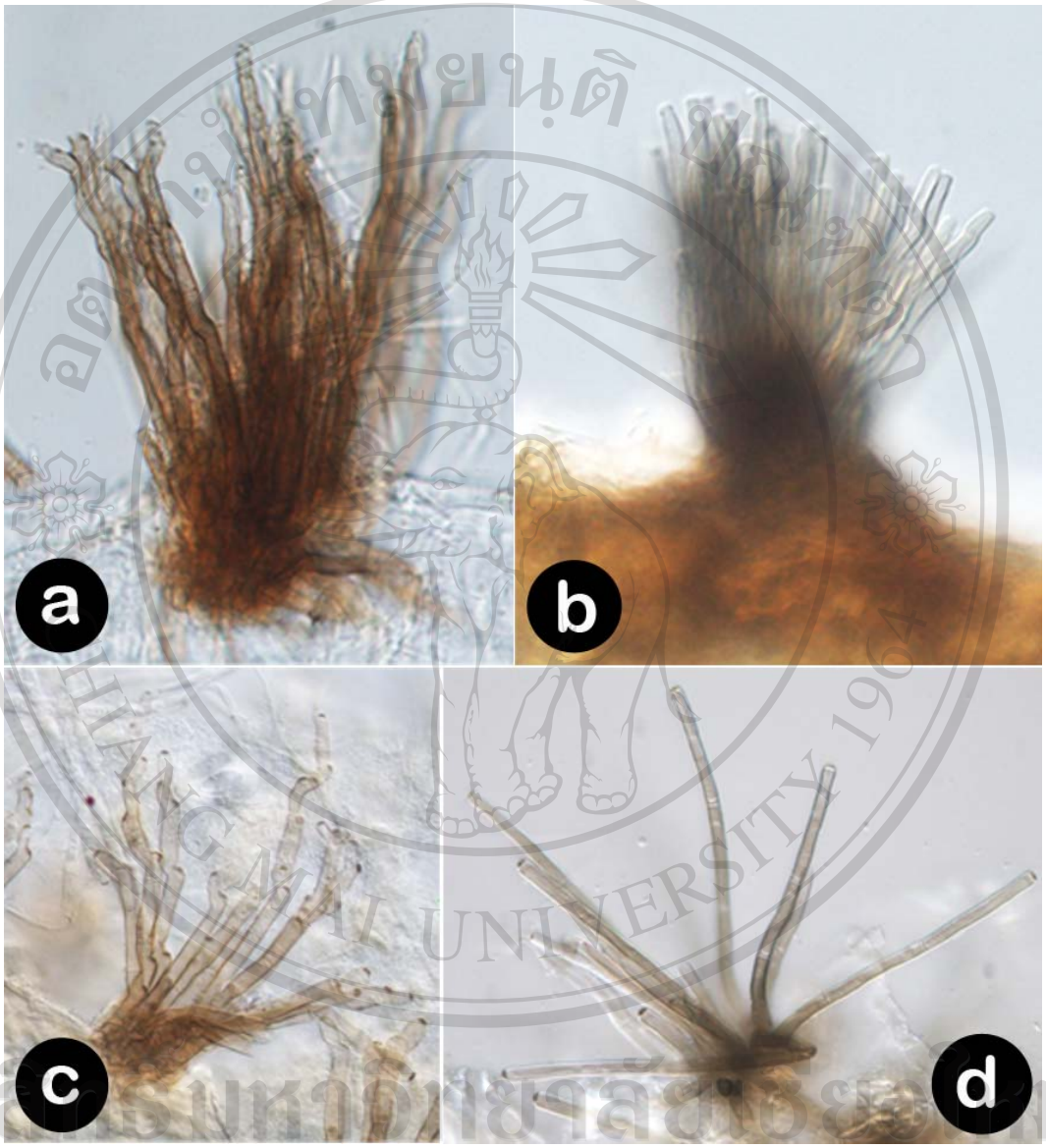

Figure 3 Various types of conidiophores and conidiogenous cells of the cercosporoid fungi. **a**. Fasciculate and non divergence. **b**. Fasciculate, non divergence, and conidiogenous loci not darkened. **c**. Fasciculate, divergence, polyblastic, with sympodial proliferation, with dark conidiogenous loci. **d**. Fasciculate, distinctly divergence, non-sympodial proliferation with dark conidiogenous loci. (40×)

The development of conidium from the conidiogenous cell or conidiophore is called conidiogenesis (Hennebert and Sutton, 1994). The conidium development may

be thallic, septate hyphae disintegrate or the initiation and elongation of conidia begins from an area as wide as the conidiogenous cell, followed by delimitation by basal septation. Thalloblastic is an introduced term for intermediate types (initiation and elongation of conidia agreeing with thallic, but the swelling occurs before delimitation). The cercosporoid fungi conidiogenesis is characterized by holoblastic (monoblastic or polyblastic), sometimes determinate but often sympodial proliferation, mostly schizolytic with single or conidia in chains. Blastic conidiogenesis is characterized by an elastic wall of the conidiogenous cells, bulging out to form a conspicuous, enlarged conidium initial. It may be holoblastic [all wall layers of the conidiogenous cells contribute towards the formation of the conidium (blastoconidia)] or enteroblastic (only the inner wall of the conidiogenous cell contributes towards the formation of the conidium). Blastic conidiogenous cells may be monoblastic (only with a single conidiogenous locus or unilocal) or polyblastic (with two or more conidiogenous loci or multilocal), formed either synchronously or, mostly; in a sympodial successions. Conidiophores (or conidiogenous cells) can be determinate (growth ceasing with the production of a terminal conidium or conidial chain) or they can proliferate [indeterminate, proliferation being sympodial or percurrent (through the open end left when the first conidium becomes detached)]. The enteroblastic nature of percurrent proliferations is usually well discernible in fairly thick walled, pigmented conidiophores. Annellations are usually inconspicuous in thin walled, pale conidiophores. Details of the proliferation are hardly to be observed by means of light microscopy. Conidial secession can be schizolytic (by cleavage at a separating septum) or rhexolytic (by rupture of the lateral wall below the basal septum or between two septa). Rhexolytically released conidia are usually

marked by conspicuous frills. Very minute frills on conidial scars and hila are not uncommon in hyphomycetes with schizolytic conidial secession. Such phenomena should not be lumped or confused with cases of rhexolytic secession. The conidia are either formed singly (conidia solitary) or in acropetal chains [catenate (development in the direction of the apex, i.e. the apical conidium is the youngest)]. Acropetal chains are either simple (monopodial, acropetal unipolar) or branched (sympodial, acropetal multipolar).

D. Conidia

Conidia are all mitospores of higher fungi. There are different concepts of the term conidium (Sutton, 1986), but this is the general lot of most historical terms. The different concepts and applications of the term "spore" are much more confused. Sutton (1993) proposed to abandon the term conidium and to replace it by mitospore. However, this proposal is not supported and applied in this thesis. "Spore" is the most general and comprehensive term, including "conidium". "Mitospore" is a neutral and broader term than "conidium". "Conidium" is well defined, concise, well established and useful under many circumstances. Both terms should alternatively be applied in appropriate cases. A "sporological" system of morphological categories for mature conidia was introduced by Saccardo in the 19th century or called the Saccardoan system. Detailed discussions and surveys are to be found in Kendrick and Di Cosmo (1979), Hawksworth *et al.* (1983), and Gams *et al.* (1987). Saccardo (1913) arranged conidia in groups based on shape, septation and pigmentation and introduced a special terminology (one celled = amerospore, two celled = didymospore, many celled = phragmospore, muriform = dictyospore, filiform = scolecospore, strongly curved to

spiral or helicoid = helicospore, stellate = staurospore). Kendrick and Di Cosmo (1979) circumscribed the terms more precisely and provided a dichotomous key.

