Fungi associated with banana leaf diseases in South Africa

BY

ANGELA KAREN JOANNA SURRIDGE

A thesis submitted in partial fulfillment of the requirements for the degree of

MAGISTER SCIENTIAE

In the Faculty of Natural and Agricultural Sciences, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa

2002

Study leaders: Dr A. Viljoen

Prof P.W. Crous

Prof F.C. Wehner

For Mum, Dad, Daniel, Aunty Barbara and Uncle Eddie

and in loving memory of my treasure, Blaxie.

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ACKNOWLEDGEMENTS

Special thanks to Riana Jacobs, Ewald Groenewald, Martin Coetzee, Nonnie Grimbeek, Eduard Venter and Dirk Swanevelder for their invaluable support during the course of this degree. Also to the personnel of the Electron Microscopy Unit at the University of Pretoria, the Banana Growers Association of South Africa and the National Research Foundation.

PREFACE

Bananas are classified in the genus *Musa*, named after Musa, physician of Augustus Caesar (Bailey 1900). The origin of banana is not known but it is accepted that primitive man in the tropical regions of South Asia developed the seedless fruit from wild varieties (Graves & Taber 1942). Further selection of seedless varieties lead to the development of the vegetatively propagated edible banana of today, which serves as staple food in over 120 countries world-wide (Jones 2000). The Latin-American-Caribbean, African and Asian-Pacific regions each produce approximately a third of the total global banana crop. Banana is an appealing crop for subsistence farmers in tropical and subtropical regions as it can be produced throughout the year and regenerates after each harvest, ensuring a constant income and food source.

Bananas were first introduced into the KwaZulu-Natal region of South Africa at the turn of the nineteenth century by Indian labourers working on sugarcane farms (Viljoen 2002). Commercial banana production began in this region and gradually expanded to the Limpopo and Mpumalanga lowveld in the 1950's. By 1981, the South African banana industry comprised 9 200 ha, and presently is established on 13 000 ha. During this time banana production has doubled from 120 000 to 240 000 tonnes per annum, with the entire crop being sold on local markets. However, due to the marked increase in production, South African banana growers have, since June 2001, started exporting their produce to countries in the middle East.

Various pathogens, including fungi, bacteria, viruses and nematodes, are known to attack banana plants. Leaf diseases pose a significant threat to banana production globally. Currently, black Sigatoka is considered to be the most threatening, but there are at least 16 other fungal pathogens infecting banana leaves. Infection results in loss of photosynthetic area and, depending on the disease and its severity, loss in yield can be as high as 100 %. Leaves are unable to produce sufficient starches and sugars necessary for optimal crop production, fruit are smaller, ripen unevenly and have an unacceptable appearance and taste. Symptoms of some of the diseases, e.g. black Sigatoka, yellow Sigatoka and eumusae leaf spot, are very similar, rendering diagnosis difficult. Rapid spread of the pathogens occurs through inoculum carried by air currents, in water and on leaf material used during packing and transport. Subsistence farmers cannot afford chemicals for the control of these diseases and more tolerant cultivars are not always acceptable to the consumer.

Very little information has been published on banana foliage diseases in South Africa. Roth (1965) isolated various fungi, including two known pathogens, from symptomatic banana leaves in the Mpumalanga lowveld. Subsequently, Van den Boom & Kuhne (1969) and Brodrick (1973) reported the presence of yellow Sigatoka and Mycosphaerella speckle, respectively, from South Africa. However, isolation, identification and molecular characterisation of the causal agents have not been attempted. A need, therefore, exists for more local information on banana leaf diseases, particularly verification of the identity of the causal organisms. This dissertation obliges accordingly by describing the identity, diversity and distribution of fungi associated with lesions on banana leaves in South Africa.

The first chapter comprises a review of fungal pathogens, saprobes and endophytes recorded from banana leaves. A brief overview of the banana plant is given to acquaint the reader with the host. The most economically important diseases are highlighted and a summary is provided of their biology and control. Less threatening diseases are briefly discussed to promote awareness of their presence and the extent of damage they cause. Chapter two describes a survey of pathogenic, saprobic and endophytic fungi isolated from lesions on banana leaves in the various banana-growing regions of South Africa. Chapters three and four report on morphological, molecular and phylogenetic studies to establish the identity of the causal agents of yellow Sigatoka and Mycosphaerella speckle, respectively, whereas Chapter five represents a first report of a speckle disease occurring only in the Levubu area.

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ANGELA KAREN JOANNA SURRIDGE

Supervisor: Dr A. Viljoen

Co-supervisors: Prof P.W. Crous

Prof F.C. Wehner

Department: Microbiology and Plant pathology

Degree: MSc

RESUMÉ

Leaf diseases are an integral part of banana production. While currently not a cause for major concern in South Africa, many of these diseases can reach epidemic proportions and cause severe crop loss. To determine the present status of leaf diseases in South Africa, a survey was conducted in the five banana-growing regions of the country. The study indicated the following:

Yellow Sigatoka, caused by *Mycosphaerella musicola*, was the most prevalent disease and occurred in all five the regions. Mycosphaerella speckle and Cordana leaf spot, caused by *M. musae* and *Cordana musae* respectively, were present in four regions. Cladosporium speckle, caused by *Cladosporium musae*, was found only in the Levubu area. Various other fungi, mainly

saprobes and endophytes, were also isolated. The most commonly encountered species included *Alternaria alternata, Colletotrichum gloeosporioides, Nigrospora oryzae, N. sacchari, N. sphaerica, Pestalotiopsis* sp., *Phoma glomerata, Selenophoma asterina* and *S. juncea.*

Following morphological identification of the pathogenic species, monoconidial isolates were established from representative isolates of each and their virulence confirmed in artificial inoculation studies. The identity of *M. musciola* and *Cladosporium musae* was verified molecularly by means of species-specific primers and/or sequencing of the ITS region. Validation of the identity of *Cladosporium musae* constitutes the first report of Cladosporium speckle on banana in South Africa. Sequence data of the ITS region of isolates from Mycosphaerella speckle lesions indicated that the symptoms are caused by two species, *M. musae* and one closely related to *M. colombiensis*, the latter previously described only from lesions on leaves of *Eucalyptus urophylla* in Colombia.

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deur

ANGELA KAREN JOANNA SURRIDGE

Leier: Dr A. Viljoen

Mede-leiers: Prof P.W. Crous

Prof F.C. Wehner

Departement: Mikrobiologie en Plantpatologie

Graad: MSc

SAMEVATTING

Blaarsiektes maak 'n integrale deel uit van piesangverbouing. Alhoewel hierdie siektes tans nie groot rede tot kommer bied in Suid-Afrika nie, kan heelwat van hulle epidemiese afmetings aanneem en ernstige oesverliese veroorsaak. 'n Opname om die huidige stand van blaarsiektes in die vyf piesang-produserende gebiede van Suid-Afrika te bepaal, het die volgende aan die lig gebring:

Geel Sigatoka, veroorsaak deur *Mycosphaerella musicola*, was die algemeenste siekte en het in al vyf die gebiede voorgekom. Mycosphaerella-spikkel en Cordana-blaarvlek, veroorsaak deur *M. musae* en *Cordana musae* onderskeidelik, was teenwoordig in vier van die gebiede.

