Diagnostic Methods
for
Black Sigatoka
Mycosphaerella fijiensis

Introduction

The Banana Industry in Australia

In 2005, the wholesale value of the Australian banana industry exceeded $320 million. More than 80% of the 20.4 million, 13kg carton crop is produced in the wet tropics of North Queensland, in the region between Tully and Cooktown. The remaining 20% are grown predominantly in the subtropical regions of the eastern coast from Nambucca Heads, in New South Wales to Nambour in Queensland. There are also small producing regions in the Northern Territory and in Kununurra and Carnarvon in Western Australia.

Approximately 95% of bananas grown in Australia are of the Cavendish subgroup (including the cultivars Grand Nain and Williams) with the remaining 5% comprising mainly the Ladyfinger cultivar but also some Goldfinger, Ducasse, Sucrier, FHIA 18, Red Dacca and Plantains. The Australian market for banana is mostly that of the fresh fruit product. Currently the only other products made from banana fruit are the small-scale production of banana puree, dried banana and banana wine. There is also a specialist manufacturer of handbags from banana fibre.

Biology

Stages of Development

Lifecycle

Most infections of *M. fijiensis* and *M. musicola* begin with spores being deposited on the susceptible cigar leaf of the banana plant. Spores will germinate within 2-3 hours of being deposited on the leaf surface if there is a water film present or if the humidity is very high. The optimal temperature for germination of *M. fijiensis* spores is 27ºC. For *M. musicola* the optimal temperature for germination of conidia is between 25-29ºC and for ascospores it is between 25-26ºC. The germ tube then grows epiphytically for several days (2-3 days for *M. fijiensis* and 4-6 days for *M. musicola*) before penetrating the leaf via stomata in a hydrotropic response through the formation of appressoria or stomatopodia over the stomata (Meredith 1970; Stover 1980).

Once inside the leaf, the infection hypha forms a large substomatal vesicle. Fine hyphae then grow through the mesophyll layers into an air chamber and then into the palisade tissue. From here the hyphae grow out into other air chambers eventually emerging through stomata in the streak that has developed. Again, epiphytic growth occurs before the re-entry of the hypha into the leaf through another stomate.

Conidia are observable from stage 2 of black Sigatoka whereas they are generally only visible from stage 4 of yellow Sigatoka. Perithecia form during stages 5 and 6 of black Sigatoka and during stage 5 of yellow Sigatoka. Overall the disease cycle is much faster for *M. fijiensis* than for *M. musicola* due to shorter time required to complete the life cycle. Generally, it has been observed, the optimal conditions for *M. fijiensis* are those where there is, on average, higher temperatures and higher relative humidity. See Table 2 for the disease development and associated structures found during the disease cycle for each of these pathogens.

Comprehensive cytological studies of the interactions between *M. fijiensis* and three banana genotypes have been undertaken (Beveraggi *et al.* 1995) (Sallé *et al.* 1989). From these studies it was found that there is a relatively long period of biotrophy before any incompatible reactions are observed in susceptible cultivars. The pathogen colonises the leaf tissue, growing intercellularly without the production of haustoria, for almost a month. During this period, little evidence of the presence of the pathogen can be seen externally. Cytological changes are visible in the parenchyma cells after about 28 days although the cells still appear healthy. There is contact between the hyphae and the cells but no localised reaction. Externally, stage 2 or the initial streak stage symptoms are visible.

After 41 days, stage 5 or second spot stage symptoms are visible externally. In the tissue sections taken from the susceptible cultivar 'Grande Naine' at this time during the studies, three distinctive zones were seen. Zone I which corresponded to the cells within the necrotic spot contained plasmolysed cells. Zone II corresponded to the yellow halo and this region contained cells with large intracellular globules. At the boundary of Zones II & III an intercellular substance, later identified as polyphenol, was noted. This substance formed intercellular bridges and host cells in contact with it showed degeneration of the cell wall.

Beyond this boundary the remaining host cells in Zone III appeared to be healthy. Hyphae were observed throughout the intercellular spaces in all of the zones (Sallé *et al.* 1989). Importantly, haustoria were never observed during the invasion of any host by *M. fijiensis*. The progression of the disease was similar in the partially resistant cultivar, 'Fougamou', except that the growth rate of hyphae in the susceptible cultivar was much higher than in the partially resistant cultivar (Beveraggi *et al.* 1995; Sallé *et al.* 1989). In the highly resistant cultivar, 'Yangambi Km5', a compatible reaction was not observed. There was no biotrophic

period; rather fungal growth was blocked at the site of penetration. Stomatal guard cells became necrotic and there was a deposition of polyphenolic substances around the outside of the cell walls of the host and the pathogen. This is consistent with a hypersensitive response (Beveraggi *et al.* 1995; Sallé *et al.* 1989).

The Causal Agents

Black Sigatoka is caused by the heterothallic ascomycetous fungi *Mycosphaerella fijiensis* Morelet (anamorph *Paracercospora fijiensis*). Yellow Sigatoka is caused by another, *Mycosphaerella musicola* Leach ex Mulder (anamorph *Pseudocercospora musae)*. Jones (2000) has a comprehensive chapter describing fungal leaf diseases of banana plants.

Disease Development and Epidemiology

Disease Cycle

The disease cycle for both *M. fijiensis* and *M. musicola* is similar with only minor differences as outlined previously. As *M. fijiensis* produces considerably less conidia and for a shorter period of time than *M. musicola*, ascospores are the main dispersal agent for this pathogen (Stover 1980). Both conidia and ascospores are important for dispersal of *M. musicola*(Stover 1971) however for both pathogens ascospores are involved in the movement of the pathogen over longer distances rather than conidia. A distinctive line spotting pattern of infection is produced when the source of inoculum is conidia dislodged by rain splashes. These run down the inside of the cigar leaf cylinder contacting the lower point of the cylinder resulting in a line of infection. The deposition of ascospores by wind currents is generally on the terminal end of these leaves resulting in a distinctive leaf tip infection (Meredith 1970; Stover 1972).

The disease cycle is much faster for black Sigatoka than it is for yellow Sigatoka, as seen by the earlier appearance of spots. Inoculation studies conducted in Honduras demonstrated that spotting associated with *M. fijiensis* infections appeared 8-10 days faster than that associated with *M. musicola* infections. Ascospore maturation time is also shorter at 2 weeks for *M.fijiensis* compared with 4 weeks for *M. musicola* (Stover 1980). A diagrammatic representation of the disease cycle for *M. musicola* is presented in Figure 1.

