

***Mycosphaerella* and Other Fungi Associated with Banana Leaf Diseases from Northern Thailand**

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ABSTRACT

*The *Mycosphaerella* species associated with banana leaf disease were initially identified by integrating the morphological characteristics and analysis of DNA sequence data; four taxa including *Mycosphaerella eumusae*, *M. musae*, *Cercospora hayi* and *Pseudocercospora callicarpae* were revealed. *Mycosphaerella eumusae* has previously been reported on banana from Thailand, whereas *M. musae*, *C. hayi* and *Ps. callicarpae* are new records. *Cordana musae* and *Deightoniella torulosa* were also frequently isolated from diseased banana leaf tissues. Other less common taxa include *Alternatia*, *Colletotrichum*, *Curvularia*, *Fusarium*, *Periconia* and *Periconiella* species. The effectiveness of chemical control of *eumusae* leaf spot of banana was determined over one month to establish the effectiveness of different chemical fungicides. Among all the treatments, rotational spraying with difenoconazole and mancozeb at weekly intervals gave the most effective and economical control.*

Key words: Banana disease, Sigatoka, *Mycosphaerella* pathogens

INTRODUCTION

Bananas are among the world's most important agricultural crops grown worldwide in 130 countries, especially in the areas of high precipitation. In 2009, total banana production in the world exceeded to 95.6 million metric tons and this number has continually increased over the years (FAOSTAT, 2009). In Thailand, banana exports comprise Pisang Mas Banana and Hom Thong Banana, with a total of 12,633 and 9,910 tons being produced, respectively (Office of Agricultural Economics, 2010).

More than 250 fungal taxa associated with *Musa* spp. have been reported including endophytes, saprobes and pathogens. Eventually, the pathogens are the most referred (e.g. Farr et al., 1989; Brown et al., 1998; Photita et al., 2002).

Sigatoka, the complex disease, is one of the most serious leaf diseases of banana, although banana wilt caused by *Fusarium* is more destructive (Ploetz et al., 2003). The complex of this disease is responsible for economically important leaf spot disease of banana and an important constraint for worldwide banana production. The Sigatoka disease complex is attributed to species of *Mycosphaerella*, including *M. musicola* (anamorph *Pseudocercospora musae*) which is the pathogen of yellow Sigatoka while *M. fijiensis* (anamorph *P. fijiensis*) which causes black Sigatoka, and *M. eumusae* (anamorph *P. eumusae*) which causes *eumusae* leaf spot disease (Jones, 2003; Crous and Mourichon, 2002). The Sigatoka disease reduces photosynthetic activity of the plant as a consequence of necrotic leaf lesions, resulting in reduced crop yield and fruit quality (Romero and Sutton, 1997). *Mycosphaerella eumusae* was recognized as a new species constituent of the Sigatoka complex of banana from part of South-East Asia, where it co-exists with the other

two species (Crous and Mourichon, 2002; Jones 2003). The identification and distribution of the different *Mycosphaerella* species associated with Sigatoka disease complex of banana is not yet fully understood. This is partly due to lack of knowledge of the species involved and the lack of useful morphological characters to distinguish different species (Arzanlou et al., 2007; Arzanlou, 2008). Identification of these fungi mainly relies on minute morphological differences of the anamorph morphology. These morphological features may not always be visible, as cultures sometimes become sterile soon after sub-culturing, and grow very slowly on synthetic media. In addition, a number of *Mycosphaerella* species known from other host plants, as well as banana, sometimes occur even in a single lesion. This makes it difficult to identify the primary pathogen responsible for the disease.

In recent years, PCR-based techniques have been developed as robust tools for diagnosis and detection of plant pathogenic fungi, and have contributed greatly to plant disease management (Waalwijk et al., 2004; Lievens and Thomma, 2005). DNA sequencing of the ITS nrDNA gene has in the past proven highly effective to distinguish among species of *Mycosphaerella* (Crous et al., 2000, 2006, 2007; Cortinas et al., 2006).