Cladosporium-spikkel, veroorsaak deur *Cladosporium musae*, is slegs in die Levubu-gebied aangetref. Verskeie ander swamme, hoofsaaklik sapro- en endofiete, is ook geïsoleer. Die algemeenste spesies was *Alternaria alternata, Colletotrichum gloeosporioides, Nigrospora oryzae, N. sacchari, N. sphaerica, Pestalotiopsis* sp., *Phoma glomerata, Selenophoma asterina* en *S. juncea.*

Na morfologiese identifisering van die patogeniese spesies is enkelspoorisolate berei van verteenwoordigende isolate van elkeen en hulle virulensie bevestig by wyse van kunsmatige infeksiestudies. Die identiteit van *M. musicola* en *Cladosporium musae* is molekulêr bevestig deur gebruik te maak van spesie-spesifieke peilstukke en/of basispaaropeenvolgingbepaling van die ITS-gebied van die rDNA geenkompleks. Bewys van die identiteit van *Cladosporium musae* verteenwoordig die eerste aanmelding van Cladosporium-spikkel op piesang in Suid-Afrika. Basispaaropeenvolgingsdata van die ITS-gebied van isolate vanaf Mycopshaerella-spikkel letsels dui daarop dat die symptome veroorsaak word deur twee spesies, *M. musae* en 'n naverwante spesie met *M. colombiensis*, laasgenoemde tot dusver beskryf slegs vanaf letsels op blare van *Eucalyptus urophylla* in Colombia.

CHAPTER 1

BANANA FOLIAGE DISEASES – AN OVERVIEW

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INTRODUCTION

The banana (*Musa*) plant is the world's largest herbaceous monocotyledon. It originates from Southeast Asia and is grown in more than 120 countries globally (Jones 2000). Bananas are the world's fourth most valuable food product, and serve as the primary source of food for over 400 million people in the tropics (Picq & Ammar-Khodja 2000). World trade totals US$ 5.3 billion annually (Sasson 1997), which represents only 10 % of the 90 billion tonnes of bananas produced worldwide each year (Ploetz 1999, Picq & Ammar-Khodja 2000). The remaining 90 % are sold on local markets by subsistence farmers.

Many fungi, bacteria, viruses and nematodes are known to attack banana plants and cause disease to the roots, corm, pseudostem, leaves and fruit. Banana plants can also be subjected to a number of disorders, mineral deficiencies, physical damage, chemical damage and genetic abnormalities (Jones 2000). Fungal diseases that affect banana foliage decrease the photosynthetic area of infected leaves, thereby reducing fruit yield and quality (Jones 2000). Chemical control of leaf diseases is expensive and not financially feasible for producers in developing countries. Some leaf pathogens also develop resistance/tolerance to particular fungicides, making them even more difficult to control (Jones 2000). International breeding programs have developed hybrids that are genetically resistant to specific leaf pathogens (Jones 2000), but these hybrids do not always have acceptable fruit and agronomic qualities.

The principal fungal leaf diseases on banana include Sigatoka-like leaf spots, speckle diseases and freckle. Several fungi are also considered to be minor pathogens on banana leaves. This overview addresses the distribution, symptoms, causal agents, disease cycle, epidemiology and control measures of banana foliage diseases. Also included is a brief summary of the endo- and epiphytes recorded to date on banana foliage.

THE BANANA PLANT

The banana plant migrated, with travellers, from Southeast Asia to the Indian peninsula, eastern Africa and the islands of the Pacific. There are approximately 1000 different types of banana varieties known in the world (Picq & Ammar-Khodja 2000). These can be subdivided into 50 groups, of which some can be eaten raw as a sweet dessert banana, or fried, steamed and cooked as a vegetable. Consumer demand for bananas began to increase during the nineteenth century, and today bananas are exported from countries in Central and South America, and South-East Asia, to markets in North America, Europe, Japan and countries of Eastern Europe and the former USSR (Picq & Ammar-Khodja 2000).

The banana plant is a perennial that rejuvenates itself approximately once a year (Picq & Ammar-Khodja 2000). It has a branched underground stem known as a rhizome or corm that gives rise to both roots and vegetative buds (Fig. 1) (Jones 2000, Picq & Ammar-Khodja 2000). The root system is relatively shallow and usually only penetrates about 60 cm vertically into the soil, but it has a horizontal spread of up to 5 m (Jones 2000). After the fruit set the mother plant is

cut down, and the new vegetative buds, known as "suckers", develop and form the pseudostem (Jones 2000). The pseudostem consists of tightly packed leaf bases with an apical meristem in the centre at soil level, giving rise to successive leaf primordia (Jones 2000). Each of these primordia differentiate into a leaf base, a petiole and a lamina. After about 25–50 leaves have been produced, the vegetative core stops producing leaves and produces, instead, an inflorescence. The peduncle of the inflorescence forces its way out of the top of the plant, lifting the bracts and allowing for the double layers of female nodes with compact fruit to emerge. Each individual banana fruit is referred to as a "finger" of the "hand", and the collective term for the "hands" is a "bunch" (Jones 2000). There are 10–15 fully functional leaves at flowering, which decrease to 5–10 leaves at harvest. The most efficient leaves for photosynthesis, counting down the plant, are leaves 2–5, as lower leaves tend to be too old (Robinson & Peterson 1999). After harvest the pseudostem dies back and is cut to ground level.

BANANA LEAF DISEASES

SIGATOKA DISEASES

Three Sigatoka diseases cause damage to banana leaves, namely yellow Sigatoka, black Sigatoka and eumusae/septoria leaf spot. Yellow Sigatoka was first recorded in Java in 1902 and later in the Sigatoka valley of Fiji (Meredith 1970). The first notable losses to the disease occurred in Fiji in 1912 and in 1924 in Australia (Wardlaw 1961, Stover 1972). Since 1930, yellow Sigatoka has disseminated rapidly due to uncontrolled movement of infected propagative material to the continents. By 1933 the Caribbean was experiencing serious crop yield losses,

and by 1937 yellow Sigatoka was taking its toll in all the banana growing areas of Columbia, the Guineas, Central America, Jamaica, Suriname, Demerara, British Honduras, French Antilles, Peru, Brazil, Mexico and the Caribbean islands (Wardlaw 1961, Stover 1972). In East Africa, by 1938, and West Africa, by 1941, the disease began to have an affect on the African banana industry (Wardlaw 1961, Stover 1972). In 1950, Ecuador became the last mass production banana area to be affected (Stover 1972). The disease now occurs in all banana-growing countries except Egypt, Israel and the Canary Islands (Carlier *et al.* 1994, Jones 2000).

Yellow Sigatoka can cause severe losses in banana production. Infection damages the leaves, thereby reducing the area of functional leaf surface and photosynthetic capacity within the plant's cells (Jones 2000). After shooting, when no new leaves are being produced, those damaged by yellow Sigatoka cannot be replaced, and the yield is affected (Jones 2000). If the disease reaches its peak just before harvest, the fruit ripen prematurely and unevenly, and some appear undersized and angular. The flesh has a buff pink colour and fruit storage time is greatly reduced (Agrios 1997). The greater and earlier the leaf damage the more pronounced the effect on yield (Jones 2000). Banana production can be reduced by as much as 50%, or even more in badly affected areas (Wardlaw 1961). The disease appears to have little or no effect on vegetative growth of banana plants in the tropics. Leaf emergence, rate of plant height increase and height of plants at shooting are not reduced as a result of infection. The reason for this is that the disease appears not to effect leaves 2–5, the main photosynthetic producers, as severely as the other leaves (Jones 2000).

Black Sigatoka was first noticed in the Sigatoka area of the Viti Levu Island in Fiji in February 1963 (Carlier *et al.* 2000a). Surveys conducted between 1964 and 1967 revealed that

black Sigatoka was spreading rapidly in the Pacific. It was reported to be present in Micronesia, New Caledonia, Papua New Guinea, Philippines, Western Samoa, Singapore, Solomon Islands, Tahiti, Taiwan, Tonga, Vanuatu, West Malaysia, Hawaiian Islands, Cook Islands and Niue. It is thought that black Sigatoka may have arrived in the Hawaiian Islands as early as 1958 and that it had been mistaken for yellow Sigatoka. Old herbarium specimens indicate that black Sigatoka was present in Taiwan from 1927 and in Papua New Guinea from 1957. In 1970, black Sigatoka was found in the Philippines on many different cultivars, but is considered to have already arrived on the island of Luzon in about 1964. Jones (1994) reported that black Sigatoka was first discovered in North Africa in 1973 and has since spread throughout East and West Africa. In 1980, both black and yellow Sigatoka were observed on the Hainan Island of China, and since then it has spread throughout southern China. In 1985 and 1993 black Sigatoka was recorded in Bhutan and Vietnam, respectively, however it has not yet been recorded in Burma, India or Bangladesh (Carlier *et al.* 2000a). In many tropical areas black Sigatoka has replaced yellow Sigatoka as the dominant leaf spot within a year. Yellow Sigatoka, however, still dominates at altitudes above 1200–1400 m in tropical regions (Mourichon *et al.* 1997).