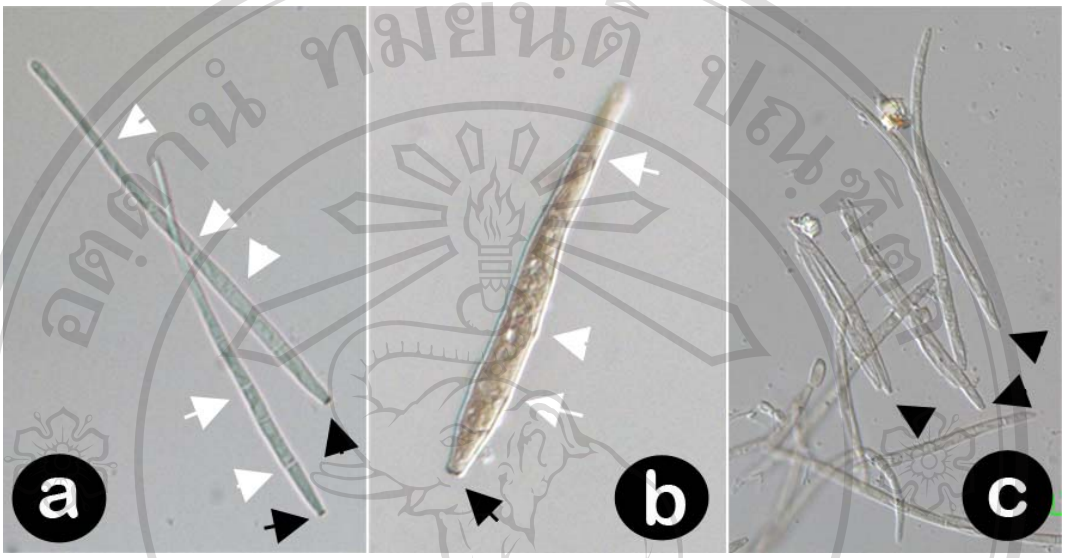

Figure 4 Various types of conidia of the true cercosporoid fungi sensu Crous and Braun (2003) found in this study (black arrows: conidia hila, white arrows: septation). **a**. Conidia of genus *Cercospora s. str*. **b**. Conidium of genus *Passalora*. **c**. Conidia of genus *Pseudocercospora*. (40×)

The general important characters of conidia of cercosporoid fungi are mostly related to the shape, septation, pigmentation, and surface (figures 4a-c). The conidia of the cercosporoid fungi are often either straight to curved, with acicular, filiform, obclavate, ellipsoidal, or combination of the shape. There are two basic types of septation, viz, euseptate (septa formed by all existing wall layers) and distoseptate/ pseudoseptate (septa formed only by the innermost layer). The term septum (septate) without specification is usually applied to eusepta (euseptate), and the cercosporoid fungi are mostly characterized by euseptate conidia. Hyaline and pigmented structures (conidiophores, conidia etc.) are usually well separated in certain taxa (genera, species) of the cercosporoid fungi, but transitional phenomena are not uncommon,

however, taxa with subhyaline to pale (yellowish green, pale olivaceous, etc.) structures often cause serious taxonomic problems. In this case, observation in unstained water mounts is crucial. The conidial of cercosporoid fungi mostly smooth, very rarely rough except the genus of *Stenella*. Different sculptures of the conidial surface can better be distinguished by means of Scanning Electron Microscopy (SEM). In some cases, closely related taxa may be separated by distinct conidial ornamentation.

Those common morphology characteristics elucidated above are general in the cercosporoid fungi taxa, however, Crous and Braun (2003) reaffirmed some primary characters that have recently been employed while treating the cercosporoid fungi as follow:

1. Structure of conidiogenous loci (scars) and hila (unthickened and almost so, but slightly darkened or refractive appears to have the same value as being unthickened). For example, thickened and darkened conidiogenous loci could be found on genera of *Cercospora* and *Passalora* (figures 4a-b), but unthickened conidiogenous loci is common character of genus *Pseudocercospora* (figure 4c).

2. Presence or absence of pigmentation in conidiophores and conidia. For example, genus *Passalora* is characterized by having pigmented (mostly brown) conidia (figure 4b) whereas genus *Cercospora* is characterized by hyaline conidia (figure 4a).

1.2.2. Review of true Cercosporoid Fungi *sensu* Crous and Braun (2003)

According to Kirk *et al.* (2001), the genus *Cercospora* Fresen. could be artificially classified as follows:

Domain: Eukaryota

Kingdom: Fungi

Form-phylum: Deuteromycota

Form-Class: Hyphomycetes

Form-Order: Hyphomycetales

Form-Family: Dematiaceae

Genus: *Cercospora*

The genus *Cercospora*, established by Fresenius (1863), is one of the largest genera of Hyphomycetes (Crous and Braun, 2003). The type species is *C. apii* Fresen. The name *Cercospora*, which is derived from the combination of the Greek "*kerkok*" (tail) and "sporos" (seed), designates the filiform conidia of the fungus. The genus has been linked to *Mycosphaerella* Johanson (*Dothidiomycetes, Capnodiales, Mycosphaerellaceae*) teleomorph that has been associated with at least 27 different Coelomycetes or Hyphomycetes anamorph genera (Kendrick and Di Cosmo, 1979). In addition, Crous *et al.* (2000) only accepted 23 genera associated to the genus *Cercospora*. Significant contributions to this group of fungi were published by Chupp (1954) who monographed *Cercospora s. lat.*, Pollack (1987) listed more than 3,000 names have already been published and proposed in the genus *Cercospora*, and Crous and Braun (2003) who re-examined and reduced the number of species name of *Cercospora s. str.* into only 659 species name, with 281 names being referred to *C. apii s. lat.*

Since Fresenius (1863) did not give the genus *Cercospora* a clear-cut definition, Saccardo (1880) defined it as having brown conidiophores and vermiform conidia which are brown, olivaceous or rarely subhyaline, but he did not mention the type species (*C. apii*) which has hyaline conidia. Saccardo considered *C. ferruginea* Fuckel as a typical of *Cercospora* and repeated this definition in *Sylloge Fungorum* (1886). Since then, two anomalous species of *Cercospora* are found to exist, i.e., those with colored conidia and those with hyaline conidia.

Spegazzini (1910) was the first to split the genus *Cercospora* and published a new generic name *Cercosporina* Speg. to accommodate those species with hyaline conidia (i.e. with the characters of *C. apii*) due to the colored conidia proposed by Saccardo (1880), and no type species was indicated for new genus. Saccardo (1913) agreed with the establishment of *Cercosporina*, and transferred 89 species from *Cercospora* (including some with colored conidia as well as those with hyaline ones) to *Cercosporina*. It caused confusion among these species. Miura (1928) was the one who actually transferred *C. apii* to *Cercosporina* and also proposed the genus *Cercosporiopsis* Miura to accommodate certain *Cercospora*-like species with colored cylindric conidia, but this genus is superfluous and illegitimate. Solheim (1930) proposed 21 sections of *Cercospora* by considering the presence or absence of external mycelium and prominent stromata, branching of conidiophores, as well as the shapes of conidia. Later, Solheim and Stevens (1931) reconsidered their reclassification of *Cercospora* by adding the character of conidial scars, and divided the genus into 38 sections and proposed the genus *Raghildiana* for the intermediate species between *Cladosporium* Link and *Cercospora* based on these characters.

Chupp (1954), in the monograph of genus *Cercospora*, made no attempt to subdivide the genus *Cercospora*, however, the monograph provided a very valuable source of reference to almost all *Cercospora* species published up to 1954, but excluded those names other than *Cercospora* or *Cercosporina.* Chupp thought that although several attempts were made to split *Cercospora,* where many new generic were proposed, there exist many intermediate species which do not allow the clear-cut classification. Chupp believed that the *Cercospora* are limited remark in their host range, and therefore, appropriate cross inoculations between species should be performed to ensure their identities. In the Chupp's monograph (1954), the character of conidial scars are taken into account, either distinctly visible or obscured, and for those prominent scars, their sizes are noted as either large, medium, or small.

Deighton (1987) continuing studies the *Cercospora* and allied genera and reclassified numerous species, and also stressed the characteristic of the conidial scars. Several allied genera of *Cercospora* were redefined or newly proposed, which fall into two distinct taxonomic categories: those in which the conidial scars are conspicuously thickened (appearing as black rims when views under light microscopy) and those in which the conidial scars are unthickened. Deighton discussed the development of taxonomic concepts and addressed problems concerning generic differentiation in a modern context. Chupp placed considerable emphasis on the presence or absence of thickening in the scars left on the conidiogenous cells after conidial secession. Two distinct taxonomic categories were recognized by Deighton (1976), one in which old conidial scars on conidiogenous cells are thickened to a greater or lesser degree and the other where scars are not thicker than anywhere else on the conidiogenous cell wall. The hilum at the base of a conidium is thickened or

unthickened or unthickened in correspondence with the scars left on the conidiogenous cell. Thickened scars of the *Cercospora* and allied genera occur in genera such as *Camptomeris* Syd., *Cercosporella* Sacc., *Cercosporidium* Earle, *Fusicladium* Bonord., *Mycovellosiella* Rangel, *Passalora, Phaeoisariopsis* Ferraris, *Phaeoramularia* Muntk.-Cvetk., *Sirosporium* Bubák and Serebrian., *Stenella* Syd., etc. Unthickened conidial scars occur in genera such as *Cercoseptoria* Petr., *Mycocentrospora* Deighton, *Pseudocercospora, Stigmina* Sacc., etc.