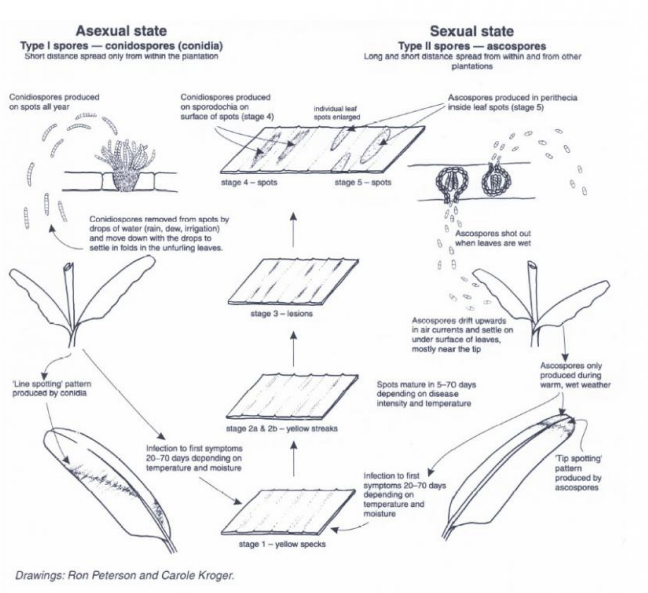


Figure 1: Life cycle of *Mycosphaerella musicola*, the fungal pathogen causing yellow Sigatoka (Reproduced with permission, Department of Primary Industries & Fisheries,Queensland).

Survival of the Inoculum

Production of perithecia and the subsequent discharge of ascospores continues for several months. Even in severely necrotic tissue, ascospore ejection can continue for more than two months, this is the case also where the leaf has been removed and placed on the ground (Carlier *et al.* 2000a). Ascospore release remains high for three weeks after removal of the leaf from the plant and then decreased rapidly over the next six weeks until the tenth week when the leaves themselves had disintegrated (Gauhl 1994). The survival of ascospores is directly related to the time it takes for the disintegration of the diseased leaf material (Stover 1980). Ascospores ejected are no longer viable after 6 hours of exposure to UV radiation (Parnell *et al.* 1998).

Spread of the Pathogen

Both *M. musicola* and *M. fijiensis* are dispersed within banana blocks by rain splash of conidia. Movement between blocks is possible through the aerial spread of ascospores ejected from the perithecia. Due to the larger amount of conidia produced by *M. musicola* than by *M. fijiensis,* conidia are considered the main means of spread for *M. musicola* while ascospores are the main method of dispersal of *M. fijiensis* (Stover and Dickson 1976).

Long distance spread may also be via the wind dispersal of ascospores. The short time that ejected ascospores can survive UV irradiation suggests that the distance viable ascospores are dispersed by this method will also be affected by the amount of cloud cover and the distance travelled through the night (Parnell *et al.* 1998). Recent population studies of both *M.fijiensis* (Rivas *et al.* 2004) and *M. musicola* (Hayden *et al.* 2005) however, suggest limited long distance dispersal-less than 50 m-of these pathogens based on the genetic structure of the populations. In many cases long distance movement, especially intercontinental movement, of the pathogen is thought to be more likely due to the direct transportation of germplasm from an infected area to a new region (Rivas *et al.* 2004).

Host Range

Currently the only known hosts of *M. fijiensis* and *M. musicola* are *Musa* spp. These species and subspecies all vary in their levels of resistance to *M. fijiensis* and *M. musicola.* There is one report in the literature of *M. musicola* having been isolated from leaf spots on a *Heliconia* species in Venezuela (Madiz *et al.* 1991).

Distribution

Relative distributions of *M. fijiensis* and *M. musicola*

M. fijiensis is found throughout the world's tropical banana growing regions from the Tropic of Cancer to the Tropic of Capricorn with the exception of Australia. *M. musicola* also causes serious yield losses in regions not affected by black Sigatoka. As this pathogen can proliferate at lower temperatures and lower relative humidity, *M. musicola* is more widespread than *M.fijiensis*. Yellow Sigatoka is often the dominant disease at higher altitudes (>1200 m) although it appears that *M. fijiensis* is becoming more adapted to higher altitudes and is gradually replacing *M. musicola* in these regions (Carlier *et al.* 2000a). Figure 2 shows the worldwide distribution of both of these pathogens.

Origin and Distribution of Black Sigatoka

Black Sigatoka, which is also known as black leaf streak in some parts of the world, is caused by the fungal pathogen *Mycosphaerella fijiensis* (Leach 1964). Black Sigatoka affects many *Musa* species throughout the world including those grown on the island of New Guinea, and in the Torres Strait Islands (TSI). *M. fijiensis* was first identified in the Sigatoka valley of Vita Levu in the Fiji Islands in 1963 (Leach 1964). Black leaf streak, as it was first known, quickly replaced Sigatoka, which was endemic in the region at that time. Surveys of the Oceanic region from 1964 to 1967 revealed that this new disease was already established in the Pacific and parts of the Pacific Rim. From its wide dispersal, it appeared that *M. fijiensis* had been present in this region for some time prior to its identification. The disease was found in the Philippines in 1970 but again it appeared that it might have been present for 15 years or more prior to this. From the examination of herbarium specimens it was concluded that *M. fijiensis* had been present on the island of New Guinea since 1957 and that it had been present in Taiwan in 1927 (Stover 1978). From this work Stover concluded that the centre of origin for *M. fijiensis* was likely to have been in PNG-Solomon Islands region.

The spread of *M. fijiensis* in the Latin Americas has been well documented as it appeared in this region

after its initial identification in Fiji in 1963. Although the pathogen may have been introduced to Honduras as early as 1969, it was only positively identified there in 1972 (Stover and Dickson 1976). It was here that the disease acquired its current name black Sigatoka. The disease slowly moved throughout Latin America and by 1981 was endemic to Central America. It has since moved south into Colombia, Ecuador, Peru and Bolivia. *M. fijiensis* has only recently reached the Caribbean. It was first identified in Cuba in 1992 (Vidal 1992) and more recently in Jamaica, the Dominican Republic, Trinidad, Grand Bahama Island and Haiti (Carlier *et al.* 2000a) (Fortune *et al.* 2005; Jones 2002) (Mourichon *et al.* 1997). The direction of the prevailing winds is believed to limit further spread by natural means of *M. fijiensis* within the Carribean (Carlier *et al.* 2000a).

Although *M. fijiensis* may have been present in Africa as early as 1973, it was first identified in Gabon in 1978 (Frossard 1980). It is believed that the pathogen was introduced into Africa from infected banana plants imported from Asia. From its first introduction, it has since spread to neighbouring countries and is now found in Cameroon, Cote d'Ivoire, the Democratic Republic of Congo, Nigeria, and Ghana. A separate introduction is believed to have brought the pathogen to the countries on the east coast of Africa (Carlier *et al.* 2000a). The disease was first identified in this region in 1987 on the island of Pemba. The disease quickly spread to the adjacent island of Zanzibar and from there to the mainland of Tanzania (Dabek and Waller 1990). By 1988 it had reached Kenya (Kung'U *et al.* 1992). The introduction of the disease to the East African countries of Rwanda, Burundi, (Sebasigari 1989), Uganda (Tushemereirwe and Waller 1993) and Malawi (Ploetz *et al.* 1992) is however thought to have been from the Democratic Republic of Congo (Carlier *et al.* 2000a). A map showing the worldwide distribution of *M. fijiensis* is presented in Figure 2.