Black Sigatoka is far more aggressive than yellow Sigatoka and affects a wider range of banana genotypes (Carlier *et al.* 2000a). It develops much faster and causes more severe damage to banana leaves. In many actively growing plants, the second leaf is often affected by the disease. Once bunching has taken place and no more leaves are produced, all remaining leaves are killed by the fungus, thereby forcing the plant to abort the bunch. Rapid destruction of the leaves of the banana plant leads to reduction in yield and premature ripening of the fruit.

During a survey for yellow and black Sigatoka on bananas in southern India, Sri Lanka, West Malaysia, Thailand and Vietnam between 1992 and 1995, an unknown pathogen causing symptoms similar to those of yellow and black Sigatoka was isolated (Carlier *et al.* 2000b, Carlier *et al.* 2000c). Since this pathogen produced a *Septoria*-like anamorph and *Mycosphaerella* teleomorph, the disease was tentatively named Septoria leaf spot (Carlier *et al.* 2000b, Carlier *et al.* 2000c). Upon further investigation it was renamed eumusae leaf spot (Crous & Mourichon 2002). Eumusae leaf spot now appears to be the dominant leaf spot disease of bananas in Thailand (Carlier *et al.* 2000c). This disease has also been found on banana leaves in Onne, Nigeria and in Mauritius (Carlier *et al.* 2000c)

Symptoms

Yellow Sigatoka exhibits five stages of symptom development on banana leaves (Table 1, Fig. 2). Initially it manifests as small (1 mm long) pale yellow flecks on the third or fourth leaf from the top of the plant (stage 1) (Fig. 2A). When conditions are favourable for infection, even the second leaf can show early symptoms (Simmonds 1959, Wardlaw 1961, Jones 1994, Jones 2000). These spots are indistinct and longitudinal, running parallel to the side veins of newly unfolded leaves (Wardlaw 1961, Agrios 1997, Mourichon *et al.* 1997). The flecks then begin to elongate into yellow green streaks, 1 x 3–4 mm (stage 2) (Fig. 2B) (Wardlaw 1961, Jones 1994, Jones 2000). Streaks enlarge to 1–2 cm in length, and their centres become dark brown or rusty red in colour (stage 3) (Fig. 2C) (Wardlaw 1961, Jones 1994, Agrios 1997). They develop definite margins with yellow, sometimes water-soaked, halos (stage 4) (Fig. 2D) (Jones 1994). Finally, the centres of lesions appear to sink and turn a light grey colour (stage 5) (Fig. 2E) (Meredith 1970, Agrios 1997, Robinson & Peterson 1999). Lesion margins, however, remain

dark brown to black, often retaining their yellow halo (Wardlaw 1961, Jones 1994, Robinson & Peterson 1999). Halos may turn dark brown as well, forming a clear ring around the mature spot (Robinson & Peterson 1999, Jones 2000). Individual spots on mature leaves are ellipsoidal and 2–5 x 12–15 mm in size (Fig. 2E) (Jones 1994). On young sucker shield leaves, the spots are usually oval or round (Jones 1994). When these spots begin to enlarge the damage to the banana plant becomes more severe. Tissue around the enlarging spots yellows and dies, and adjacent spots begin to coalesce, leading to larger areas of dead tissue on the leaf (Fig. 2F) (Simmonds 1959, Wardlaw 1961, Agrios 1997, Jones 2000). With high infection intensity the necrotic leaf areas turn white-grey and the brown borders of individual lesions become indistinct (Fig. 2F) (Robinson & Peterson 1999, Jones 2000). Mature spot symptoms are found on older leaves and infection severity appears to increase on older leaves (Jones 2000). In very severe cases, the affected leaves can die within a few weeks, leaving less than the minimum of nine leaves required for maturation of the fruit (Robinson 1996). On dead leaves distinct brown-black lesion margins are visible on the dead brown background (Jones 1994, Jones 2000).

Initial symptoms of black Sigatoka appear as chlorotic or red-brown streaks less than 0.25 mm in diameter on the lower surface of the leaf (Jones 1994). These initial streaks elongate to 20 x 2 mm and are parallel to the leaf veins, often so close that they overlap (Carlier *et al.* 2000a). During the second stage of symptom development, streaks turn a very dark brown-black in colour with a water-soaked halo (Fig. 3A) (Carlier *et al.* 2000a). Lesions become fusiform or elliptical with depressed centres, and they are surrounded by a yellow halo maintaining its water-soaked appearance (Jones 1994). The third stage results in the centre of the lesion turning a pale grey colour with a black margin surrounded by a yellow halo (Fig. 3B) (Carlier *et al.* 2000a). Where spots are coalescing entire sections of the leaf become necrotic and die, but lesions are still

visible due to their pale centres (Fig. 3C and D) (Carlier *et al.* 2000a). On healthy plants that are still growing, streaks are seen on the third, fourth and fifth leaves, whereas on stressed plants symptoms are often seen on the first and second leaves (Carlier *et al.* 2000a). On resistant cultivars, symptoms are usually only seen on the lower leaves, dependent on the resistance levels of the cultivar (Carlier *et al.* 2000a). Susceptible cultivars, however, may loose all their leaves and bunches will drop to the ground before maturing (Jones 1994).

Eumusae leaf spot lesions have an appearance very similar to that of yellow and black Sigatoka (Carlier *et al.* 2000c). It seems that in the past this disease has been mistaken for either of the Sigatoka diseases, as its causal organism has only recently been identified (Carlier *et al.* 2000c). Brown streaks expand into large spots that darken, the centres turn grey and develop a distinct dark brown border, and are ovoid or elliptical in shape when mature (Fig. 4A) (Carlier *et al.* 2000b, Carlier *et al.* 2000c, Crous & Mourichon 2002). Leaf spots are larger and more rounded (Fig. 4B) than the streaks seen in black and yellow Sigatoka infections, they closely resemble the lesions caused by Phaeoseptoria leaf spot (Carlier *et al.* 2000c). On growing plants, streaks are present on the third, fourth and fifth leaves and streaks as well as spots are present on the fifth and older leaves (Crous & Mourichon 2002). At high infection densities lesions coalesce and cause large areas of leaf tissue to become necrotic. Grey spots are visible in the necrotic area and a yellow halo precedes the expanding necrosis (Fig. 4C) (Carlier *et al.* 2000c). Where coalescence has occurred, streaks on the upper surface change from brown to black while those on the lower surface remain brown (Crous & Mourichon 2002).

Causal agents

Yellow Sigatoka is caused by *Mycosphaerella musicola* Leach ex J.L. Mulder & R.H. Stover (anamorph *Pseudocercospora musae* (Zimm.) Deighton), a heterothallic ascomycete in the order *Dothideales*, family *Mycosphaerellaceae*. It reproduces by means of ascospores in pseudothecia (Fig. 5A) and conidia in sporodochia (Fig. 5B) (Agrios 1997). Pseudothecia form after fertilisation of receptive hyphae by compatible spermatia (Agrios 1997), and contain 10–27 asci each (Stover 1972). They are more abundant in mature spots on the upper than on the lower leaf surface (Jones 2000). Sporodochia appear during the brown spot stage (stage 4) and last throughout the mature spot stage (stage 5) of yellow Sigatoka symptom development (Agrios 1997). Sporodochia are produced on both sides of the leaf, but are more abundant on the upper surface where wind and/or rain dispersal occurs (Agrios 1997). They develop in the sub-stomatal air chamber and emerge through the stomatal pore (Jones 2000). Up to 100 conidiophores can form in a sporodochium at one time, each producing conidia (Jones 1994). Infection by either ascospores or conidia results in the same type of spot and disease development (Agrios 1997).