The character of conidial scars, stressed by Deighton as an unambiguous taxonomic criterion, have been adopted by recent workers of many countries in the classification of the *Cercospora* and allied genera such as Pons and Sutton (1988) and Braun (1988a, 1988b, 1989, 1990). Braun (1993) concluded that the *Cercospora* generic conception adopted by Chupp (1954) was too wide, and this genus could be safely redefined into various additional genera to provide a better workable system. Braun (1993) also established generic separation of *Cercospora* on diverse criteria including ontogeny, pigmentation, and ornamentation of conidia, conidiophores and conidiomata. Pons and Sutton (1988) described *Distocercospora* N. Pons and B. Sutton for *Cercospora*-like Hyphomycetes with distoseptate scolecospores conidia. On the other hand, Braun (1993) separated *Pseudocercospora*-like species with percurrent proliferating conidiogenous cells and *Mycosphaerella* teleomorphs from *Stigmina*, and published the new genus *Cercostigmina* U. Braun. Although Deighton (1967) separated *Passalora* and *Cercosporidium* on account of the presence or absence of a substomatal stroma, Braun (1995) redefined *Cercospora, Passalora,* and *Phaeoisariopsis*. Braun discussed the status of these genera and noted that small stromata were also developed in the type species of *Passalora*. Therefore, the degree

of the development of stroma-like hyphal aggregations in the sub stomatal cavities should not be used for generic differentiations with the *Cercospora* and allied genera.

In the recent publication, Crous and Braun (2003) re-examined and represented a compilation of more than 3,000 names that have been published or proposed in *Cercospora*. They separated the cercosporoid genera mainly based on a combination of characters, of which the structure of conidiogenous loci (scars) and hila, and the presence and absence of pigmentation in conidiophores and conidia. Crous and Braun (2003) only recognized 659 *Cercospora* species from more than 3,000 *Cercospora* names that published by several earlier authors. Crous and Braun (2003) retreated and reexamined 5,720 names that related to the *Cercospora* and allied genera and proposed 455 taxonomic novelties within 10 genera including *Cercospora, Dactylaria* Sacc., *Fusicladium, Mycosphaerella, Passalora, Scolecostigmina* U. Braun, *Semipseudocercospora* J. M. Yen, *Sirosporium, Sporidesmium* Link, and *Stenella*.

In Thailand, numerous species were reported by several researchers such as Sontirat *et al.* (1980) who enumerated 21 species of *Cercospora* in Thailand, Giatgong (1980) listed 47 identified and 13 unidentified species of *Cercospora* in *The Host Index of Plant Diseases in Thailand*. Petcharat and Kanjanamaneesathian (1989) reported 49 species of *Cercospora* on infected plants in Thailand. However, their reports were mainly based on the generic concepts introduced by Chupp (1954) who using the characteristics of conidia, conidiophores, stromata and symptoms on the host plants. Therefore, these reports must be reclassified and validated refer to the Deighton's system (1959, 1967, 1971, 1973, 1974, 1976, 1979, and 1983) and Crous and Braun (2003) as an acceptable concept that is used by most workers in the recent

years. In addition, further reports of new species, new records and additions of the cercosporoid fungi in Thailand were also published by Ellis (1976), Manoch *et al.* (1986), Pons and Sutton (1988), Barreto and Evans (1994), Crous (1998), Crous and Braun (2003), Lumyong *et al.* (2003), Braun *et al.* (2006) and Hunter *et al.* (2006).

A. *Cercospora* Fresen.

Type species: *C. apii*

Teleomorph: *Mycosphaerella*

The species of *Cercospora* are usually phytoparasitic and cause leaf spots (figure 5A). Mycelium internal; hyphae septate, branched, almost colorless to pigmented. Secondary mycelium absent. Stromata absent to well developed. Conidiophores solitary to fasciculate, emerging through stomata or erumpent through the cuticle, straight to curved, geniculate to geniculate sinuous, simple or occasionally branched, continuous to septate, pigmented, rarely hyaline or subhyaline (figure 5B). Conidiogenous cells intercalary, terminal or conidiophores reduced to a single conidiogenous cell, polyblastic, sympodial. Conidiogenous cells scars conspicuous, thickened, and darkened (figure 5B). Conidia solitary, scolecosporous, acicular, cylindric-fusiform, slightly obclavate, transversely euseptate, usually pluriseptate, hyaline, occasionally subhyaline; hilum conspicuously thickened and darkened (figure 5C).

Notes: Braun (1995) discussed that *Cercospora* comprise *Cercosporella*-like fungi with scolecosporous conidia, pigmented conidiophores, and conspicuously thickened, darkened conidial scars. Some *Cercospora* species which have hyaline or subhyaline conidiophores may be confused with *Cercosporella* spp. Therefore, Braun

(1995) introduced *Cercospora* subgenus *Hylocercospora* to accommodate this unusually species. Colorless *Cercospora* spp. possesses thickened, darkened conidial scars fully agreeing with typical *Cercospora* scars.

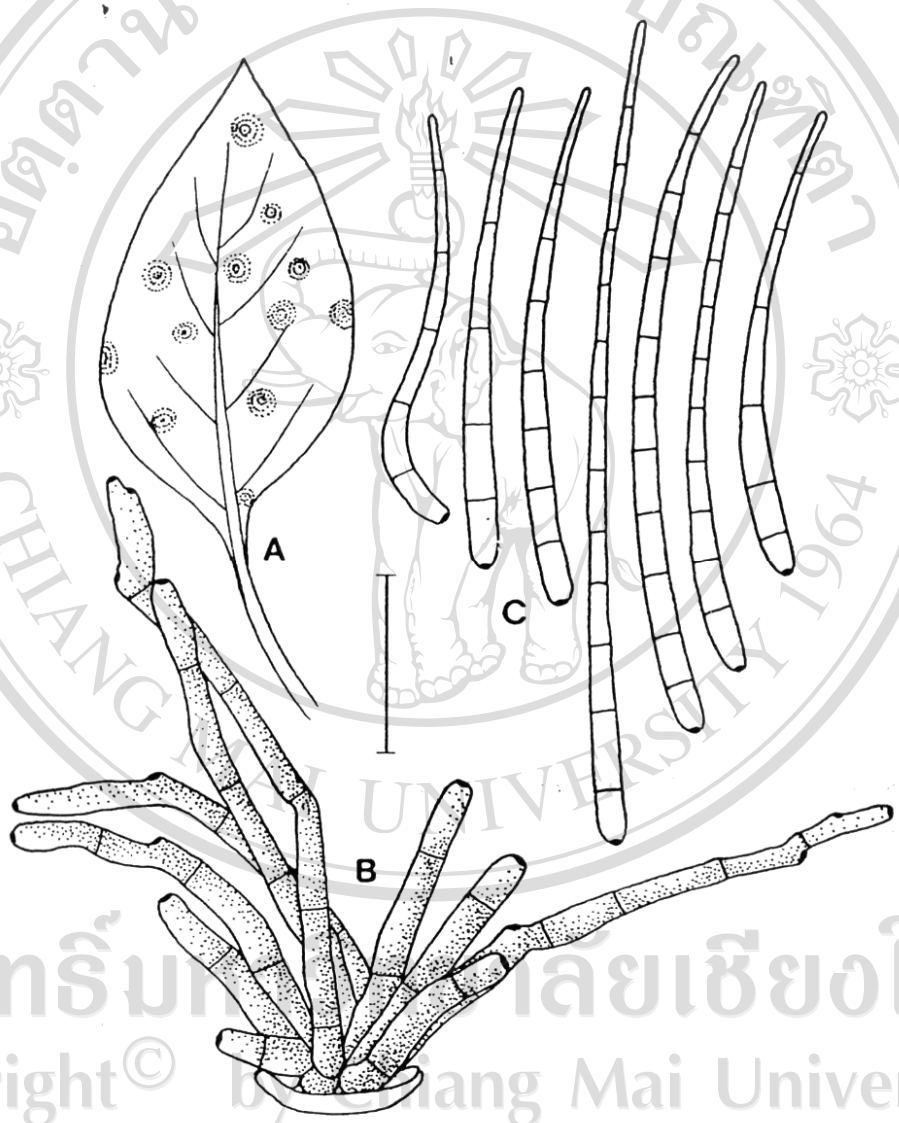

Figure 5 *Cercospora capsici* Heald and F. A. Wolf on *Capsicum annuum* L. **A**. Symptom. **B**. Conidiophores. **C**. Conidia. (scale bar = 30 µm) (Shin and Kim, 2001).