The distribution of *M. fijiensis* in Asia is still unclear (see section on Other Leaf Spots) although there are several substantiated reports of this pathogen from this region. The pathogen has been confirmed as being present in Southern China (Carlier *et al.* 2000a; Mourichon and Fullerton 1990), Vietnam, Thailand, Taiwan and Singapore. Black Sigatoka has also been confirmed as being present in parts of Malaysia; West Malaysia, Jahore, Langkawi and East Malaysia and also as being present in parts of Indonesia, namely Halmahera, Java, Kalimantan and West Sumatra.

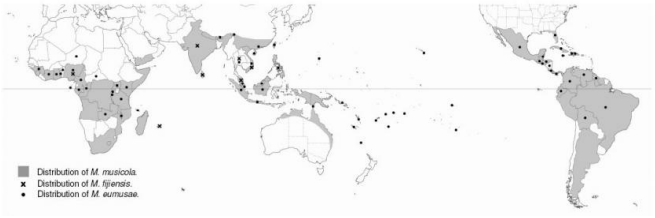


Figure 2. World map showing the global distribution of *Mycosphaerella fijiensis*, *Mycosphaerella musicola* and *Mycosphaerella eumusae*.

Black Sigatoka in Australia

Currently, mainland Australia is a designated 'black Sigatoka free' region. However, there have been nine previous incursions of the disease in North Queensland, the first detected in 1981 and the most recent incursion in 2001 in the Tully Valley. A Pest Free Area was declared on the 20th December 2004.

Although *M. fijiensis* was only detected on bananas on the Torres Strait Islands during a plant disease survey in 1981 its spread would indicate that it had been present in this region for a considerable period of time prior to its detection. However, as no disease surveys had been undertaken in the region prior to 1981, it cannot be certain how long the disease had been present for. The pathogen had most likely been introduced on banana plants brought over from Papua New Guinea where it is known to be endemic.

Black Sigatoka was also detected in Bamaga, on mainland Australia, during the 1981 disease survey. Although the disease was widespread through the township, only 25% of plants were infected due to the resistance of many of the banana cultivars grown. Many plants found in Bamaga belonged to the ABB genotype. The ABB genotype contains subgroups with many cultivars having higher resistance to *M. fijiensis* compared with other genotypes, such as the AAA subgroup of which Cavendish is a member. Another factor that impeded the spread of the disease was this region had a low density of banana plants and is situated in the dry tropics of Australia. Without additional water and nutrients, banana plants do not grow well in this region. As an initial measure to prevent the spread of black Sigatoka, the disease was declared quarantinable under the Commonwealth Quarantine Act of 1908. The region was then proclaimed a quarantine region enabling enforceable restrictions on the movement of banana material out of the area.

Due to the proximity of this region to Queensland's major commercial banana production areas, the Queensland Department of Primary Industries and Fisheries (DPI&F) first attempted eradication in October of 1981. The Commonwealth Government and Australia's banana growing States sponsored this eradication program. This original attempt at eradication involved the destruction of all banana plants in the Bamaga region as well as all banana plants on Thursday Island, Badu Island and Kubin Village on the western side of Moa Island. Replanting was left for a period of 6 months in order to avoid reinfection from any ascospores discharged from the banana trash. In 1984, *M. fijiensis* was again detected on some banana plants during routine plant disease surveillance by the DPI&F. This time the disease was restricted to the Bamaga area. It was not found on Thursday, Badu or Moa Islands (Jones 1984) indicating that the eradication program on these islands had been successful.

Failure of the initial eradication program in Bamaga was thought to be due to several factors. Regrowth from eradicated plants may have provided a source of inoculum or some banana plants may have been missed during eradication despite thorough surveys of the area. Another possibility is that local residents may have hidden plants prior to the eradication program and then returned them to Bamaga after the reintroduction of clean planting material, and so providing a source of inoculum (Jones 1989). At that time it was decided not to proceed any further with eradication attempts and the disease was instead managed and contained in Bamaga. This was possible due to quarantine restrictions preventing the movement of banana material out of the Torres Strait Islands and Bamaga to other parts of Australia and also due to the remoteness and isolation of the region at the time. Trials were undertaken in the region to evaluate the resistance of several banana cultivars to black Sigatoka (Jones 1984). A program was also introduced during this time by the Queensland Banana Industry Protection Board (BIPB) to replace all the bananas in the Bamaga area with more resistant cultivars such as Bluggoe and Tu8 (Jones 1989).

In 1988, a second attempt at eradication was undertaken due to increased pressure from the banana industry. A small commercial plantation of Cavendish bananas had been planted at Bamaga and the access road had been upgraded leading to an increase in traffic to the region. The industry feared that black Sigatoka would move south into the large commercial growing regions devastating the industry. Eradication of the pathogen was effected through the destruction of all banana plants in the area, replanting with resistant lines such as Tu8, Bluggoe and Ducasse, and the favourable climate of a long, hot, dry season. Another incursion in this region detected in 1999 has been linked to regrowth of plants from the 1988 eradication program and also to the importation of plants directly from Thursday Island (Davis *et al. 2000). Again, all susceptible plants were removed and buried. Black Sigatoka has not* been detected in the region since the 1999 eradication. There have been six further small incursions since 1988 (Figure 2) all in isolated, banana growing regions. On each occasion, officers from DPI&F have managed the eradication of the disease by the removal and destruction of all banana plants in a buffer zone of up to a 50 km around the site of the infection. The area was then allowed to remain free of banana plants for at least 4 months before replanting with cultivars more resistant to black Sigatoka. Eradication was also assisted by the natural climatic conditions. Many regions in Cape York experience long, hot and dry seasons interspersed with a short, wet season. Banana plants do not survive the dry season in this area without irrigation and *M. fijiensis* does not proliferate under these conditions (Peterson 2002).

All of these incursions have been attributed to the movement of diseased plants between sites of infection. The close proximity of the Torres Strait Islands to the mainland and the strong cultural ties between various communities residing in this region suggest a logical ingress for the pathogen. The initial movement of diseased banana plant material and suckers from the Torres Strait Islands could have brought the pathogen to mainland Australia and then the subsequent movement of planting material transported it between sites of infection on the mainland.