Mycosphaerella fijiensis Morelet is the causal agent of black Sigatoka on banana foliage. Its anamorph was initially placed in *Cercospora*, but is currently known as *Pseudocercospora fijiensis* (M. Morelet) Deighton (Carlier *et al.* 2000a). *Mycosphaerella fijiensis* also reproduces by means of conidia formed in a stroma (Fig. 6A) and ascospores formed in ascostroma (Fig. 6B). In the case of *M. fijiensis,* ascospores are the dominant means of reproduction. Ascostroma are found on both leaf surfaces but more abundantly on the upper surface (Carlier *et al.* 2000a). Ascospores are two celled, slightly constricted at the septum. Conidia are formed singly at the conidiophore apex only later becoming lateral with the development of the conidiophore (Carlier

et al. 2000a). Four mature conidia may be attached to a conidiophore at any time (Carlier *et al.* 2000a).

Mycosphaerella eumusae Crous & Mourichon (anamorph *Pseudocercospora eumusae* Crous & Mourichon) causes eumusae leaf spot lesions (Crous & Mourichon 2002). It produces two distinct types of fruiting structures on the upper surface of leaf lesions (Carlier *et al.* 2000b, Carlier *et al.* 2000c). The asexual stage comprises pycnidia that are amphigenous, hypophyllous, immersed, erumpent, flask-shaped and ostiolate when young (Carlier *et al.* 2000b, Carlier *et al.* 2000c, Crous & Mourichon 2002). Contained within the pycnidia are conidia that are pale brown to sub-hyaline, fusiform and 3–5 septate (Carlier *et al.* 2000b, Carlier *et al.* 2000c). The sexual stage manifests as globose perithecia each with a with short protruding ostiole, dark brown in colour (Carlier *et al.* 2000b, Carlier *et al.* 2000c). Asci are aparaphysate, fasciculate, bitunicate, subsessile, obovoid and straight or slightly curved with eight spores. Ascospores are tri- to multiseriate overlapping, hyaline, guttulate, thick-walled, straight, obovoid with obtuse apices (Crous & Mourichon 2002). They are uniseptate and widest in the middle of the apical cell, however the basal cell may be longer (Carlier *et al.* 2000b, Carlier *et al.* 2000c, Crous & Mourichon 2002).

Mycosphaerella fijiensis, *M. musicola* and *M. eumusae* are morphologically very similar, and can be distinguished on conidial and conidiophore characteristics only (Table 3) (Mourichon *et al.* 1997, Carlier *et al.* 2000a, Carlier *et al.* 2000b). *Mycosphaerella fijiensis*, unlike *M. musicola* and *M. eumusae*, does not form sporodochia and produces few conidiophores on the lower leaf surface mainly (Meredith 1970). *Mycosphaerella musicola* and *M. fijiensis* have been shown, using restriction fragment length polymorphism (RFLP) and internal transcribed spacer

(ITS1) region sequence analyses, to be two distinct species (Carlier *et al.* 1994). It has also been proven that *Mycosphaerella* species can be easily distinguished by random amplification of polymorphic DNA (RAPD) using 10-mer or more primers (Johanson *et al.* 1994). Johanson *et al.* (1994) indicated that *M. musicola* and *M. fijiensis* differ significantly at DNA level. Johanson and Jeger (1993) developed a set of species-specific primers for molecular detection of *M. fijiensis* and *M. musicola* from infected leaf material. Johanson *et al.* (1994), used the PCR-based technique of RAPD analysis to differentiate between *M. fijiensis* and *M. musicola*. Certain sequences present in one fungus but not the other show up during DNA amplification using a sequence specific primer for each (Johanson *et al* 1994, Molina *et al.* 2001). Etienne *et al.* (1997), developed an enzyme linked immunosorbent assay (ELISA), based on polyclonal antibodies, to detect the *Mycosphaerella* species causing both yellow and black Sigatoka on bananas.

Disease cycle and epidemiology

Spread of Sigatoka leaf diseases over long distances is thought to occur via the movement of infected banana germplasm, suckers and leaves, and by means of wind-borne ascospores (Mourichon *et al.* 1997). Once introduced into a plantation, the Sigatoka pathogens are disseminated within the plantation by means of conidia and ascospores (Fig. 7) (Jones 1994, Mourichon *et al.* 1997). Conidia are the primary agents of infection in *M. musicola,* and ascospores in *M. fijiensis* (Jones 2000). Conidia are usually dislodged and disseminated by raindrops or wind (Jones 2000), and infect the unfurling heart-leaves of the same and nearby plants (Jones 2000). Older leaves are less susceptible to infection than younger leaves (Jones 1994). Perithecia are abundant in lesions of recently dead leaf tissue. Ascospores are forcibly

ejected from perithecia when necrotic tissue was saturated for approximately 48 hours during periods of high relative humidity after rain or dew (Jones 1994). They are disseminated within a plantation or over long distances (up to 50 km) by wind currents, and result in the characteristic apical spotting of infected leaves.

Relative humidity and temperature play an important role in the life cycle of Sigatoka pathogens. Under moist conditions, ascospore germination occurs on the lower leaf surface within 2–3 hours of initial attachment, whereas conidia may take a little longer (Jones 2000). Optimal temperature for germination of conidia is 25–29 °C and 25–26 °C for ascospores in *M. musicola* (Jones 2000). In *M. fijiensis*, this temperature is about 1–2 °C higher (Carlier *et al.* 2000a). After germination, the fungus grows epiphyllically for up to 6 days before producing an appressorium and penetrating the leaf (Jones 1994). Conditions required for stomatal penetration include humidity near saturation point and temperatures above 20 °C. Hyphae, therefore, may pass over some stomata before appressorium formation is initiated (Stover 1972). Germ-tubes collapse in hot dry weather and will only elongate under moist conditions. Free water on the leaves enables an infection, most often on the lower leaf surface (Jones 1994). Factors contributing to symptom development are cultivar resistance, infection intensity and environmental conditions (Jones 1994). Heavy infection of a susceptible cultivar, under favourable conditions, results in streaks progressing to spots within 10–15 days, with extensive leaf death following soon afterwards (Jones 1994). If conditions are unfavourable, disease symptoms can take as long as 105 days to appear (Jones 2000). Yellow Sigatoka follows a seasonal pattern that begins when night temperatures rise above 18°C and relative humidity increases to >92 % (Jones 2000). Temperatures required for black Sigatoka infection to take

place are 2–3 °C higher (Carlier *et al.* 2000a). Besides climate, other factors can affect disease development and intensity, such as the physiological state of the plant and light intensity. Healthy plants are in a better position to resist disease, lesions/symptoms can be repressed by shade (Jones 2000).

The epidemiology of *M. eumusae* has not yet been studied and no research has been conducted on the disease cycle in the field. However, its *Mycosphaerella* teleomorph and similar morphology and symptoms indicate that its epidemiology and disease cycle may correspond with those of *M. fijiensis* and *M. musicola*.

Control

Sigatoka leaf diseases can be managed using integrated disease control measures. These measures are based primarily on cultural, chemical and genetic control. Sigatoka diseases in commercial plantations are predominantly controlled by the use of fungicides, while control on subsistence farms involves mainly cultural practices.

Cultural control

The primary objective of cultural control is the reduction of fungal inoculum levels in banana plantations. Regular removal and destruction of leaves showing Sigatoka symptoms is, therefore, recommended (Meredith 1970). In the tropics, this practise should take place throughout the year. In the sub-tropics, deleafing should be carried out 4–6 weeks before the wet season. Since ascospores can survive in dead leaves hanging from banana plants for several weeks, deleafing reduces the ascospore production by about 85 % (Jones 2000). Banana leaves

can be buried or piled on top of each other to reduce ascospore spread even further (Meredith 1970, Jones 2000). Planting density affects the ventilation and relative humidity within a plantation. When bananas are planted too closely, the humidity is increased which favours infection by the pathogen (Meredith 1970). Irrigation should be by means of drip and not overhead sprinkle systems, as the latter increases dissemination of the pathogen within the plantation.