B. *Passalora* Fr.

Type species: *P. baccilligera* Fr. and Mont.

Teleomorph: *Mycosphaerella*

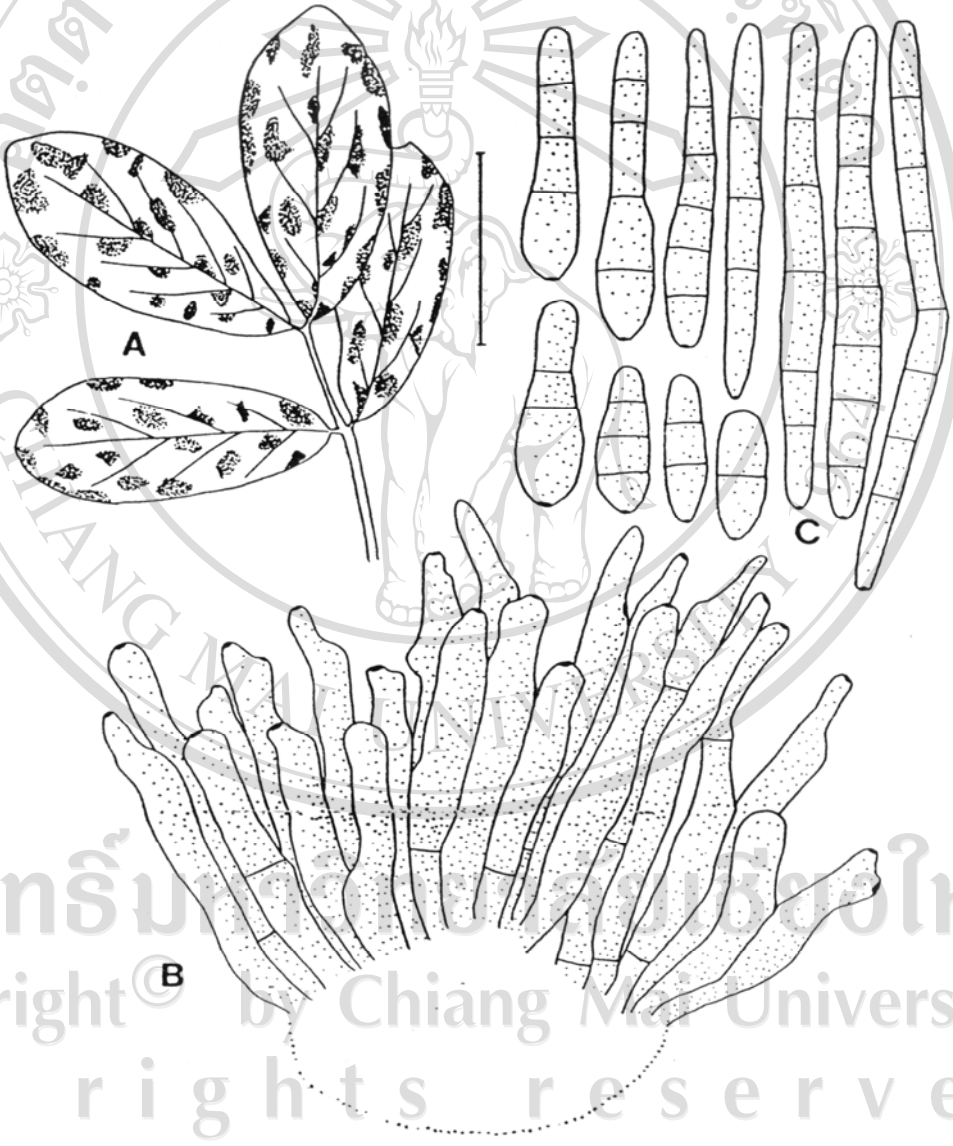

Figure 6 *Passalora personata* (Berk. and M. A. Curtis) S. A. Khan and M. Kamal on *Arachis hypogaea* L. **A**. Symptom. **B**. Conidiophores and **C**. Conidia. (scale bar = 30 μm) (Shin and Kim, 2001).

Phytopathogenic and mostly causing leaf spots, sometimes almost indistinct (figure 6A). Caespituli mostly amphigenous, conspicuous, punctiform to effuse. Mycelium internal. Hyphae septate, branched, hyaline to pigmented. Conidiophores arranged in loose to dense fascicles, sometimes in almost sporodochial fascicles, emerging through stomata or erumpent through the cuticle, straight to curved, subhyaline to pigmented, geniculate to geniculate-sinuous, pluriseptate (figure 6B). Conidiogenous cells integrated, terminal or conidiophores reduced to a single conidiogenous cell, polyblastic, sympodial. Conidiogenous cells scars conspicuous, slightly thickened, somewhat darkened (figure 6B). Conidia solitary, ellipsoid-ovoid, obclavate, broadly subcylindric to fusiform, mostly broad, pigmented, pluriseptate; hilum slightly thickened, somewhat darkened (figure 6C).

Notes: Deighton (1967) separated *Passalora* and *Cercosporidium* on account of the presence or absence of substomatal stromata. However, Arx (1983) discussed the status of these genera and explained that small stromata are also developed in the type species of *Passalora.* They are very small, not very conspicuous. Therefore, Arx (1983) reduced *Cercosporidium* to synonymy with *Passalora*. The degree of the development of stromata-like hyphal aggregations in the substomatal cavities should not be used for generic differentiations within the cercosporoid fungi. Hence, Deighton (1990) and Braun (1995) agreed with Arx (1983), preferred to merge *Passalora* with *Cercosporidium*.

C. *Pseudocercospora* Speg.

Type species: *P. vitis* (Lév.) Speg.

Teleomorph: *Mycosphaerella*

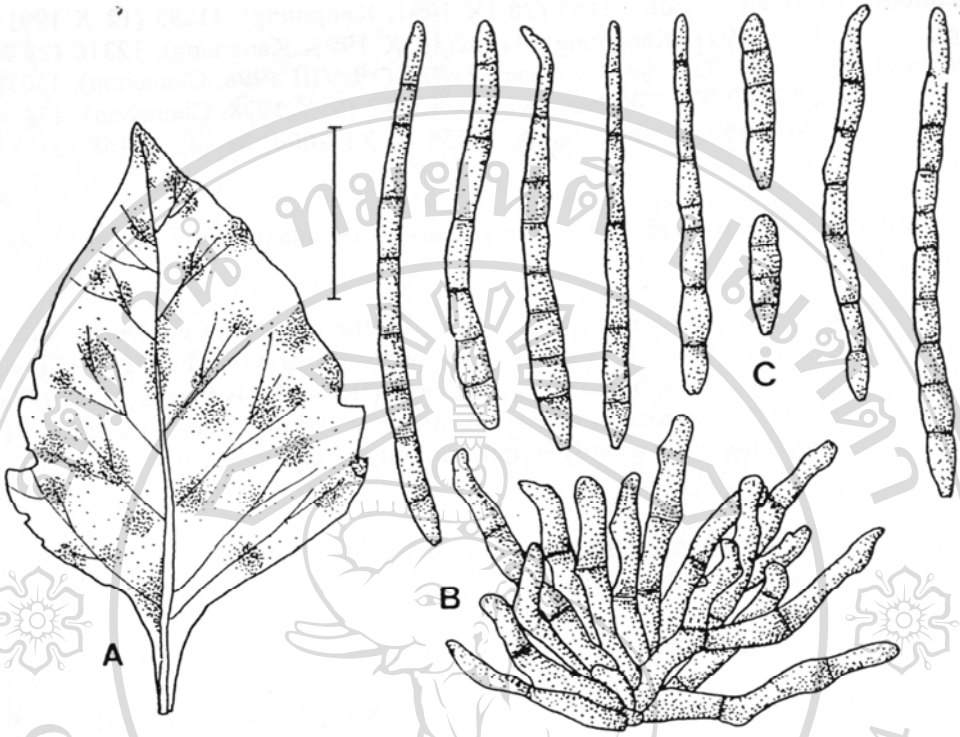

Figure 7 *Pseudocercospora atromarginalis* (G. F. Atk.) Deighton on *Solanum nigrum* L. **A**. Symptom. **B**. Conidiophores. **C**. Conidia. (scale bar = 30 μm) (Shin and Kim, 2001).