The most recent and most serious of incursions was in Tully in 2001. This incursion differed from the previous ones in two ways: (i) this incursion was in the largest commercial banana production area in Australia and (ii) Tully has one of the highest rainfalls in Australia. The prior eradication strategy of removing all banana plants in the region was revised as this now involved 4400 ha of banana plants. An alternative strategy was developed which relied heavily on deleafing of all banana plants to achieve a zero disease level in the region. Constant surveillance also enabled scientists to monitor the disease. Diagnosis of diseased leaf material was however more difficult than usual. Heavy rains in the area at the time had washed away all of the fungal structures used to differentiate *M. fijiensis* from the endemic *M.*. Molecular diagnosis was a useful tool used to differentiate between the two musicola pathogens and was one of the many factors contributing to the success of the eradication campaign.

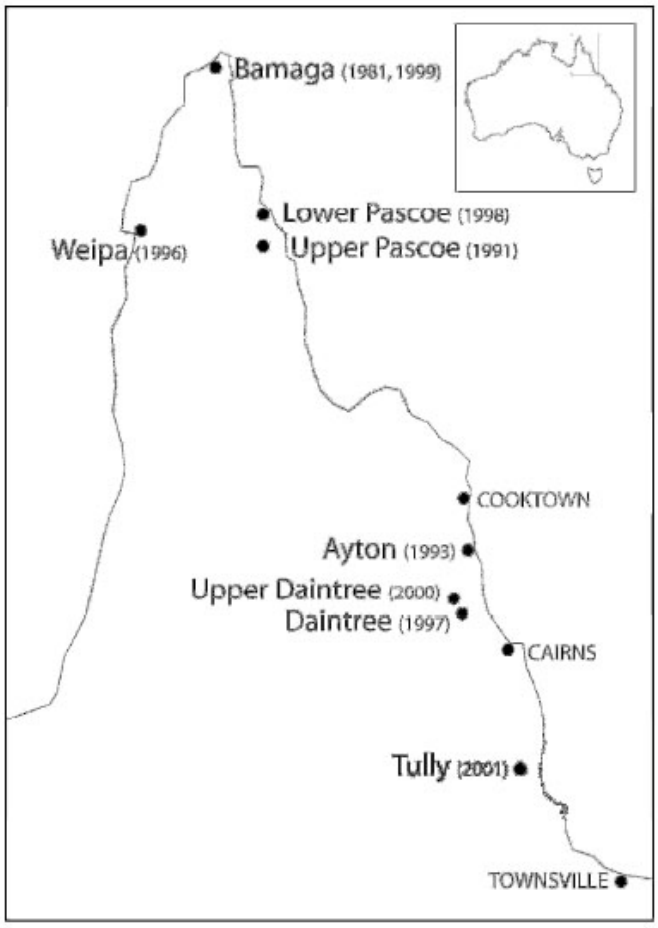


Figure 3: Map of Queensland showing the locations of all *Mycosphaerella fijiensis* incursions on mainland Australia since 1981. All incursions were eradicated by the Queensland Department of Primary Industries and Fisheries (DPI&F).

Yellow Sigatoka

Yellow Sigatoka is caused by the fungal pathogen *Mycosphaerella musicola* Leach which is closely related to *M. fijiensis*. The pathogen is considered to have a worldwide distribution. It has not however been reported in the banana growing regions of the Canary Islands, Egypt and Israel (Jones 2000) and its exact distribution through Asia is still unclear.

Stover (1962) hypothesised the mode of spread of *M. musicola* worldwide (Stover 1962). Working from disease records, Stover proposed that *M. musicola* was moved from Java where Zimmermann first described the anamorph of this pathogen, *Pseudocercospora musae* in 1902, to Fiji on banana leaf material used as packing material in shipping containers. *M. musicola* was first identified in Fiji in 1913 by Massee (Massee 1914). From here Stover proposed that the pathogen moved to the east coast of Australia on the prevailing winds in around 1924. At this time there was a disease epidemic in the Fijian banana plantations of the Sigatoka Valley which was causing inoculum levels to be exceptionally high. Once in Australia the disease quickly spread throughout banana plantations, many of which had been left unmanaged due to the severe banana bunchy top disease (BBTD) epidemic. Stover hypothesised that the combination of exceptionally high levels of inoculum during the epidemic years of the late 1920s and early 1930s, coupled with unusual climatic conditions and air turbulence, could have resulted in enough viable ascospores surviving the long journey on the tradewinds to cause the disease outbreaks in Africa and South America.

This hypothesis was tested using RFLP markers to study the genetic structure of the global population of *M. musicola* (Hayden *et al.* 2003). Hayden (2003) found that Stover's hypothesis was supported by the fact that many alleles found in the Indonesian population were detected in the Australian, African and South American populations. The hypothesis however was not supported when the Australian population was compared with the African and the South American populations. Both of these populations possessed alleles not present in the Australian population. Further, Hayden concluded from the genetic differentiation data that it is likely that the African and South American *M. musicola* populations arose from the Indonesian population in a separate founder event from that of the Australian population.

In Australia, *M. musicola* was found to have spread to the banana growing regions of New South Wales by 1927 (Simmonds 1928). *M. musicola* is now endemic throughout all bananagrowing regions in Queensland and northern New South Wales. In Western Australia it was first detected in Kununurra in 1990 although it is thought to have been present for some time before this first report (Shivas and Kesavan 1992). It is now identified as a common pathogen to the banana growing regions of the Kimberleys. The pathogen has also been detected in banana growing regions in the Northern Territory.

Detection

Symptom Description

Black Sigatoka

Black Sigatoka causes large necrotic lesions on the leaves of the banana plant and early drop (collapse) of the entire leaf (Figure 4). The resulting loss of photosynthetic capacity leads to slower filling of fingers, reduced yields and finger size and premature ripening of fingers. Field losses vary from 30-50% depending on the climatic conditions (Gauhl *et al.* 2000; Stover 1983) and are presently 5-10% in even well-managed plantations with good control strategies (R. Romero, pers. comm.). In subsistence crops of plantain, yield loss has been estimated to be up to 33% during the first crop cycle and up to 76% in the second (Mobambo *et al.* 1996).

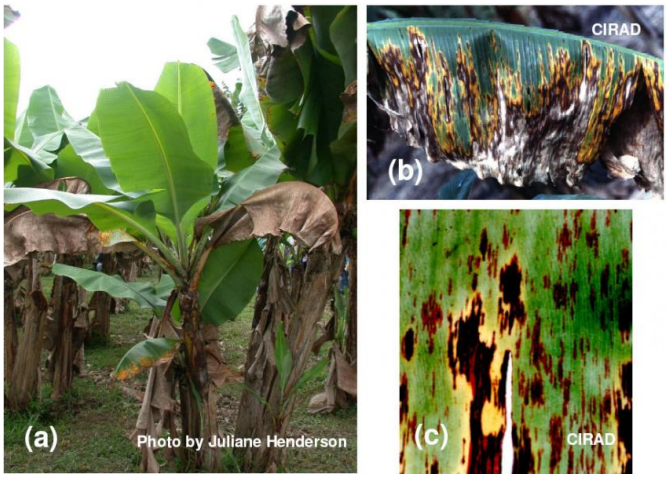


Figure 4(a): Black sigatoka infection in a managed banana plantation in Costa Rica (Photo by Juliane Henderson), **(b)** heavily diseased leaf (Image courtesy of CIRAD) and **(c)** underside of a diseased leaf (Image courtesy of CIRAD).