Sigatoka disease control is almost exclusively based on fungicide, dithiocarbamates, benzimidazoles, azoles, and strobilurins fungicides have been used to control the disease (Romero and Sutton, 1997; Marin et al., 2003).

The main objectives of this study were to determine the fungal species associated with Sigatoka leaf spot found in five provinces in northern Thailand (plantation areas) and to evaluate the effectiveness of some fungicides against Sigatoka leaf spot disease.

MATERIALS AND METHODS

Fungal isolation and morphological study

Symptomatic banana leaves were collected at various locations in northern Thailand within Chiang Mai, Chiang Rai, Mae Hong Son, Nan and Phrae provinces. Fungal isolation and morphological study were done following the methods of Cheewangkoon et al. (2008).

DNA isolation, amplification and analyses

Genomic DNA from *Mycosphaerella* and related anamorphs were extracted from mycelia of fungal colonies cultivated on MEA using the UltraClean™ Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, CA, USA). The Primers V9G and LR5 were used to amplify part of the nuclear rDNA operon spanning the 3' end of SSU, ITS1, ITS2 and the 5' end of LSU. PCR conditions, sequence alignment and subsequent phylogenetic analysis followed the methods of Cheewangkoon et al. (2008). Alignment gaps were treated as new character states. Sequences were compared with the sequences available in NCBI's GenBank nucleotide (nr) database using a megablast search and results are discussed in the relevant species notes where applicable. Alignment gaps were treated as new character states.

Chemical control

Four treatments of fungicide combinations were conducted for disease control trials, mancozeb 80%WP alone and three combinations of mancozeb 80%WP with propiconazole 25%EC, carbendazim 50%SC and difenoconazole 25%EC (Table 1). Fungicides treatments were applied at four consecutive foliar spraying with the intervals of 7 days rotationally to a *Musa* AAA (cultivar SK and KU2) plantation. Non-sprayed plants were used as control. Fungicides treated areas demonstrated efficacy equivalent to the control treatments. Assessment of the severity of infection was taken after the chemical application and thereafter severities of infection were evaluated using a scoring system as shown in Figure 1.

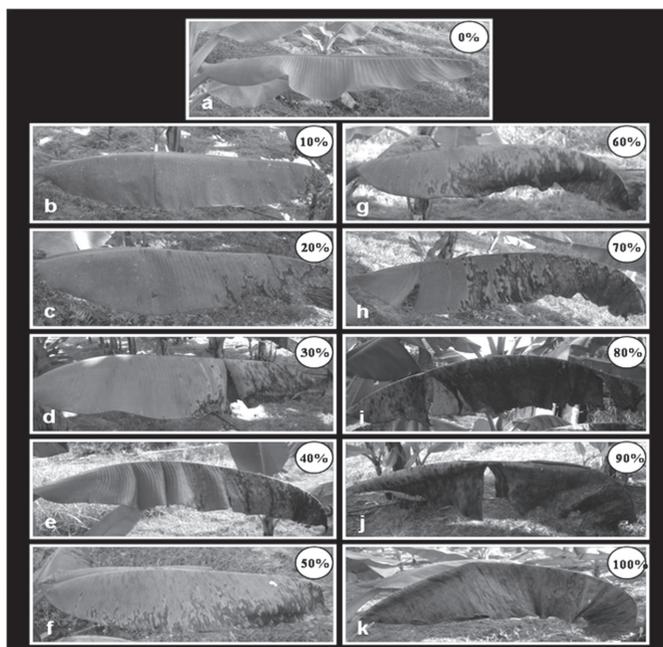


Figure 1. Severity levels of Sigatoka disease; a. 0% of infection, b. 10% of infection, c. 20% of infection, d. 30% of infection, e. 40% of infection, f. 50% of infection, g. 60% of infection, h. 70% of infection, i. 80% of infection, j. 90% of infection, k. 100% of infection.