Phytopathogenic, mostly causing leaf spots (figure 7A). Mycelium internal, as well as external, repent, sometimes climbing leaf hairs or forming ropes. Stromata absent to well-developed. Conidiophores solitary, arranged in loose to dense fascicles, sometimes synnematous or arising from superficial hyphae, lateral or terminal, aseptate to pluriseptate, pigmented (figure 7B). Conidiogenous cells integrated, terminal or conidiophores reduced conidiogenous cells, polyblastic, sympodial, geniculate to sinuous. Conidiogenous cells scars inconspicuous (figure 7B). Conidia solitary, very rarely in short chains, obclavate-cylindric, subcylindric, filiform,

acicular-filiform, straight to curved, hyaline to subhyaline, one to pluriseptate, smooth, hilum unthickened and not darkened (figure 7C).

Notes: *Pseudocercospora* was introduced by Spegazzini (1910). Deighton (1976) re-introduced the concept of this forgotten genus considerably to include a diameter range of cercosporoid with inconspicuous scars. Deighton (1976) reduced *Helicomina* L. S. Olive, *Ancylospora* Sawada, and *Cercocladospora* G. P. Agarwal and S. M. Singh to synonymy with *Pseudocercospora*. Deighton (1976) distinguished *Cercoseptoria* Petr. from *Pseudocercospora* by having narrow, acicular conidia, but both genera cannot be properly differentiated (Deighton, 1987; Braun, 1988b). Arx (1983) merged *Cercoseptoria*, which is characterized by having pigmented conidiophores, with the colorless genera *Pseudocercosporella* and *Thedgonia* B. Sutton. Presently *Cercoseptoria* is accepted as a synonym of *Pseudocercospora* (Hsieh and Goh, 1990; Guo and Hsieh, 1995; Crous and Braun, 1996; Braun and Melnik, 1997), which is also supported by the molecular data reported by Crous *et al.* (2000).

D. *Stenella* Syd.

Type species: *S. araguata* Syd.

Teleomorph: *Mycosphaerella*

Species of this genus usually plant pathogenic, often symptomless or causing leaf lesions. Primary mycelium internal, secondary mycelium external, superficial, always present, hyphae branched, septate, hyaline to pigmented, verruculose (figure 8). Stromata lacking to well-developed. Conidiophores solitary, arising from superficial hyphae, lateral or terminal, or fasciculate, arising from internal hyphae or

stromata, erect, aseptate to pluriseptate, pigmented, very pale olivaceous to medium dark brown, smooth to verruculose, wall thin to somewhat thickened (figure 8). Conidiogenous cells integrated, terminal to intercalary or conidiophores reduced to conidiogenous cells. Conidiogenous loci conspicuous, somewhat thickened and darkened, pileate to planate (figure 8). Conidia solitary or catenate, scolecosporous to filiform, sometimes obclavate, euseptate, aseptate to pluriseptate, colorless to pigmented, usually verruculose, thin-walled, hilum slightly thickened and darkened (figure 8).

Notes: *Stenella* was introduced by Sydow (1930) and recognized again by Ellis (1971, 1976) who reduced *Biharia thirum* (Thirumalachar and Mishra, 1953) to synonymy with this genus. Deighton (1979) followed this concept of *Stenella* and differentiated it from *Mycovellosiella* based on the formation of verruculose superficial hyphae, and usually rough walled with catenate conidia. However, many species with conidia formed singly have been assigned to *Stenella*, and therefore, the verruculose creeping hyphae remain the only reliable basis for the differentiation of the two genera. According to David (1997), the scars of *Stenella* are pileate and differ from the planate *Cercospora*-type scars. Based on the molecular phylogenetic data reported by Crous *et al*. (2000, 2001), *S. araguata* clusters separately from other species of *Stenella,* suggesting that the genus is polyphyletic within *Mycosphaerella* anamorphs. Further molecular studies have indicated, however, that *Stenella* should be retained as a separate genus from *Passalora* (Pretorius *et al.*, 2003; Taylor *et al.*, 2003). Therefore, Crous and Braun (2003) have included this genus in the true cercosporoid fungi alongside *Cercospora*, *Passalora*, and *Pseudocercospora*.

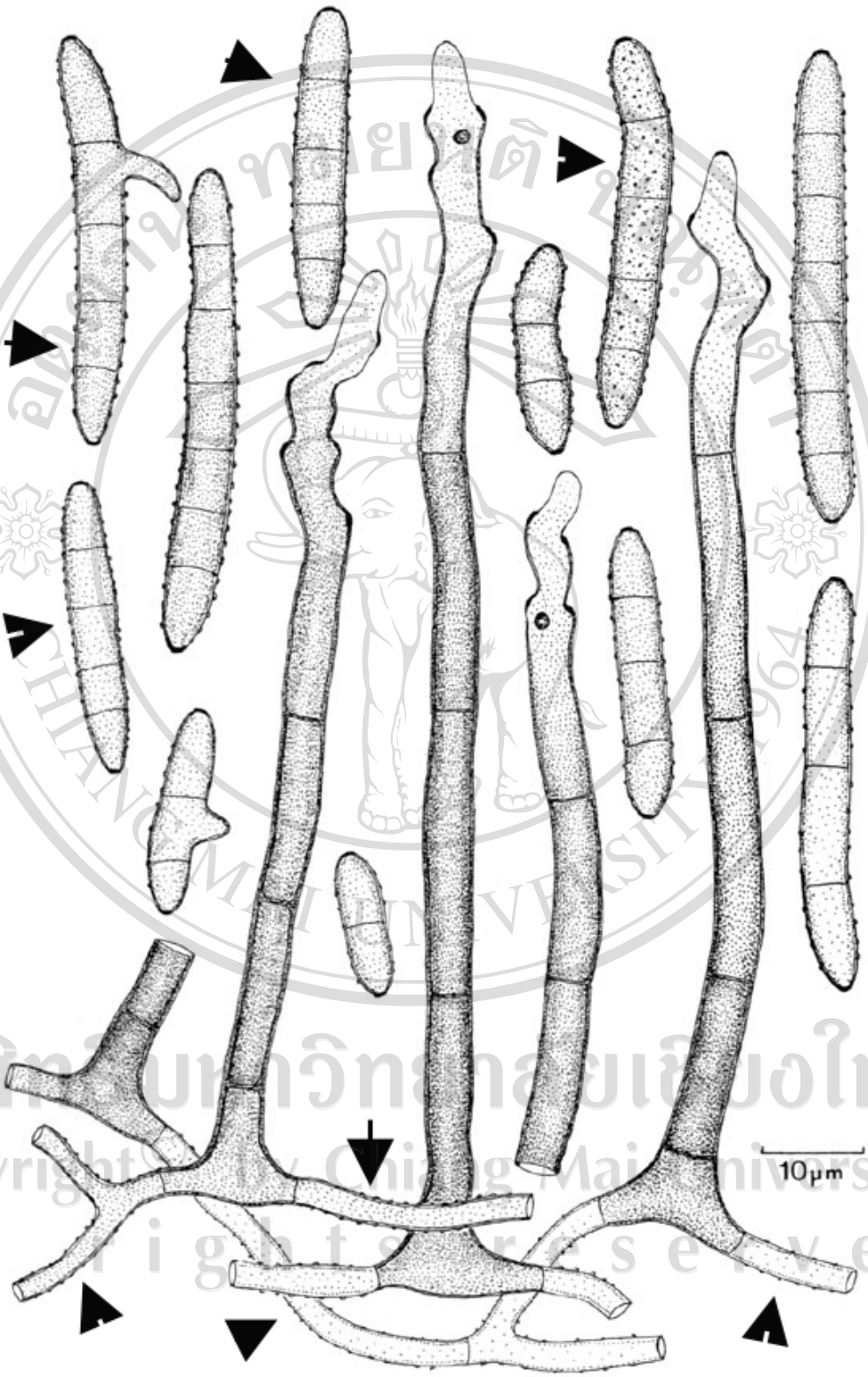

Figure 8 *Stenella osyridina* R. K. Chaudhary and S. K. Singh on *Osyris wightiana* Wall. (verruculose conidia and secondary mycelium showed by black arrows) (Chaudhary *et* al., 1996).

1.2.3. Phylogeny and Evolution of Cercosporoid Fungi

Every living organism contains DNA, RNA, and proteins. Closely related organisms generally have a high degree of agreement in the molecular structure of these substances, while the molecules of organisms distantly related usually show a pattern of dissimilarity. With the advent of molecular technique, particularly since the finding of fungal ribosomal RNA genes amplification and direct sequencing technique by White *et al.* (1990), nucleotide sequences sampled from genome have been commonly employed in recent years by systematists to investigate the phylogeny of various groups of fungi, and consequently, the progress in molecular phylogenetic of Kingdom Fungi has been accelerated rapidly.