There are six recognised stages in symptom development (Fouré 1987; Meredith and Lawrence 1969). A brief description of each stage follows:

Stage 1: Initially, tiny specks < 0.25 mm and white to yellowish in colour that quickly turn a reddish brown, appear on the abaxial surface (underside) of the leaf laminar. This first stage is also known as the 'initial speck stage').

Figure 5(a): Stage 2 symptoms or 'First Streak Stage'. Conidia and conidiophores may be present at this stage. Note that Stage 1 symptoms (initial speck stage) are barely visible at <0.25mm. (Image reproduced with permission, Department of Primary Industries and Fisheries, Queensland)

Stage 2: The tiny reddish brown specks elongate and widen, becoming streaks approximately 2mm X <1 mm. This stage is also referred to as the 'initial streak stage'. The streaks are more clearly visible on the abaxial surface of the leaf laminar than the adaxial surface (upper side) of the leaf. Conidia and conidiophores may be present (Figure 5(a)).

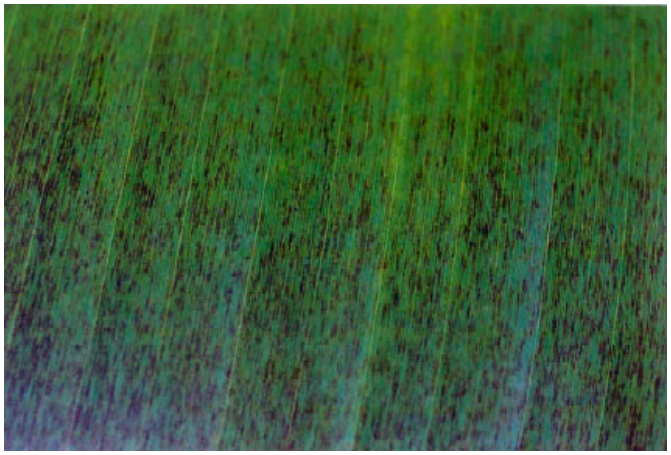


Figure 5(b): Stage 3 symptoms or 'Second Streak Stage'. Streaks are now almost black. Conidia and

conidiophores are present. (Image reproduced with permission, Department of Primary Industries and Fisheries, Queensland)

Stage 3: The streaks continue to expand in size and change colour to a very dark brown, almost black, colour. This is also referred to as the 'second streak stage'. Where infection is heavy, the streaks overlap to give a black appearance to large areas of the leaf. The streaks are clearly visible from the adaxial side of the leaf. Conidia and conidiophores are present at this stage (Figure 5(b)).

Figure 5(c): Stage 4 symptoms or 'First Spot Stage'. The streaks are becoming more elliptical and have a watersoaked border. (Image reproduced with permission, Department of Primary Industries and Fisheries, Queensland)

Stage 4: The streaks continue to enlarge and become more elliptical in shape as it broadens and a water-soaked border may develop around the edges. This stage is known as the 'first spot stage' (Figure 5(c)).

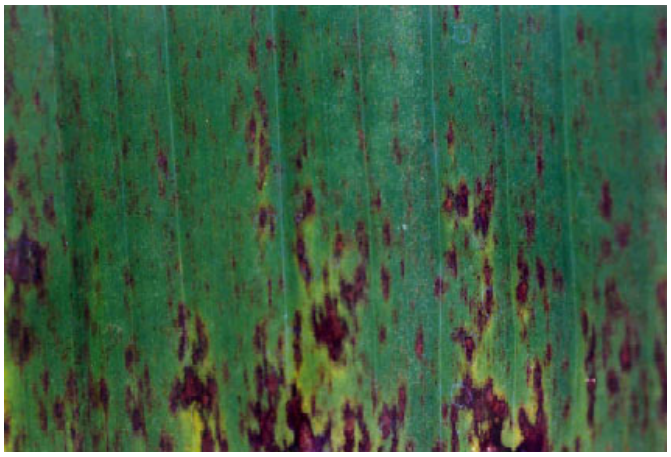


Figure 5(d): Stage 5 symptoms or 'Second Spot Stage'. Note the blackening in the centre of the spots. The watersoaked border begins to develop a yellow halo. (Image reproduced with permission, Department of Primary Industries and Fisheries, Queensland)

Stage 5: This stage also known as the 'second spot stage' is characterised by the central region of the spot becoming slightly depressed. The water soaked border may develop a yellow halo around it. Where infection is heavy, large areas of leaf tissue collapses. (Figure 5(d)).

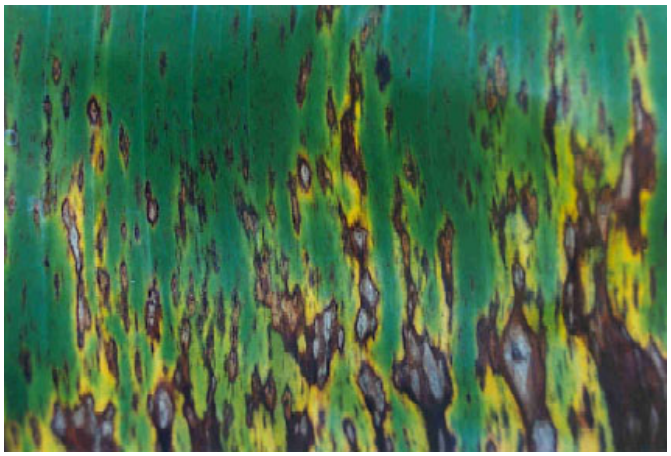


Figure 5(e): Stage 5 & 6 'Third Mature Spot' symptoms. Multiple lesion stages are present. Note the pale grey centres of the Stage 6 lesions. (Image reproduced with permission, Department of Primary Industries and Fisheries, Queensland)

Stage 6: The final stage, also referred to as the 'third spot stage', is when the centre of each spot becomes dry and pale grey to beige in colour. Perithecia and ascospores are present in stage 6 lesions. Surrounding

each of the spots is a distinctive black border. Where infection is heavy the large areas of the leaf become necrotic. The spots remain visible even after the death and desiccation of the leaf due to the dark border encircling each of the individual spots (Figure 5(e)).