RESULTS

Banana leaf samples presented several symptoms such as pale brown oval patches, ranging from one to several centimeters in diameter. The spots formed towards the leaf margins and were surrounded by light yellow and light grey. *Cordana musae* and *Deightonella torulosa* were often isolated from this symptom or associated with wounds caused by other fungi and insects. *Mycosphaerella* and cercosporoid fungi were often found on symptoms showing narrow specks, about 1 millimeter in length on the upper surface of the leaf. The speck developed into several millimeters long and expands to become elliptical in shape and became light brown. The blotches darkened and eventually become dark, irregularly-shaped, speckled areas.

Base on morphological characters, eight fungal taxa were isolated (Figure 2) and identified to species level.

Alternaria alternata (Fr.) Keissl., Beih. bot. Zbl., Abt. 2 29: 434 (1912)

Cochliobolus geniculatus R.R. Nelson, Mycologia 56: 778 (1964)

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc., Atti Inst. Veneto Sci. lett., ed Arti, Sér. 3(2): 670 (1884)

Cordana musae (Zimm.) H?hn., Zentbl. Bakt. ParasitKde, Abt. II 2(60): 7 (1923)

Fusarium oxysporum Schltdl., Fl. berol. (Berlin) 2: 139 (1824)

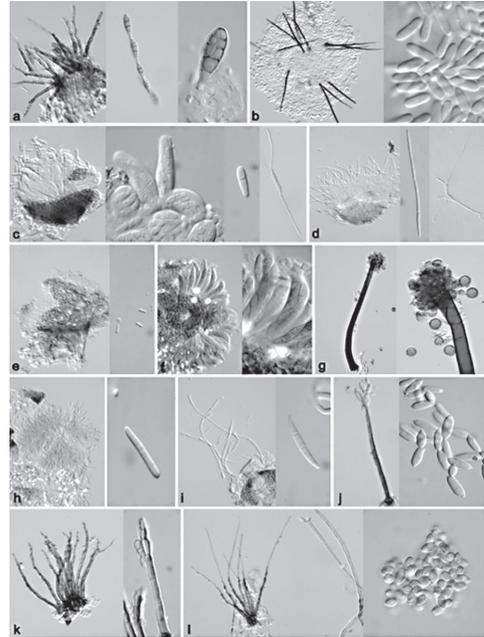
Periconia byssoides Pers., Syn. meth. fung. (Göttingen) 1: 18 (1801)

Periconiella smilacis M.B. Ellis., Mycol. Pap. 111: 31 (1967)

Phaeoseptoria musae Punith., Kew Bull. 31(3): 469 (1977)

Sphaerulina musicola J.M. Yen, Bull. trimest. Soc. mycol. Fr. 89(1): 70 (1973)

Figure 2. The microfungi occurring on *Musa* spp. leaves. a. conidiophores and conidia of *Alternaria alternata*, b. conidia in *Colletotrichum gloeosporioides*, c. ascomata, ascus, ascospore and spore germination in *Mycosphaerella eumusae*, d. *Pseudocercospora callicarpae* conidium and spore germination. e. *Mycosphaerella* sp. spermatia, f. *Sphaerulina musicola* ascomata, ascus and ascospore, g. *Periconia byssoides* conidiophores and conidia, h. *Phaeoseptoria musae* conidia, i. *Fusarium* sp. conidiophores and conidia, j. *Periconiella smilacis* conidiophores and conidia, k-l. conidiopores and conidia in *Cladosporium* sp.



Phylogenetic analysis

To resolve the species of *Mycosphaerella* and related cercosporiid fungi infect banana leaves, ITS nrDNA sequence data was obtained. The manually adjusted ITS alignment contained 19 taxa (including the outgroup sequence) and, of the 506 characters used in the phylogenetic analysis, 228 were parsimony-informative, 59 were variable and parsimony-uninformative and 246 were constant. Neighbour-joining analysis using the three substitution models of the sequence data yielded trees with similar topology and bootstrap values; 1000 equally most parsimonious trees were obtained from the heuristic search, one of which is shown in Figure 3 (TL=206, CI=0.883, RI=0.907, RC=0.802, HI=0.117). The phylogenetic tree derived from the ITS region showed that some of the isolates belong to known species (*Cercospora hayi*, *Mycosphaerella eumusae*, *Mycosphaerella musae* and *Pseudocercospora callicarpae*) whereas other two (CPC19006 and CPC19009) appeared to be not closely related to any of the species in GenBank.