Resistant cultivars

Resistant cultivars offer practical control for small-scale subsistence farmers who cannot afford chemical control measures (Mourichon *et al.* 1997). International breeding programmes have made substantial progress in developing resistant cultivars of both dessert and cooking bananas (Jones 1994). Many cooking banana cultivars are resistant to Sigatoka diseases and can be used to replace susceptible plantain species (Carlier *et al.* 2000a). However, consumer taste discernment plays a role in the acceptability of new cultivars on the market.

Biological control

Biological control is, as yet, not a viable option for control of Sigatoka. However, epiphyllic fungi on the leaves of banana have been reported to inhibit the germination of *M. musicola* spores (Meredith 1970).

Chemical control

The first fungicide used for the control of Sigatoka diseases was Bordeaux mixture (Jones 2000). This was effective in preventing infection by conidia but not infection of the unfurling cigar leaf by ascospores. Zineb, copper oxychloride suspended in mineral oil, or mineral oil on

its own, give good control of yellow Sigatoka (Agrios 1997, Jones 2000). The contact dithiocarbamate fungicides, maneb and mancozeb, give reasonable control of yellow Sigatoka when applied in oil-water emulsions (Jones 2000). Mancozeb and mineral oil is currently the only fungicide registered for the control of Sigatoka in South Africa (Nel *et al.* 1999). Recently, highly effective ergosterol-inhibiting fungicides have been developed that have revolutionised disease control (Jones 1994). Fungicides of this group most commonly used for yellow Sigatoka control are the triazoles (e.g. propiconazole, systemic, and flusilazole, non-systemic). The strobilurine fungicide group, especially azoxystrobin, showed exceptional control of yellow Sigatoka and a reasonable control of black Sigatoka when used in integrated control programmes (Knight *et al.* 2002).

Fungicides can be applied to banana leaves as a fine mist by mist-blowers on the ground or aerially by helicopters/fixed-wing planes. It is important to alternate schedules between contact and systemic fungicides. Continuous use of systemic fungicides can lead to resistance, and it is recommended that less that six sprays of the same systemic fungicide should be applied in one season. Because of the higher day and night temperatures throughout the year in the tropics, more applications are necessary for the control of Sigatoka diseases. More sprays are necessary for the control of black than yellow Sigatoka. In subtropical countries, the number of sprays are reduced significantly because of the cooler night time temperatures and dryer conditions during winter. A disease monitoring and forecasting system is often used to predict climatic conditions favourable for new infections to occur. By using such systems, fungicide applications can be reduced from 25–40 down to about 10–12 per year (Jones 1994). Fungicidal sprays in the subtropics and tropics are often combined with additional control strategies in an integrated disease management programme.

SPECKLE DISEASES

Several diseases cause speckle symptoms on banana foliage, namely Mycosphaerella speckle, Cladosporium speckle, leaf speckle and tropical speckle (Jones 2000). These are considered to be of minor economic importance, as they usually do not cause extensive damage. However, under favourable climatic conditions they may become severe. Mycosphaerella speckle and Cladosporium speckle have been reported to cause leaf death in the subtropical regions of Australia (Jones 2000) and West Africa (Frossard 1963), respectively. Since 1981, leaf speckle has been considered to be a major disease of Cavendish bananas in Taiwan (Jones 2000). Tropical speckle is not regarded as a significant disease in any of the areas in which it is found. Of these diseases, Mycosphaerella speckle has predominantly been found in sub-tropical regions, while the other three are restricted to tropical areas.

Mycosphaerella speckle

Mycosphaerella speckle symptoms comprise light brown to tan coloured irregular blotches on the lower leaf surface, and smoky patches on the upper surface (Fig. 8) (Stover 1972, Jones 2000). Lower surface symptoms darken eventually to purple-black, irregularly shaped, speckled areas visible on both leaf surfaces. These blotches coalesce to form large necrotic areas that dry out and bleach with time to grey and yellow on the lower and upper leaf surfaces, respectively. Symptoms are seldom present above the fifth leaf on a banana plant (Stover 1972), and extensive leaf tissue death is usually not seen above leaf eight on actively growing plants. Nevertheless, when bunch emergence begins and leaf production ceases, severe defoliation can occur before harvest.

Mycosphaerella speckle is caused by *Mycosphaerella musae* (Speg.) Syd. & P. Syd.. The asexual stage of the fungus has not been observed on banana leaves, though *Cercospora* type conidia are produced in culture. Ascospores land on the leaf surface and produce germ tubes that grow five times faster than those of *M. musicola* (Stover 1972). Perithecia develop in large quantities on the lower leaf surface as the necrotic leaf tissue dries out (Jones 2000). At this stage, perithecia, similar to those of *M. musicola*, become visible in dead tissue and asci are forcibly released when perithecia are wet (Stover 1972, Jones 2000). Mycosphaerella speckle development is favoured by high humidity and symptoms can appear within 45 days in a saturated atmosphere (Jones 2000). In an unsaturated environment, symptoms only occur after 80–120 days. *Mycosphaerella musae* is always more prevalent in sheltered areas like hollows or moist areas in plantations, and the disease develops more quickly on senescing or injured leaves. Infection does not take place on younger leaves, and advanced symptoms of the disease are seen mainly on older leaves (Jones 2000). Temperatures below 20 °C retard disease development, and plant growth, resulting in speckle symptoms being more pronounced in winter in subtropical areas (Jones 2000).

Fungicides of the fixed copper and dithiocarbamate groups are used as oil-in-water emulsions for the control of *M. musae* (Stover 1972). Copper oxychloride, maneb and zineb have given good control when applied to the underside of leaves 4–6 (Stover 1972, Jones 2000). Control has also been successful when protectant fungicides were sprayed onto the lower surfaces of leaves 4–6. Fungicides such as mancozeb and propiconazole, those currently used in Sigatoka control, also provide acceptable control of Mycosphaerella speckle (Jones 2000). Leaves killed

by the fungus should be removed to reduce the level of inoculum and prevent inoculum build-up (Jones 2000).

Cladosporium speckle

Cladosporium speckle is caused by *Cladosporium musae* E.W. Mason, a weak pathogen affecting mainly mature leaves in humid climates (Stover 1972, Jones 2000). Although the disease is considered to be a minor problem, it can cause damage to susceptible cultivars under favourable environmental conditions (Jones 2000). Badly diseased leaves dry out and drop prematurely, lowering the photosynthetic capacity of the plant (Jones 2000). Cladosporium speckle has been reported in Australasia-Oceania, Asia, Africa and the Latin American-Caribbean region (Jones 2000).

Cladosporium speckle manifests as a diffuse grey-brown blotching on the upper surface of older leaves (Jones 1994). These lesions become yellow-orange and then necrotic with age (Fig. 9A) and are more commonly found along leaf margins (Jones 1994). Conidiophores that arise from a profuse epiphyllic mycelium on the adaxial surface can easily be observed with a hand-lens (Wardlaw 1961). Initial symptoms in West Africa appear as pale brown pencil-mark-like spots, approximately 0.3 x 1.5 mm and can be seen 3–4 weeks after unfurling of the youngest leaf (Stover 1972, Siboe 1994, Jones 1994, Jones 2000). Spots elongate into lesions of 15 x 30 mm that coalesce turning yellow, then violet-black (Fig. 9B) (Stover 1972, Jones 1994, Jones 2000). In Uganda, Malaysia and Thailand symptoms are similar to those described above. Initial symptoms are small spots that elongate into grey and later brown streaks, and can be easily mistaken for black Sigatoka (Jones 2000). At high levels of infection the grey lesions coalesce

forming a larger orange region surrounded by a yellow halo (Jones 2000). These regions later turn a violet-black colour before the leaf tissue dies (Jones 2000). Lesions have been observed on leaf midribs and entire leaves can become necrotic (Jones 2000). Symptoms in Central America are initially a diffuse grey-brown blotching on the upper surfaces of the oldest leaves. This later turns yellow-orange and then becomes brown and necrotic (Jones 2000).