In the cercosporoid fungi, until present time, only a few molecular phylogenetic analyses have been published worldwide. One of the first significant phylogenetic analyses was arguably published by Stewart *et al.* (1999) who reported the monophyletic of *Cercospora*, *Passalora,* and *Pseudocercospora* based on ITS region of partial rDNA sequence analysis, and reaffirmed that *Ramulispora* Miura and *Mycocentrospora* Deighton are not related to *Mycosphaerella* teleomorph. Stewart *et al.* (1999) also reduced *Paracercospora* Deighton as a synonym of *Pseudocercospora*. However, because of limited taxa and no other species with *Mycosphaerella* teleomorph were included in the analysis, it was not possible to determine the phylogenetic relationship of the cercosporoid species to other anamorphs genera.

Similar to the cercosporoid fungi, the taxonomy and phylogenetic of *Mycosphaerella* teleomorph is also complicated (von Arx, 1983; Crous *et al.*, 2000).

Due to the large number of associated anamorphs, Crous and Wingfield (1996) noted that *Mycosphaerella* was a polyphyletic assemblage of presumably monophyletic anamorph genera. Goodwin *et al*. (2001), based on the analysis of a large number of anamorphs of *Mycosphaerella* using ITS region of rDNA sequence, also found that the genus *Mycosphaerella* was not monophyletic. The interesting results from Goodwin *et al*. (2001) are, *Cercospora s. str.* formed a highly supported monophyletic group, and the *Cercospora* species produced the toxin cercosporin were suggested to have a single evolutionary origin. Crous *et al*. (2007), based on the analysis of Large Sub Unit (LSU) region of ribosomal DNA (28SrDNA), reaffirmed that *Mycosphaerella* was polyphyletic. Crous *et al*. (2007) also generated *Teratosphaeriaceae* Crous and U. Braun as a new family in the Order *Capnodiales* to accommodate many extreme-tolerant species.

Although the *Mycosphaerella* complex encompasses thousands of names, it may appear strange that it is only now that more clarity is obtained regarding the phylogenetic relationships among taxa in this group. This is partly due to the fact that these organisms are cultivated with difficulty, and also that the first to address the taxonomy of this complex based on DNA sequence data was only relatively recently published (Stewart *et al*., 1999; Crous *et al*., 2007). However, significant results have still been successfully produced from the relatively limited publications in the *Mycosphaerella* complex, such as the synonymous of *Paracercospora*, *Phaeoisariopsis*, *Stigmina*, and *Cercostigmina* to *Pseudocercospora*, *Mycovellosiella* and *Phaeoramularia* to *Passalora*. Furthermore, in relation to the morphological structure of the cercosporoid fungi, one of the important achievement from molecular phylogenetic in the cercosporoid fungi, that is, conidiomatal structure has not

significant contribution to the phylogenetic tree related from the analysis (Crous and Braun, 2003; Verkley and Starink-Willemse, 2004). Therefore, the separation of Coelomycetes genera with acervuli and Hyphomycetes with sporodochia in anamorphs of *Mycosphaerella* complex is but one aspect that needs further study via molecular systematics. Crous and Braun (2003) also concluded that conidial catenulation, septation, and proliferation of conidiogenous cells were less importance in separating species at generic level, and the morphological characters, viz, pigmentation (*Cercospora* vs. *Passalora*), scar structure (*Passalora* vs. *Pseudocercospora*), and verruculose superficial hyphae (*Stenella* vs. *Passalora*), are significant with molecular phylogenetic analysis at generic level. In general, all these information have showed that, in some cases, generic concepts of anamorphs based on morphology and conidium ontogeny, particularly in the cercosporoid fungi, conform well with phylogenetic relationships, although this is not true in all cases due to convergence evolution (Crous *et al*., 2007).

1.2.4. Role of Cercosporin in Cercosporoid Fungi Pathogenesis of Host Plants

A toxin is a poisonous substance produced by living cells or organisms that is active at very low concentrations (Daub and Ehrenshaft, 2000). Toxins can be small molecules, peptides, or proteins and are capable of causing disease on contact with or absorption by body tissues by interacting with biological macromolecules such as enzymes or cellular receptors. Toxins vary greatly in their severity, ranging from usually minor and acute to almost immediately deadly. Of those, one widespread but somewhat neglected group of nonspecific toxins is the photosensitizing perylenequinones synthesized by phytopathogens in at least eight different genera of

fungi including the *Cercospora* (Daub and Ehrenshaft, 2000). These toxins are unique because they require light for toxicity to their host plants and use activated oxygen species to damage cells. Perylenequinones belong to a chemically diverse and large group of both natural and synthesized compounds known as photosensitizers. Photosensitizers absorb light energy and are converted to an energetically activated state, which then reacts with molecular oxygen to form both radical and nonradical species of activated oxygen. Activated oxygen species have near-universal toxicity, as they target macromolecules common to all cells such as lipids, proteins, and nucleic acids (Daub and Ehrenshaft, 2000). This generalized toxicity, coupled with the ability of these fungi to harvest energy from light, an energy source absolutely required by plants, makes these toxins a potent pathogenesis mechanism that poses significant problems for plants (Daub and Ehrenshaft, 2000).

The toxin cercosporin, one of the perylenequinones produced by members of the highly successful genus of phytopathogens, *Cercospora*, was first isolated in 1957 from mycelial cultures of *C. kikuchii* (Tak. Matsumoto and Tomoy.) M. W. Gardner as an interesting pigment (Kuyama and Tamura, 1957). Production of cercosporin *in vitro* is strongly influenced by medium composition, temperature, and light, and that optimal conditions are highly isolate-specific (Jenns *et al*., 1989). There are a number of compelling rationales for these investigations. From the phytopathologist's point of view, cercosporin plays a consequential role in plant-pathogen interactions. Phytopathogenic species in the genus *Cercospora* are pervasive and economically detrimental to their hosts, which include some of the world's most valuable crops. Numerous lines of evidence, both direct and correlational, lend credence to the observation that a significant portion of the success of this group of pathogens can be

attributed to their synthesis of cercosporin. Beyond cercosporin's involvement in plant disease, however, another strong inducement for studies of cercosporin is its near-universal toxicity and the lack of knowledge about cellular resistance to photosensitizing compounds (Daub and Ehrenshaft, 2000). The preponderance of cells and organisms tested are sensitive to cercosporin. In fact, cercosporin is perhaps the very epitome of a non-host–specific toxin because it is lethal not only to plants, but also to bacteria, most fungi, and animals (Daub and Ehrenshaft, 2000). The only organisms that show high levels of resistance to cercosporin are *Cercospora* species and other fungi that produce perylenequinone toxins (figure 9), such as *Hypocrella bambusae*, *Cladosporium* spp., and *Elsinoe* spp. Although levels as low as 1µM cercosporin kill plant cells, cultures of *Cercospora* species produce mM levels of cercosporin in culture are unaffected.