Yellow Sigatoka

Figure 6: (a) Banana plant infected with *Mycosphaerella musicola*. Note later stage lesions are always present in the lower leaves which are older while the newer leaves show the earlier stage symptoms. Symptom development can be used in conjunction with other tools to assist with diagnosis. The photographs in **(b)** and **(c)** show advanced lesions on leaves. (Images (a) & (c) reproduced with permission, Department of Primary Industries & Fisheries, Queensland (b) courtesy of CIRAD & INIBAP)

Yellow Sigatoka disease is similar to black Sigatoka (Figure 6). There are, however, some distinguishing diagnostic features for yellow Sigatoka. The disease ultimately has the same effect on yields as black Sigatoka, although yellow Sigatoka disease development is slower, enabling it to be controlled through deleafing and the use of fungicides.

Yellow Sigatoka can be differentiated from Black Sigatoka at the early stages of lesion development (Stages 1 and 2) on visual symptoms. At later stages, examination of the conidiophores and conidia requires compound microscopy. There have been several descriptions of the development of individual lesions of Sigatoka disease over the years which are well summarised in Meredith (1970) (Meredith

1970). Brun's description (Brun 1958) is similar to that of Leach (Leach 1946) except that Brun excludes Leach's 5th stage (second spot stage). A brief description of each stage as per Brun follows:

Figure 7(a): Stage 1 lesions of yellow Sigatoka characterised by the light green dots and dashes which are about 1 mm in length. (Image reproduced with permission, Department of Primary Industries and Fisheries, Queensland)

Stage 1: This stage is characterised by the appearance of very small light green dots or dashes of approximately 1 mm in length. (Figure 7(a)).

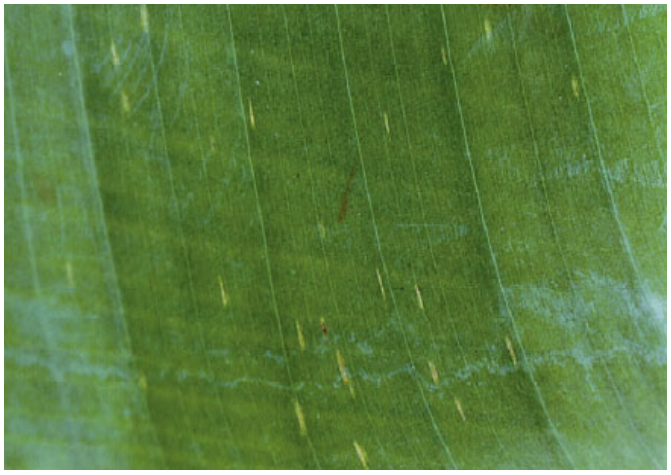


Figure 7(b): Lesions associated with Stage 2a (early) of yellow Sigatoka. Note light green streaks which are the characteristic lesions at this stage. (Image reproduced with permission, Department of Primary Industries and Fisheries, Queensland)

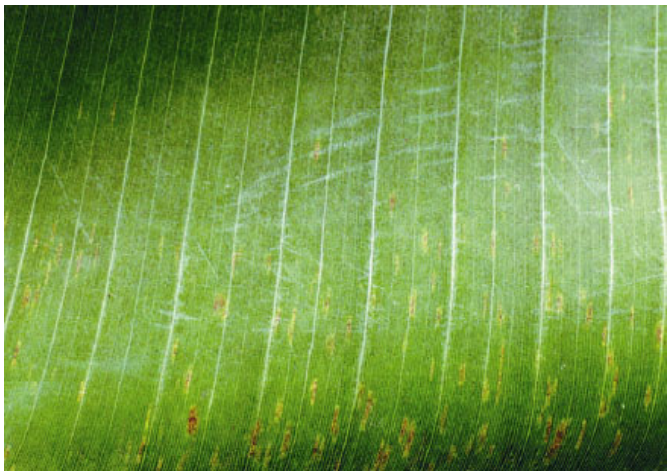


Figure 7(c): Lesions associated with Stage 2b (late) of yellow Sigatoka. Note the change in colour of the streaks from light green to rusty brown. (Image reproduced with permission, Department of Primary Industries and Fisheries, Queensland)

Stage 2: The small dot or dash of Stage 1 elongates into a light green streak several millimetres long. (Figure 7(b)&(c))

Figure 7(d): Stage 3 symptoms associated with yellow Sigatoka. Note that the streaks from stage 2 have now elongated and widened. (Image reproduced with permission, Department of Primary Industries and Fisheries, Queensland)

Stage 3: At this stage there is a change in the colour of the streak to a rusty brown. The streak becomes elongated and widens slightly. The border of the streak is ill defined. (Figure 7(d)).

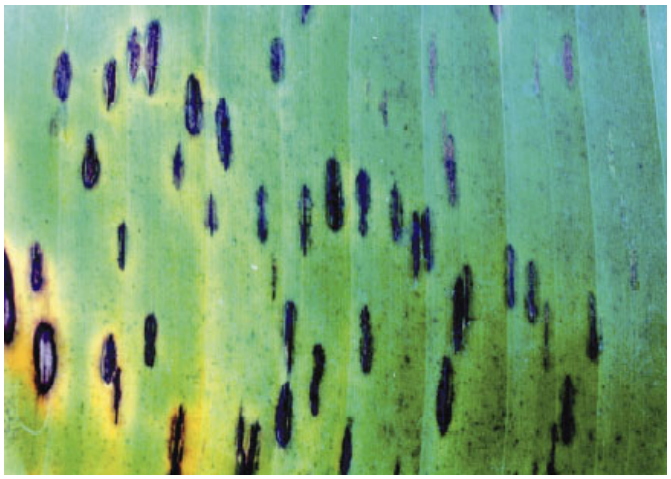
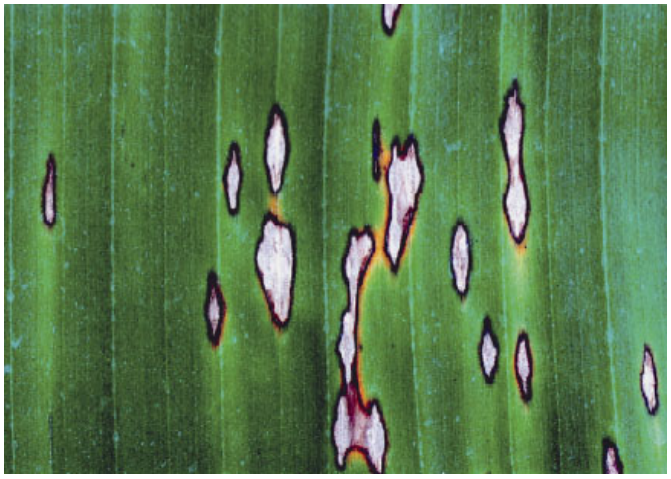


Figure 7(e): Stage 4 symptoms associated with yellow Sigatoka. Note that the Stage 3 streaks have now become spots. Conidia and conidiophores may be present from this stage. (Image reproduced with permission, Department of Primary Industries and Fisheries, Queensland)

Stage 4: The streak becomes more elliptical and is a definite spot with a sunken dark brown centre. It is often surrounded by a yellow halo. At this stage the conidia and conidiophores are produced. (Figure 7(e))



Stage 5: The final stage has a grey dried out centre and an obvious black margin. This black margin can

still be seen even after the leaf has dried out. (Figure 7(f)) The stages of both yellow and black Sigatoka are summarised in Table 1.