Cercospora hayi Calp., Studies on the Sigatoka disease of Bananas and its fungus pathogen, Atkins Garden and Research Laboratory, Cuba: 63 (1955)

Mycosphaerella eumusae Crous & Mour., Sydowia 54(1): 36 (2002)

Mycosphaerella musae (Speg.) Syd. & P. Syd., Annl's Mycol. 15(3/4): 206 (1917)

Pseudocercospora callicarpae (Cooke) Y.L. Guo & W.X. Zhao, Acta Mycol. Sin. 8(2): 118 (1989)

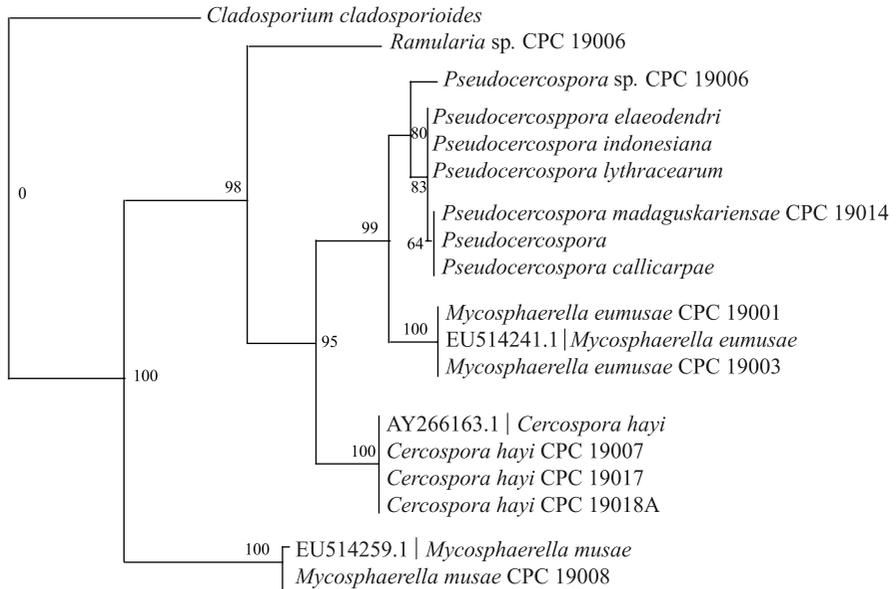


Figure 3. One of eight equally most parsimonious trees obtained from a heuristic search with 100 random taxon of ITS sequence alignment. Bootstrap support values (65% and higher) from 1000 replicates are shown at the nodes. The tree was rooted to sequences of *Cladosporium cladosporioides*.

Fungicide application to control leaf disease

Control treatment of both KU2 and SK varieties showed high percentage of infection were 33.29 and 44.65% respectively which differ from other treatments significantly. Applied mancozeb with propiconazole, carbendazim and difenoconazole (T1, T2 and T3) resulted more effective than applied mancozeb alone in reducing the percentage of infection in both banana varieties (Table 1). However, KU2 responded to the fungicide application than SK.

Table 1. Percentage of infection after chemical application.

Banana variety	Percentage of infection
<i>Musa</i> AAA (KU2)	
T1 propiconazole 25%EC + mancozeb 80%WP	9.46* de
T2 carbendazim 50%SC + mancozeb 80%WP	3.52 e
T3 difenoconazole 25%EC + mancozeb 80%WP	3.56 e
T4 mancozeb 80%WP	26.27 bc
T5 control	33.29 ab
<i>Musa</i> AAA (SK)	
T1 propiconazole 25%EC + mancozeb 80%WP	15.31 cde
T2 carbendazim 50%SC + mancozeb 80%WP	16.57 cd
T3 difenoconazole 25%EC + mancozeb 80%WP	11.69 de
T4 mancozeb 80%WP	21.28 bcd
T5 control	44.65 a
LSD (<i>P</i> = 0.01)	12.55
CV (%)	57.45

*Means within a column followed by the same letter are not significantly different based on the Least Significant Difference (LSD).