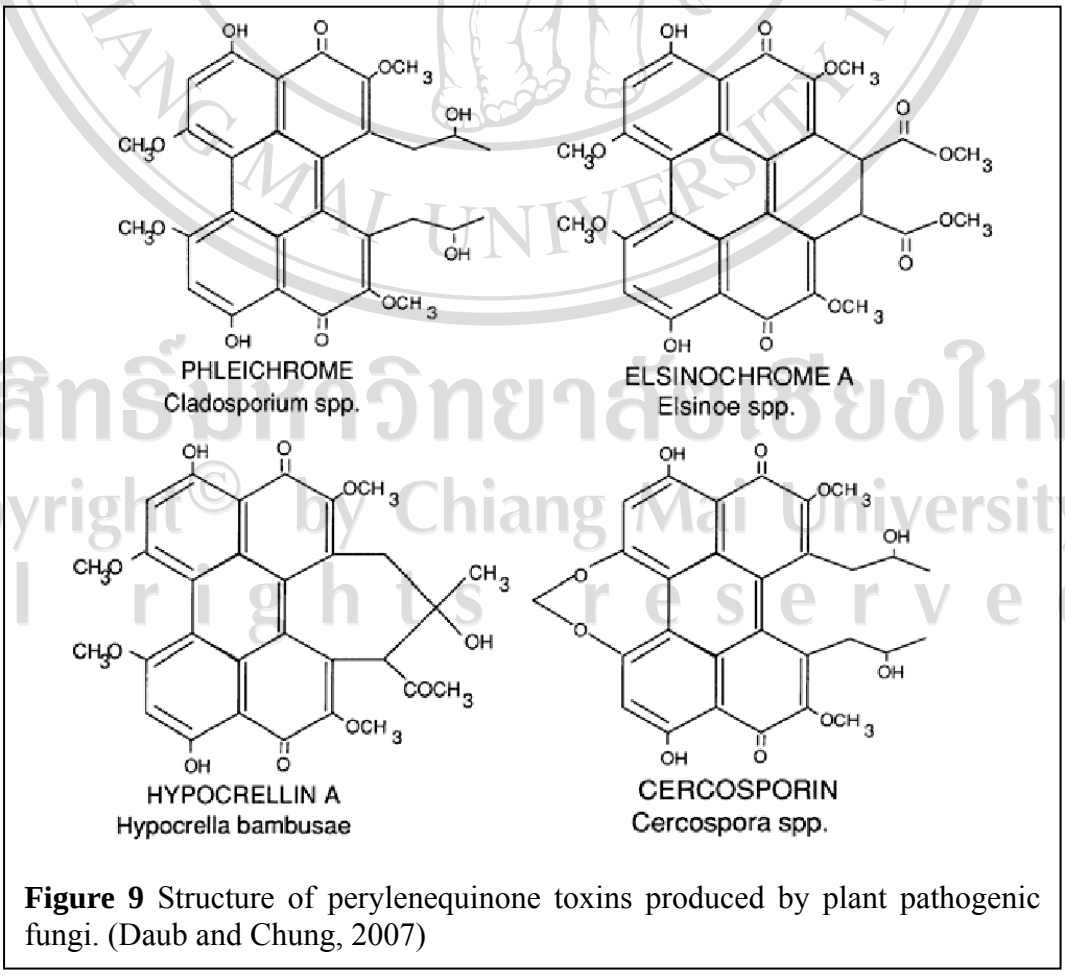

Figure 9 Structure of perylenequinone toxins produced by plant pathogenic fungi. (Daub and Chung, 2007)

The biosynthesis pathway of fungal toxins is an area of current major interest, and compared to the progress made on biosynthesis of other toxins, including polyketides such as aflatoxin and T-toxin, the pathway for cercosporin biosynthesis is poorly understood (Daub and Chung, 2007). Cercosporin offers a significant advantage over most plant pathogenic toxins, however, as it is red in color, allowing for easy visual identification.

Progress on defining the pathway of cercosporin biosynthesis has been limited. Early labeling experiments by Okubo *et al*. (1975) indicated that cercosporin is produced *via* a polyketide mode of synthesis, i.e. via the condensation of acetate and malonate subunits. Okubo *et al*. (1975) also suggested that cercosporin is formed from a condensation of two identical polyketomethylene chains to form a molecule with bilateral symmetry (figure 10). No pathway intermediates, however, have ever been isolated, and only very recently has progress been made on the isolation of synthesis mutants and genes involved in synthesis. More detail is known about the physiology of cercosporin synthesis, which appears to be regulated in a complex and hierarchical fashion, with light being the primary signal for initiation of biosynthesis. Cercosporin biosynthesis is affected by nutrient conditions, temperature, and light (Daub and Chung, 2007). Generally, cercosporin accumulation is enhanced if cultures are grown at temperatures slightly below that required for optimum growth (Jenns *et al*., 1989). Medium components and carbon:nitrogen (C:N) ratios also play a role, often with the same conditions enhancing production in one isolate while having a negative or no effect on another (Jenns *et al*., 1989).

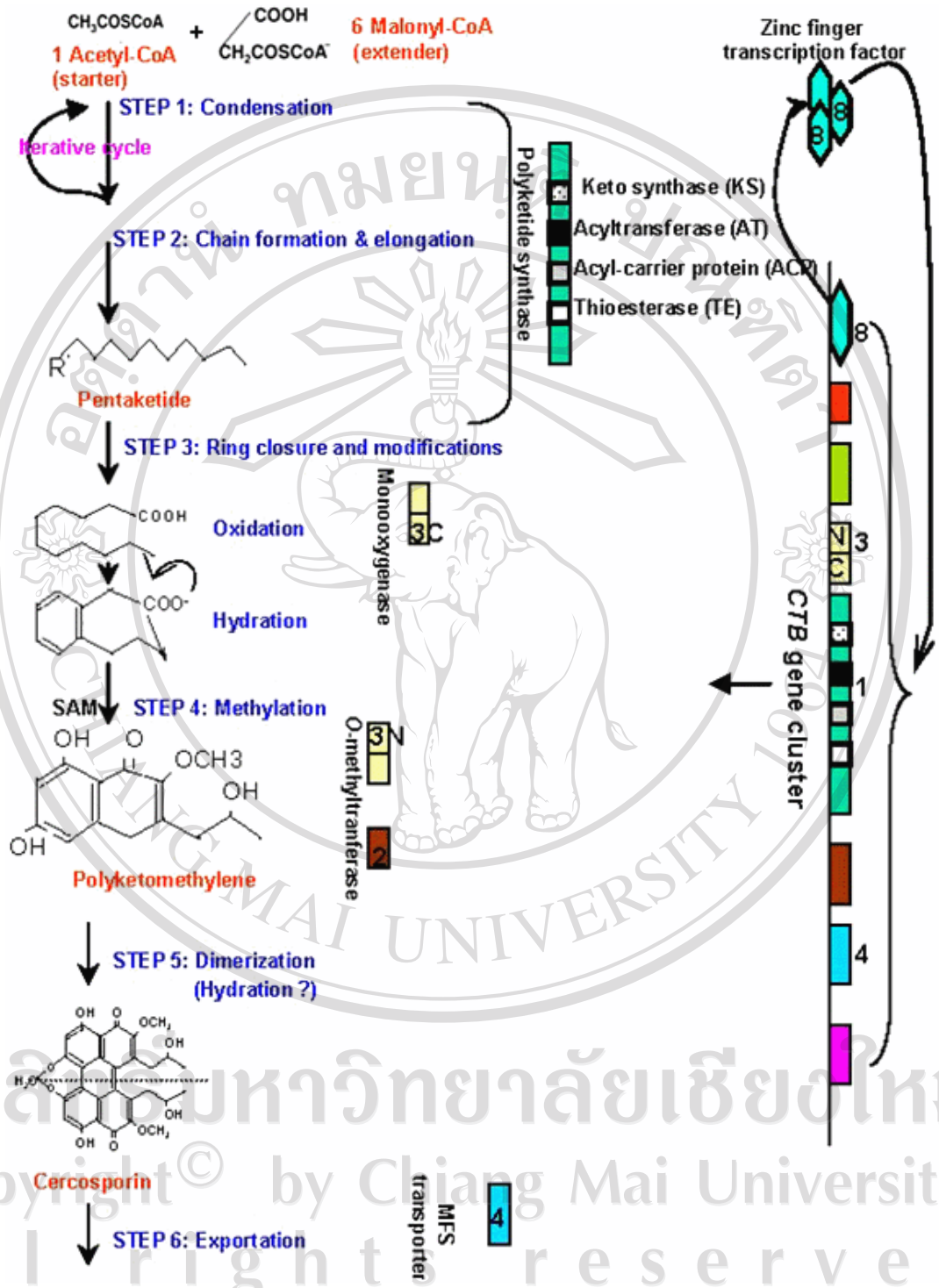

Figure 10 Schematic illustration of a proposed cercosporin biosynthetic pathway. The putative functions of the *CTB* gene products in relation to cercosporin production are indicated (Daub and Chung, 2007).

Cercosporin is unique among the well-characterized fungal toxins, as it is classified as a photosensitizer (Daub, 1982). The term photosensitizer defines a large group of structurally diverse compounds that are activated by visible wavelengths of light and generate activated oxygen species toxic to living cells (Spikes, 1989), a process often referred to as photodynamic action. The potent and broad-spectrum toxicity of photosensitizers is due to their production of activated oxygen species, which occurs when the photosensitizer molecule is converted, through absorption of light energy, to a long-lived, electronically excited triplet state (figure 11).