Cladosporium speckle development is favoured by high humidity (Jones 2000). Conidia of *C. musae* are carried on air currents and germinate in moisture on the leaf surface (Jones 1994, Jones 2000). Older leaves are the first to be infected, transferring inoculum to younger ones (Jones 2000). In Kenya, severe disease was reported to be present from the sixth leaf of naturally infected plants that have not yet flowered. Complete leaf damage, however, was observed on flowered plants at harvest (Siboe 1994). Infection severity is recorded as being varied between cultivars, with dessert bananas being the most susceptible and cooking bananas the most resistant (Siboe 1994).

Fungicides used in the control of yellow and black Sigatoka are effective against Cladosporium speckle of banana (Jones 2000). In Thailand the disease is controlled by fortnightly applications of benomyl during the rainy season (Jones 2000). Early removal of diseased leaves is recommended in Ethiopia to limit inoculum buildup(Jones 2000). On the Ivory Coast, 500 g of metallic copper per hectare is applied every 15 days in the Sigatoka oil spray (Stover 1972). Maneb sprays are preferred to copper for leaf diseases, as copper sprays may cause injury (Stover 1972). Excessive plant populations favour infection due to increased humidity. Reduced planting density, therefore, reduces speckling (Stover 1972).

Leaf speckle

Leaf speckle is a minor disease on banana foliage occurring in South-East Asia. However, in 1981 it was described as a threat to commercially grown "Cavendish" bananas in Taiwan (Jones 2000). Leaf speckle symptoms have also been recorded in Australia, Malaysia, Thailand and Vietnam. They appear as small dark brown specks densely aggregated on the lower leaf surface (Jones 2000). These develop into streaks that run parallel to the leaf veins up to 4 x 0.3 mm in size. Tan coloured blotches on the upper leaf surface are concentrations of these streaks. The affected areas eventually yellow and become necrotic (Fig. 10).

Acrodontium simplex (F. Mangenot) de Hoog (*Hyphomycetes*) causes leaf speckle on banana leaves (Jones 2000). Conidia usually cover the terminal portion of the conidiophore (Ellis 1967). Disease development of the leaf speckle pathogen is affected by climatic conditions (Jones 2000). Incubation is up to 35 days in hot, wet summer months and 60 days in cool, dry winter months (Ellis 1967, Jones 2000). Inoculum builds up on unsprayed Cavendish cultivars after shooting. In southern Taiwan climatic conditions influence disease development. Fungicides used in the control of yellow and black Sigatoka, containing dithiocarbamates in oil, are effective against leaf speckle (Jones 2000).

Tropical speckle

Tropical speckle has been observed on banana leaves in hot humid environments world-wide, but it is not perceived as a threat as it has no effect on either plant growth or yield (Jones 2000). Tropical speckle is found in the Asian-Pacific, Latin-America, the Caribbean and in Africa.

Tropical speckle manifests as two types of symptoms occurring on the same leaf. The first is initially a circular, chlorotic blotch, up to 4 cm in diameter, which progresses to form tan blotches abaxially and dark brown-black pinprick specks heavily distributed adaxially (Fig. 11A). Conidiophores are clearly visible within lesions and form a bristle-like, dense mass on the adaxial surface. The second is a dark, irregular blotch on lower surface consisting of black specks with extensive discoloured areas less distinct on the upper surface (Fig. 11B). Visible conidiophores are velvety within lesions and along the midrib and peduncle.

Two fungi are thought to be the cause of tropical speckle, namely *Veronaea musae* M.B. Ellis and *Periconiella musae* Stahel ex M.B. Ellis (*Hyphomycetes*). There are several differences between the two organisms. Conidiophores of *V. musae* are unbranched and shorter than those of *P. musae* that are branched and longer. Conidia of *V. musae* are oval and have tiny papillae at the point of attachment to the conidiophore, while those of *P. musae* are more elliptical in shape. *Periconiella musae* grows slower than *V. musae* on PDA although they have similar colony characteristics. Tropical speckle inoculum germinates within 24 hours of landing on the host and produces hyphae on the lower leaf surfaces (Jones 2000). It forms a loose epiphyllic network of branched hyphae, 1–2 µm thick (Wardlaw 1961). Club-shaped stomatopodia, that are darker than

the hyphae, form and infection tubes enter the leaf via stomata. Infection hyphae grow through the stomatal air space into palisade tissue, and side branches enter cells. The fungus does not spread further than the tissue surrounding the colonised stoma. Necrosis, therefore, is confined to the area of infection, resulting in a speckled appearance (Ellis 1967). Tropical speckle agents are considered as weak parasites. In Australia and Papua New Guinea symptoms only appear in unsprayed plantations in high rainfall and shady moist areas, and in Central America on lower leaves during high rainfall seasons. Fungicides used in the control of yellow and black Sigatoka are effective against tropical speckle of banana (Jones 2000).

FRECKLE

Freckle disease attacks leaves and fruit of banana in Africa, Australasia-Oceania, Caribbean, the Philippines (abaca), South and East Asia, Taiwan and West Malaysia (Jones 2000). In some regions of Taiwan and the Philippines, freckle is regarded as being more serious than black Sigatoka. On local markets, freckle blemishes on fruit are not a problem. Heavy leaf infections, however, can cause complete defoliation and reduction in yield.

Freckle disease of bananas is caused by *Guignardia musae* F. Stevens (*Ascomycetes*) (anamorph *Phyllosticta musarum* (Cooke) Aa (*Coelomycetes*)). The pycnidial stage is usually present on the host and about five pycnidia are contained within small spots (Meredith 1968). Symptoms manifest as two types of leaf spotting on the upper surfaces of older leaves (Meredith 1968). The first is a very small, dark brown-black spotting less than 1 mm in diameter, giving the leaf a sooty appearance and rough texture. This symptom is formed by spots clustering in

lines and running diagonally or horizontally across a leaf or fruit surface (Figs. 12A, B). The second symptom is a 4-mm diameter distinct brown-black spot with a grey-fawn centre. These spots coalesce into large black areas or streaks on which pycnidia are prominent under a raised epidermis (Fig. 12C). Diseased leaves and fruit feel rough to the touch. In severe cases leaves turn yellow, wither and die prematurely (Jones 1994). Symptoms are seen on petioles, midribs and transition leaves.

During the wet season, ascospores of *G. musae* are forcibly released from perithecia and conidia of *P. musarum* exuded as white gelatinous tendrils from pycnidia. They are carried in water droplets across leaves and fruit where infection occurs. Germination occurs within 2–3 hours of initial contact with the host at a temperature of about 24 °C. Twelve hours later a lateral swelling differentiates into an appressorium in grooves on the leaf surface between adjacent host cells. At 24–72 hours, a single epidermal cell is invaded, many of these invasions cause a scattered pattern that begins to discolour due to penetration by infecting appressoria. Pycnidia develop in lesions 3 weeks after inoculation and thick intercellular hyphae accumulate 2–3 cell layers below the epidermis. They differentiate, enlarge and break through the epidermis causing secondary leaf infections to intensify symptoms and resulting in streaks. In Taiwan the incubation period varies from 20 days in warm wet weather, to 60 days in cool dry weather, and conidia present in water drop from leaves to fruit (Jones 2000).

Propiconazole has been shown to be the most effective means of controlling freckle (Jones 2000). Mancozeb is effective as well, with benomyl giving minimal control and mineral oil none. In the Philippines, propiconazole and flusilazole, used in the control of black Sigatoka,

also control freckle. In Taiwan, removal of lower diseased leaves reduces inoculum levels and bagging of bunches reduces fruit infection (Jones 2000).