DISCUSSION AND CONCLUSION

This study collected and described fungi associated with banana leaf diseases from northern Thailand. Those taxa have been previously reported as endophytes, pathogens and saprobes (Millör, 1980; Kulik, 1984; Andrews et al., 1985; Brown et al., 1998; Photita et al., 2002). Although *Deightoniella torulosa* and *Cordana musae* were isolated from banana leaf lesions in this study, the lesions were quite large but the disease was not severe. It is possible that the banana leaves may contain latent pathogens as an endophytic stage in their life cycles which have often been isolated and described as weak pathogens which become pathogenic when host plant is stressed (Sinclair and Cerkauskas, 1996; Brown et al., 1998; Photita et al. 2002, 2004). *Periconiella smilacis* was isolated only as an endophyte in this study as it occurred on greenish tissue of host plant. This species differs from *P. musae* which was reported by Photita et al. (2003) as a common endophyte on banana leaf conidial size and conidiophores branching. *P. musae* has also been reported as weak pathogen (Ploetz et al., 2003). *Colletotrichum gloeosporioides* is identified to species complex since molecular data is needed to determine the specific species (Hyde et al., 2009; Cai et al., 2009; Phoulivong et al., 2010; Wikee et al., 2011).

Deightoniella torulosa were also reported as saprobe by Photita et al. (2002), Its mode of life was undetermined in this study, Photita et al. (2002) and this study however also indicate that the endophytic stage may be important in the life cycles of some banana pathogens. Photita et al. (2004) suggested that some pathogens have a latent phase within the host tissue and some saprobes can be facultative parasites. It is therefore quite feasible that endophytes, pathogens and saprobes of some plants may be the same species. Alternatively the pathogens may stay alive on dead plant tissue.

The genus *Mycosphaerella* has been linked to approximately 30 anamorph genera. Many of these anamorph genera resulted from a reassessment of cercosporoid forms (Crous and Braun, 2003; Crous et al., 2007). Three major species of *Mycosphaerella*, namely *M. fijiensis* (black Sigatoka disease), *M. musicola* (yellow Sigatoka disease), and *M. eumusae* (eumusae leaf spot disease), are known to cause major economic losses on banana (Jones, 2003; Crous et al., 2002). Besides these three major species, several other *Mycosphaerella* species have been described from banana, most of which are not known from culture. Traditionally, *Mycosphaerella* systematics relied mainly upon host plant association and morphological characters of anamorphs and teleomorphs (Crous, 1998; Arzanlou et al., 2007), criteria which were repeatedly shown to be unreliable (Crous et al., 2004; Crous and Groenewald, 2005; Crous et al., 2009). Due to the co-occurrence of species with similar morphologically on the same leaf or even on the same lesion, accurate identification based solely on morphology is almost impossible. This study has demonstrated that morphological characters and molecular techniques are complementary and necessary to uncover the diversity and geographical range of *Mycosphaerella* species occurring on banana. Although three *Mycosphaerella* species have been identified (*Cercospora hayi*, *M. eumusae*, *M. musae* and *Pseudocercospora callicarpae*) there are still two undescribed isolates which are phylogenetically distinct from species in GenBank.

Control of Sigatoka disease mainly has been achieved through the application of chemical compounds (Romero and Sutton, 1997; Ploetz, 2003; Marin et al., 2003). Different generations of fungicides such as dithiocarbamates, benzimidazoles, azoles, and more recently strobilurins, are being used to control the Sigatoka disease in banana plantations (Romero and Sutton, 1997; Marin et al., 2003). A field experiment to control leaf spot (*Mycosphaerella eumusae*) of banana was conducted to find out the effectiveness of different chemical fungicides. Mancozeb reduced less the percentage of severity infection when applied alone in both KU2 and SK cultivar. However, mancozeb in combination with propiconazole, carbendazim or difenoconazole resulted in reducing the percentage of severity infection in both banana cultivars. This result is an indication that application of protectant fungicide plus a systematic fungicide is effective for Sigatoka disease (*M. eumusae*).

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