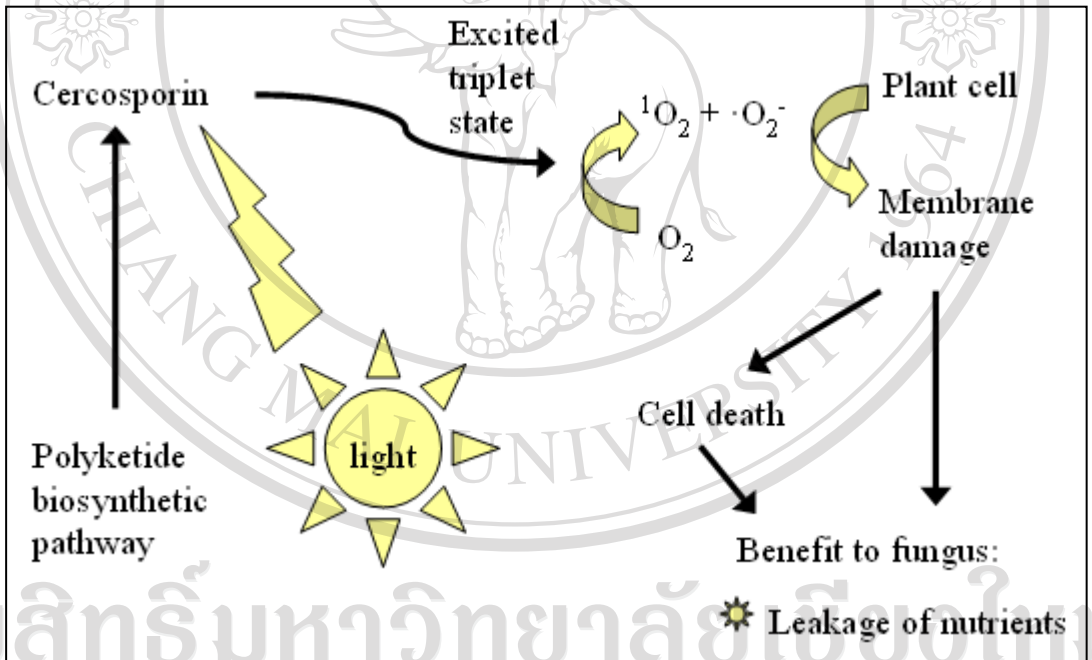

Figure 11 The mode of action of cercosporin. Cercosporin is activated by light and, in the activated state, reacts with oxygen to produce toxic oxygen species such as singlet oxygen ($^1O_2$) and superoxide (O_2^-). Production of reactive oxygen species leads to peroxidation of lipids in the plant cell membranes. It is hypothesized that this membrane damage allows for leakage into the intercellular spaces of nutrients that are used by the fungus to colonize the leaf (Daub and Chung, 2007).

The cercosporin's photodynamic properties were clearly described by Yamazaki *et al*. (1975). Cercosporin kills plant cells only in the light, with a linear relationship between light intensity and cell death (Daub, 1982). The *Cercospora* species are a highly successful group of plant pathogens, causing leaf spot and blight diseases on a diversity of crop species worldwide. Among these diseases are cercospora leaf spot of sugar beet (*Beta vulgaris* L.) caused by *C. beticola* Sacc., gray leaf spot of corn (*C. zeae-maydis* Tehon and E. Y. Daniels), purple seed stain of soybean (*C. kikuchii*), frogeye leaf spot of tobacco (*C. nicotianae* Ellis and Everh.), and brown eye spot of coffee (*C. coffeicola* Berk. and M. A. Curtis). One reason for the success of these pathogens is likely due to their production of cercosporin. Several lines of evidence support a critical role for cercosporin in *Cercospora* pathogenesis. The most compelling, and perhaps most significant, evidence for a role of cercosporin and cercosporin-like toxins in disease comes from studies on the effect of light on infection of plants by the *Cercospora* species and other perylenequinone producing fungi. Studies of the cercospora diseases of coffee, sugar beet, and banana have correlated disease severity with light exposure. Symptoms caused by *C. coffeicola* on coffee were less severe when plants were grown close together. Analysis of the effects of shading revealed that fungal penetration of plant's stomata was reduced and that fewer lesions developed on shaded leaves (Daub and Ehrenshaft, 2000).

1.2.5. Fungicides Resistance in Cercosporoid Fungi

Due to the destructive effect of the cercospora disease on some economically important crops such as cereal, maize, banana, and sugar beet, several attempts such as combination of tillage, rotation, disease resistance, and fungicide sprays (Franc *et*

al., 2001) have been carried out in order to control or limit the damage resulted from the cercospora disease worldwide. Despite the many achievements of modern agriculture, including the use of resistance cultivars of crops, plant disease control has now become heavily dependent on fungicides particularly a group of mitosis-inhibiting fungicides such as benzimidazole, benomyl, thiabendazole, etc., to combat a wide variety of fungal diseases that threaten agricultural crops (De Waard *et al*., 1993).

Benzimidazole fungicide, a heterocyclic aromatic organic compound, has been used extensively in plant disease management for approximately 30 years and showed great efficacy in controlling plant pathogenic fungi (Davidse, 1986). However, numerous tolerance cases of the cercosporoid fungi species, particularly *C. beticola* on sugar beet (*B. vulgaris*), *P. fijiensis* (M. Morelet) Deighton (tel. *M. fijiensis* M. Morelet) on banana (*Musa paradisiaca* L.), *C. zeae-maydis* on Corn (*Z. mays*), have been arised and reported as consequences of the fungicides long term application by the farmers (Butters and Holloman, 1999; Cañas-Gutiérrez *et al.*, 2006; Davidson *et al*., 2006). The mechanism of tolerance to benzimidazole fungicides has also been examined in a number of different filamentous fungi such as *Colletotrichum* spp. (Buhr and Dickman, 1994; Nakaune and Nakano, 2007), *Venturia inaequalis* (Cooke) G. Winter (Koenraadt *et al.*, 1992), etc. Benzimidazoles act primarily by binding to fungal tubulin and interfering with mitosis and the fungal cytoskeleton (Davidse, 1986). Most often benzimidazole tolerance is due to mutations in the β-tubulin gene which reduce benzimidazole binding (Reijo *et al.*, 1994). This loss of binding affinity has been associated with one or several single nucleotide polymorphisms (SNPs) in the β-tubulin gene that cause changes in several amino acids probably located at the

fungicides's binding site (Koenraadt *et al.*, 1992). The most common SNPs described to be associated with benzimidazole resistance are located at codons 50 (McKay *et al.*, 1998), 198 and 200 (Koenraadt *et al.*, 1992), and 240 (Albertini *et al.*, 1999) of the β-tubulin gene. These mutations can be used to rapidly identify tolerant strains with nucleic acid-based methods (Luck and Gillings, 1995). Some mutations conferring benzimidazole tolerance also confer sensitivity to N-phenylcarbamates (Koenraadt and Jones, 1993). This sensitivity has been used in some areas to manage benzimidazole-tolerant fungal isolates (Elad *et al.*, 1995), however, use has been limited because combined resistance to both benzimidazoles and N-phenylcarbamates has also been found (Elad *et al.*, 1992; Josepovits *et al.*, 1992).

Although the reportes of the cercosporoid fungi resistance to systemic fungicides in Thailand have never been recorded until now, however, the possibility of cases of cercosporoid resistances occur is arguably high, due to various systemic fungicides have been widely used by the farmers in this country for a long time, and they also usually increase the dosage of the fungicides over the recommended concentration whenever they find the standard dosage of fungicides could not control the diseases. This simple practical approach will possibly generates the fungicide resistance in the cercosporoid fungi and also causes significant negative effects to the environments (e.g. pollutes environment, reduces biodiversity and soil quality), farmers (health effects), and consumers (health effects).

1.3. Aims of the Study

In order to have a better understanding regarding diversity, distribution on various hosts (from crops to ornamental plants), phylogenetic relationship, and ecology of the true cercosporoid fungi and other similar taxa in Thailand, the following four main objectives are designed in this thesis:

1. To assess the diversity and host range distribution of the true cercosporoid fungi in northern parts of Thailand.
2. To provide a comprehensive database of the true cercosporoid fungi in Thailand.
3. To analyse the evolution of the true cercosporoid fungi and its associations with hosts.
4. To provide a literature guide for the identification of the true cercosporoid fungi in Thailand.

1.4. Outline of the Thesis

This thesis is generally divided into five major chapters in order to address all of the main objectives. In the first chapter, research rationale and objectives that forming the fundamental throughout this thesis are described and elucidated, and the current understanding of the cercosporoid fungi, including important morphological characteristics for identification, current status of classification based on conventional and molecular phylogenetic analysis, factor related to their pathogenesis of the host plants, and also current status on their response to the application of systemic fungicides worldwide are reviewed. Diversity and taxonomic description of taxa of the true cercosporoid fungi found during this research are presented and illustrated in

chapter 2. Molecular phylogenetic analysis based on the ITS region of nuclear ribosomal DNA sequence of several important taxa, and evolution of their association with hosts are analyzed and elucidated in chapter 3. Morphology and phylogenetic study of one new species of cercosporoid fungi causing leaf spot on exotic weed, *Christella parasitica*, are presented in the chapter 4. In the final chapter, chapter 5, a general discussion and conclusions to the study are provided, and the thesis concluded with publications from this study.

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