MINOR LEAF DISEASES

Some diseases of banana leaves are considered to be of minor economic importance. They are usually caused by opportunistic pathogens, but the symptoms they cause can cover entire leaves. However, these diseases seldom result in a reduced yield as with Sigatoka and speckle diseases. Some minor diseases of banana leaves include Cordana leaf spot, Phaeoseptoria leaf spot, black cross leaf spot, Deightoniella leaf spot, Drechslera leaf spot, Malayan leaf spot, Pestalotiopsis leaf spot, Pyricularia leaf spot and rust.

Cordana leaf spot

Cordana leaf spot is caused by *Cordana musae* (Zimm.) Höhn. and *Cordana johnstonii* M.B. Ellis, two species that can be morphologically differentiated on the grounds of conidial size and shape, with *C. johnstonii* having longer and wider conidia than *C. musae* (Jones 2000). It has a world-wide distribution and has been reported as a serious problem on plantains in Central America during and after wet seasons (Wardlaw 1961, Stover 1972, Jones 2000). Epidemics have occurred on the "Williams" cultivar in Southern New South Wales, Australia (Jones 2000). Cordana specimens collected in Australia since the 1930's showed that *C. johnstonii* was limited to New South Wales, Lord Howe Island and Norfolk Island, and *C. musae* to Queensland and the Northern Territory, suggesting that *C. johnstonii* is better adapted to cooler environments than *C.*

musae (Jones 2000). Characteristic Cordana leaf spot lesions are large, oval to fusiform, pale brown and necrotic (Fig. 13) (Jones 2000). Leaf spots caused by *C. johnstonii* are smaller, more regular in outline and more tapered than the larger oval spots of *C. musae* (Jones 2000). Cordana leaf spot often infects leaf margins where the lamina is more likely to tear due to senescence or nutritional deficiency (Jones 2000). Quite frequently, *C. musae* also infects around lesions caused by pathogens such as, *M. musicola*, *M. fijiensis* and *M. eumusae* (Stover 1972, Jones 1994, Jones 2000).

Cordana johnstonii sporulates profusely on the adaxial surface and *C. musae* on the abaxial surface of leaf spots on green leaves and leaves in leaf litter during cool misty weather (Jones 1994, Jones 2000). Conidia are forcibly released under conditions of decreasing vapour pressure at dawn when humidity drops (Stover 1972, Jones 2000). The conidia germinate and produce germ tubes and appressoria that penetrate the leaf surface (Jones 2000). Germination and infection time is 6–48 hours, depending on temperature, and symptoms are produced within 4–10 days (Jones 2000). Under laboratory conditions the optimum temperature for conidium production on living leaves is 22 °C and on detached leaves 22–25 °C (Jones 2000). *Cordana musae* is usually not economically important and therefore does not warrant control (Jones 2000). However, when outbreaks arise, the fungicides used in the control of yellow and black Sigatoka provide adequate control of this fungus (Stover 1972, Jones 2000).

Phaeoseptoria leaf spot

Phaeoseptoria musae Punithalingham is the organism responsible for Phaeoseptoria leaf spot of banana (Jones 2000). It is thought that *Mycosphaerella* is possibly the perfect stage. Conidia are dispersed by rain splashes and ascospores are forcibly ejected during wet weather. Phaeseptoria leaf spot affects only older leaves, causing ellipsoidal to ovoid spots with pale centres, a dark brown border and a yellow halo (Punithalingham 1983). At high densities lesions coalesce, forming large irregularly shaped necrotic areas with off-white to yellow centres. Phaeoseptoria leaf spot causes severe blight of banana leaves in Australia, Cameroon Colombia, East Malaysia Ghana, Guyana, Honduras, Kenya, India, Tanzania, Trinidad and Uganda (Jones 2000). There is no information available on control measures for the disease.

Black cross leaf spot

Black cross leaf spot, caused by *Phyllachora musicola* C. Booth & D.E. Shaw, has been reported from American Samoa, Australia, Fiji, Indonesia, New Caledonia, Niue, Papua New Guinea, Philippines, Tonga, Vanuatu and Western Samoa (Jones 2000). An asexual state has not been observed, but it is thought to be *Scolecobasidium* (Hyde 1992). Black cross leaf spot is found on the lower surface of older leaves as large, black, four-pointed crosses, stars or diamonds (Fig. 14A). Upper surface symptoms are usually black dots, but in Western Samoa they are yellow diamond-shaped spots interspersed with dark brown lines (Fig. 14B). The typical black crosses on abaxial surface are the mature stroma of *P. musicola*. During humid conditions, white masses of spore exudate collect along the arms of the stromatic black cross (Jones 2000).

Ascospores are carried in water drops and spread the disease between leaves. Ascospores become airborne when forcibly ejected from perithecia, dispersing disease over wider areas (Wardlaw 1961). Commercial AAA cultivars (Cavendish) are fairly resistant to the disease so no control measures are practised. In Western Samoa planting in sunny places rather than in shade is recommended.

Deightoniella leaf spot

Deightoniella leaf spot is caused by *Deightoniella torulosa* (Syd.) M.B. Ellis. The disease has been recorded on senescing or injured leaves in Bermuda, Brazil, Central America, Ceylon, Ethiopia, Ghana, India, Jamaica, Peru, Philippines, Sierra Leone, Suriname and Trinidad (Jones *et al.* 2000a). Lesions are most prevalent along the leaf blade and on older lower leaves. The disease is mainly reported from Cavendish cultivars in plantations, though various abacá and enset varieties have been reported to be susceptible (Jones *et al.* 2000a). Initial symptoms are tiny, black, necrotic spots (1–2 mm) that become oval with a black border (may be confused with Cordana leaf spot at this stage) (Fig. 15) forming a smoky colouring over a tan background. Mature spots reach up to 25 mm in diameter, coalesce and form bands of necrotic tissue along leaf margins (Stover 1972). Stem lesions are similar to speckle symptoms having a defined black margin, 1–2 mm wide, and a yellow halo that encompasses each lesion. Mature lesions brown and dry out, leaving the original black spots visible. This results in a speckle-like spotting on petioles and pseudostem. *Deightoniella torulosa* is found in living and dead leaf tissue. Inoculum is produced during rain and dew periods when spores are forcibly discharged and become airborne when the humidity drops (Ellis 1957). They germinate in surface water and symptoms

develop within days. Spores are not transmitted long distances and viability is lost within 4 days at humidities less than 95 % (Wardlaw 1961). No control measures are required on banana, however dead leaf litter should be removed to prevent inoculum build-up. Only resistant abaca and enset cultivars should be planted in areas where the disease is known to occur (Jones *et al.* 2000a). Additional control measures encompass reduction of planting density, harvesting before rotting becomes severe as well as cutting down and burning badly diseased plants (Jones *et al.* 2000a).

Drechslera leaf spot

Drechslera gigantea (Heald & F.A. Wolf) S. Ito is the causal agent of Drechslera leaf spot. The disease is found in Central America, Ethiopia and Jamaica (Jones *et al.* 2000b). Symptoms manifest as tiny, sunken, red spots with pale green or yellow borders (Meredith 1968). These spots become oval toward the leaf veins, centres turn dark brown and dry to a white/grey surrounded by a thin defined dark brown margin with a pale yellow-green halo (Fig. 16A). Spots measure 16 x 8 mm on leaves, midribs and petioles. Symptoms on enset may coalesce and form large necrotic areas of severe blight symptoms on the unfurling, first and second leaves (Fig. 16B). The fungus also causes eyespot on banana leaves of suckers less than 2 m tall, during wet weather and heavy dewfall. Conidia are forcibly expelled under decreased humidity and surface moisture favours germination and infection (Stover 1972). Early removal of diseased leaves is recommended to reduce inoculum levels in plantations (Jones *et al.* 2000b).