Table 1. Summary of the different lesion stages associated with yellow and black Sigatoka leaf spot of bananas.

Lesion Stage	Yellow Sigatoka	Black Sigatoka
Stage 1	Very small light green dot or dash up to 1 mm long	Small pigmented spot of white or yellow, similar to yellow Sigatoka stage 1
Stage 2	Light green streak several millimetres long	Brown streak, visible on underside of leaf, later visible on leaf upper surface as yellow streak; colour changes progressively to brown, then black on upper leaf surface
Stage 3	An elongated rusty brown spot with an poorly defined border	Enlarged stage 2, streaks become longer
Stage 4	A mature spot with a dark brown sunken centre; often surrounded by a yellow halo, conidiophores and conidia are produced at this stage	Appears on leaf underside as brown spot, as a black spot on upper leaf surface
Stage 5	Spot has developed a grey, dried out centre and a peripheral black ring which is evident even after the leaf has dried out	Elliptical spot is totally black on the underside of the leaf, surrounded by a yellow halo
Stage 6		Centre of spot dries out, turns grey and is surrounded by a well-defined margin and a bright yellow halo

Other Leaf spots

A third leaf spot disease, Eumusae leaf spot (Figure 8), has recently been described. This disease is caused by the pathogen, *Mycosphaerella eumusae*, anamorph *Pseudocercospora eumusae* which is very closely related to *M. fijiensis* and *M.musicola* (Carlier *et al.* 2000c; Crous and Mourichon 2002). Distribution of this pathogen is still uncertain however originally it was found serendipitously in Asia in West Malaysia, Thailand, Vietnam, Southern India and Sri Lanka during a survey initiated by INIBAP to determine the distribution of *M. fijiensis* and *M. musicola* within South and South-east Asia. Samples collected as Sigatoka leaf spots were found to be associated with this new pathogen (Carlier *et al.* 2000b; Carlier *et al.* 2000c; Crous and Mourichon 2002). Little is known about *M. eumusae* however, one isolate was found infecting a banana known to be resistant to *M. fijiensis*. Morphologically it is similar to *M. musicola.*

Several other leaf spot diseases may produce lesions with a similar appearance to those of *M. fijiensis* and *M. musicola* however these other pathogens can be easily distinguished using light microscopy as they are morphologically quite different.

Figure 8: Banana leaves with lesions associated with Eumusae leaf spot (ELS) caused by the fungal pathogen *Mycosphaerella eumusae*. (Images reproduced with permission, INIBAP & CABI, UK)

Identification

Handling/Preservation

Reagents for DNA extraction

CTAB extraction buffer (100 mL)

Stock Component	Volume Added	Final Concentration
CTAB	2g	2% (w/v)
5M NaCl	28.4mL	1.42M
0.5M EDTA	4mL	20mM
1M Tris-HCl (ph8.0)	10mL	100mM
PVP-40	2g	2% (w/v)
Ascorbic Acid (MW = 176.12)	88mg	5mM
DIECA (MW = 171.3)	68mg	4mM

Dissolve components completely in MilliQ water, warming up to 70ºC if required. Do not heat above 70ºC, as some components are heat labile. Divide into smaller aliquots and store out of the light. Discard unused buffer after 6 weeks.

****DO NOT AUTOCLAVE THIS BUFFER - HEAT LABILE****

5M NaCl Stock Solution

- Place 146.1g NaCl in 1 litre beaker and make up to 500mL with MilliQ water

- Stir until dissolved
- Divide into smaller aliquots and autoclave (121°C/15 mins)

0.5M EDTA (pH 8.0)

- Place 143.1g of EDTA in a 1 litre beaker and add 400mL of MilliQ water
- Stir vigorously whilst adjusting the pH of the solution to 8.0 with NaOH (approximately 10g of solid NaOH)**
- Make up to 500mL with MilliQ water
- Divide into smaller aliquots and autoclave (121°C/ 15 mins)

*** The disodium salt of EDTA will only go into solution when the pH has been adjusted to 8.0 with NaOH. ***

1M Tris-HCl (ph8.0)

- Place 60.55g Tris Base in a 1 litre beaker and add 400mL of MilliQ water
- Stir whilst adjusting the pH of the solution to 8.0 with concentrated HCl (42mL/L final solution)**
- After solution has returned to room temperature make up to 500mL and make final pH adjustment
- Divide into smaller aliquots and autoclave (121°C/ 15 mins)

*** This solution will become very warm after the addition of the concentrated HCl but this is normal. ***

Chloroform-Isoamyl Alcohol (24:1)
To make up 100mL

- mix 96mL (24 parts) chloroform with 4mL (1 part) isoamyl alcohol.

*** This solution must be made up and used inside a fume cabinet. ***
Chloroform is toxic and a possible carcinogen.

Reagents for Agarose Gel Electrophoresis

0.5 X Tris-borate (TBE) Running Buffer

If not purchasing the concentrated TBE make a 5 X stock solution as follows:
54g Tris base
27.5g boric acid
20mL 0.5M EDTA (pH 8.0)

- Add the above to 800 mL of distilled water and stir until dissolved
- Make up to a final volume of 1.0L

Store at room temperature and discard if precipitation occurs. To make a working solution of 0.5 X TBE dilute 1:10 with MilliQ water.

6 X Gel-Loading Buffer

Four different gel-loading buffers are provided below. Personal preference and availability of individual components will dictate choice.

1. 0.25% bromophenol blue
0.25% xylene cyanol FF
40.0% (w/v) sucrose
Dissolve above components in MilliQ water and store at 4° C.

2. 0.25% bromophenol blue
0.25% xylene cyanol FF
15% Ficoll 400
Dissolve above components in MilliQ water and store at room temperature.

3. 0.25% bromophenol blue
0.25% xylene cyanol FF
30.0% (w/v) glycerol
Dissolve above components in MilliQ water and store at 4° C.

4. 0.25% bromophenol blue
40.0% (w/v) sucrose
Dissolve above components in MilliQ water and store at 4° C

Morphological Methods

 The Causal Agents Black Sigatoka is caused by the heterothallic ascomycetous fungi Mycosphaerella fijiensis Morelet (anamorph Paracercospora fijiensis). Yellow Sigatoka is caused by another, Mycosphaerella musicola Leach ex Mulder (anamorph Pseudocercospora musae). Jones (2000) has a comprehensive chapter describing fungal leaf diseases of banana plants. Taxonomy of M.fijiensis and M. musicola Morphology Although M. fijiensis and M. musicola are extremely closely related, and some stages of the disease appear similar, the morphology of the conidia and conidiophores can be routinely used to differentiate the two pathogens. Conidia produced by M. fijiensis are pale to medium olive green with paler tips, 1-10 septate, obclavate to cylindro-obclavate and straight or curved. Conidia produced by M. musicola however are a paler shade of olive green, 0-6 septate, cylindrical to obclavate-cylindrical, and also either straight or curved. The conidia of M. fijiensis are also discernable by the thickened basal hilum at the base. Conidiophores of Paracercospora fijiensis can be either straight or bent, are pale to medium brown, 0-5 septate, often geniculate and are generally unbranched. A diagnostic characteristic are the distinctive scars present on the tip of the conidiophores. Conidiophores of P. musae differ in that they are straight, hyaline, mostly without septa, geniculation or branching scars are not present. Another difference between the two pathogens is the location of the conidiophores. When P. fijiensis is the infecting pathogen they are mainly found on the abaxial surface of the leaf whereas conidiophores of P. musae are abundant on both surfaces. Conidiophores are also present much earlier in the lesions associated with black Sigatoka, as early as stage 2 lesions, whereas they are generally not present until the disease has progressed to stage 4 lesions for yellow Sigatoka. Images of these structures are presented in Figures 9 and 10. The structures of Mycosphaerella eumusae are also presented in Figure 11 for comparison. The morphology of the anamorphs of M. fijiensis and M. musicola is summarised in Table 2. Figure 9 (a) Fruiting bodies of Paracercospora fijiensis found in lesions associated with black Sigatoka. Note the small number of conidia produced by these

in comparison with M. musicola and M. eumusae. (Image courtesy of CIRAD & INIBAP) Figure 9(b) Conidia produced by the fruiting bodies of Paracercospora fijiensis Note the basal thickening which is not found in Pseudocercospora musae or Pseudocercospora eumusae. (Image courtesy of CIRAD & INIBAP) Figure 10 (a): Fruiting bodies of Pseudocercospora musae found in lesions associated with yellow Sigatoka disease. (Image courtesy of CIRAD & INIBAP) Figure 10 (b): Conidia of Pseudocercospora musae. No basal thickening is present. Up to 50 conidia may be associated with a single sporodochia. (Image courtesy of CIRAD & INIBAP) Figure 11 (a): Fruiting bodies of Pseudocercospora eumusae found in lesions associated with Eumusae leaf spot. Conidiophores are found mainly on the abaxial leaf surface. (Image courtesy of CIRAD & INIBAP) Figure 11 (b): Conidia of Pseudocercospora eumusae. Conidia are shorter (21.2-41.6 x 2.5 m) than those of P. musae (10-109 x 2-6 m). (Image courtesy of CIRAD & INIBAP)   Table 2. Comparison of the morphology of Paracercospora fijiensis and Pseudocercospora musae.   <table style="text-align: center;" width="100%" border="0" cellpadding="0" cellspacing="0"><tr style="height: 119px;" valign="top" align="left"><td style="border: 1px solid #000000; "></td><td style="border: 1px solid #000000; ">Paracercospora fijiensis </td><td style="border: 1px solid #000000; ">Pseudocercospora musae </td></tr><tr style="height: 119px;" valign="top" align="left"><td style="border: 1px solid #000000; ">Conidiophores </td><td style="border: 1px solid #000000; "> * first appear at stage 2 or initial streak stage
* can be either straight or bent, are pale to medium brown, 0-5 septate, often geniculate, generally unbranched with distinctive, slightly thickened spore scars that are diagnostic for this pathogen
* mainly found on the abaxial surface of the leaf
* emerge singly or in small groups </td><td style="border: 1px solid

#000000; ">* first appear at stage 4 or first spot stage
* are straight, hyaline, mostly without septa, geniculation or branching and they do not have any spore scaring
* abundant on both surfaces</td></tr><tr style="height: 119px;" valign="top" align="left"><td style="border: 1px solid #000000; ">Conidia </td><td style="border: 1px solid #000000; ">* pale to medium olive green with paler tips, 1-10 septate, obclavate to cylindro-obclavate and straight or curved
* thickened basal hilum</td><td style="border: 1px solid #000000; ">* a paler shade of olive green, 0-6 septate, cylindrical to obclavate-cylindrical, and also either straight or curved
* no basal thickening present</td></tr></table> Microscopic Diagnosis of Disease It is difficult for the less experienced plant pathologist to confidently identify black or yellow Sigatoka based on leaf spot symptoms alone. In fact there are several diseases that can, atdifferent stages and under certain conditions, appear to be identical. The development and appearance of symptoms can differ for each of the Sigatoka diseases as a consequence of various biotic and abiotic factors such as the prevailing weather conditions, nutritional state of the plant and inoculum levels present. They will also vary due to the different levels of resistance among different cultivars of Musa spp. These differences are used to classify banana cultivars into resistance groupings. A preliminary diagnosis can sometimes be made based on the leaf spot symptoms. When used in conjunction with light microscopy a definitive diagnosis can generally be made. Microscopy is therefore an indispensable tool in the diagnosis of these pathogens. Although morphologically these two pathogens are very similar, there are some small but significant differences between them. Generally, these differences can be observed on microscope slides prepared directly from the diseased leaf tissue. Leaf tissue with suspected early stage Sigatoka leaf spot lesions should be incubated overnight at 100% relative humidity at around 25ºC. Generally the samples will already be contained inside a sealed plastic bag from collection which achieves this high humidity without additional incubation. The high humidity will ensure a profusion of conidia and conidiophores for identification. As noted previously, conidia are present much earlier in M.fijiensis infections and can be observed as early as stage 2 lesions whereas conidia can only be seen from stage 4 lesions in M. musicola infections. The Pathogen Images of the identifying structures (conidiophores and sporodochia) are presented in Figures 9 and 10. Note the scaring and basal thickening in the conidiophores of M. fjiensis. Images of the closely related M. eumusae are presented as well for comparison (Figure 11).

Molecular Methods

Molecular methods are currently under review.

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Use of Reference Material

Sources of Reference Material Fungal cultures and DNA
Please contact the Queensland Department of Primary Industries & Fisheries Plant Pathology Herbarium (BRIP) for isolates of M. musicola and M. fijiensis to be used as positive controls in these assays.
Please note that only cultures of M. musicola, and heat-treated or -irradiated plant tissue infected with M. fijiensis and M. musicola, are held by the Plant Pathology Herbarium. Plant Pathology Herbarium,
Qld Dept of Primary Industries & Fisheries,
Plant Pathology Building,
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Further Reading

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