Malayan leaf spot

Malayan leaf spot, caused by *Haplobasidion musae* M.B. Ellis, affects banana leaves in Fiji, Papua New Guinea, Tonga, West Malaysia and Western Samoa (Jones 2000). Infection occurs on young leaves soon after they emerge (Jones 2000). Adaxial symptoms are diamond shaped, grey-white spots, with the longer axis parallel to leaf veins. Abaxial symptoms display a velvety mass of mycelium (Ellis 1957). In West Malaysia spots are ellipsoidal or round, with spotting in white, grey or brown. Lesions are paler on the adaxial surface and have dark purple borders. In Papua New Guinea there is no typical diamond shape, lesions vary in size, are round and ellipsoidal with well-defined dark borders and grey centres on the leaf blade and midrib (Fig. 17). At high infection intensities, tissue surrounding lesions yellows and large necrotic areas develop with the dark borders and grey centres of the original lesions still visible (Jones 2000). Shade and cool temperatures favour development of symptoms (Jones 2000). Short daylight hours, high humidity and high rainfall render leaves more susceptible when the plant is near flowering at cooler temperatures. The disease occurs in West Malaysia at altitudes between 1372 m and 1525 m, and in Papua New Guinea well above 1000 m. In western Samoa disease incidence increases with altitude, and in Tonga the cooler climate at sea level is conducive to the disease (Jones 2000). Leaves sprayed with oil predispose banana plants to infection by *H. musae*. Maneb applied in water is recommended to delay symptom appearance in areas were disease is severe (Jones 2000).

Pestalotiopsis leaf spot

Pestalotiopsis leaf spot, that occurs in Central America and Jamaica, is caused by *Pestalotiopsis palmarum* (Cooke) Steyaert (Jones 2000). Symptoms manifest as yellow-brown circular spots between leaf veins. Spots develop until they reach vascular tissue, then spread and linearly extend lesions (Meredith 1968). Grey-fawn spots can develop around tears or abrasions in leaf margins, with necrosis progressing outwards and possible extension to the midrib. Concentric zones are visible within spots, beginning at the centre, with a narrow dark brown band surrounded by a bright yellow-orange halo (Stover 1972). No control strategies are currently available.

Pyricularia leaf spot (enset)

Pyricularia leaf spot occurs in Sidamo and North Omo in Ethiopia (Tessera & Quimio 2000). It forms circular, oblong or spindle-shaped lesions with dark borders. Leaves, midribs, petioles and leaf sheaths are affected (Figs. 18A, B) and lesions coalesce and form large necrotic areas (Tessera & Quimio 2000). A *Pyricularia* sp. has been isolated and found to be pathogenic to enset and not to the AAA (Cavendish) banana cultivars. Most enset cultivars are susceptible to the fungus, but older plants are not affected. Pyricularia leaf spot can become severe on young enset seedlings and lead to leaf death. Older plants are not badly affected, and removal of infected leaves is recommended to prevent disease spread (Tessera & Quimio 2000).

Rust

Two different fungi cause rust on banana leaves, *Uromyces musae* Henn and *Uredo musae* Cummins (Jones 2000). Uredosori form on the lower surfaces of older leaves as lesions elongate, broaden and coalesce, causing the leaf to yellow. At high infection densities necrosis sets in and powdery, light brown masses of uredospores cover lesions (Fig. 19). Uredosori act as points of entry for secondary infections (Wardlaw 1961). Wind blown uredospores are thought to transfer the fungus between plants (Jones 2000). They germinate in surface water and invade the leaf tissue. Cavendish cultivars are susceptible to rust when sprayed with mineral oil. Rust symptoms are found on banana leaves in Australia, Congo, Fiji, Malaysia, Nigeria, Papua New Guinea, Philippines, Wallis Islands and Western Samoa (Jones 2000). No control is warranted for rust. Severe outbreaks that have occurred on Cavendish plantations can be related to the use of oil or benomyl to control leaf spot diseases (Jones 2000).

ENDO- AND EPIPHYTES OF BANANAS

Numerous fungi are associated with the foliage of banana plants. Some of these fungal taxa have been reported as endo- or epiphytes on *Musa* species, particularly on wild banana (*Musa acuminata* Colla) (Table 4). Fungi isolated most commonly include *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., *Curvularia* spp., *Fusarium* spp., *Nigrospora* spp., *Pestalotiopsis* spp., *Phomopsis* spp., xylariaceous taxa and unknown sterile species (Brown *et al.* 1998, Photita *et al.* 2001, Photita *et al.* 2002). Some of these, e.g. *C. gloeosporioides*, can cause disease in banana fruit, whereas others, such as *Phoma* and *Phomopsis* species, may become

problematic under environmental stress (Brown *et al.* 1998). However, many asymptomatic endophytic colonisers exist mutualistically with their hosts, possibly benefiting the latter by protecting them from attack by pathogens (Petrini 1993, Dorworth & Callan 1996).

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Table 1: Stages of development of yellow Sigatoka lesions on banana leaves (Stover 1972, Jones 1994, Agrios 1997, Robinson & Peterson 1999, Jones 2000).

Stage	Symptom
1 (speck/fleck)	Yellow-green flecks shorter than 1 mm.
2a (Initial streak)	Flecks enlarge to 3–4 mm x 1 mm streaks, the lesion becomes yellow.
2b (Final streak)	Streaks become a dark brown colour.
3 (Initial spot)	Streaks enlarge and become spots, definite margins that may become water-soaked darken to brown.
4 (Brown spot)	Spots have a definite dark brown margin, sometimes with a yellow halo, the lesion centre begins to sink in. Conidia are produced on the surface of the lesion.
5 (Mature spot)	Sunken lesion centres turn grey but retain their brown border often with a halo. Ascospores are produced in the grey central area of the mature spot.

Table 2: Differences between conidial and ascospore infection of banana leaves by *Mycosphaerella musicola* (adapted from Simmonds 1959, Jones 2000).

Characteristic	Conidia	Ascospores
Development	Night temperature ≥ 18°C	Night temperature ≥ 21°C
Leaf stage	Stage 4 and 5	Stage 5
Fruiting structures	On both sides of leaves	Mainly on upper surface
Dispersal	Rain or dew	Rain
Spread	Water-splashes (airborne)	Carried on wind-currents
	Short distance spread	Long distance spread
Infection Moisture	High humidity	Film of water on leaf
Germination	24–48 hr	2–3 hr
Penetration	4–6 d	48–72 hr
Survival		47–48 weeks
Production time	Wet season, continuous	Middle-late wet season
Production area	Leaf surface	Internal to leaf
Transmission	Water	Ejected, airborne
Infection	Heart leaf	Tips of 2nd and 3rd leaves
Pattern	"Line spotting"	"Tip spotting"

Table 3: Morphological differences between the anamorph structures of *M. musicola, M. fijiensis* and *M. eumusae* (Crous & Mourichon 2002).

Species	Sporodochia	Conidiogenous cells	Conidiophores	Conidia
M. fijiensis	Sporodochia absent, produces few conidiophores on the lower leaf surface	Up to 25 x 2–4 µm at apex, 1–3 thickened scars.	Hypophyllous fascicles, pale brown, 0–5 septate, straight to geniculate, occasionally branched, subcylindrical, 16.5–62.5 x 4–7 µm. One or more scars are present near tip	Sub-hyaline, obclavate to cylindric obclavate, 1–10 septate, slightly thickened and darkened hila, 10–120 x 2.5–5 µm.
M. musicola	Amphigenous, dark brown substomatal stromata.		Pale brown, aseptate, unbranched, straight to bottle-shaped, 5–25 µm long, lack any visible scarring.	Smooth, pale olivaceous, cylindrical to cylindric obclavate, straight to curved, 0–8 septate, subtruncate to obclavate ends, no significant scarring, 10–80 x 2–6 µm.
M. eumusae	Epiphyllous, dark brown substomatal stromata.	Truncate ends	Sub-hyaline to pale olivaceous, pale brown at the base, sub-cylindrical, 0–3 septate, 10–25 x 3–5 µm, longer and more septate than *M. musicola*.	Sub-hyaline to pale olivaceous, sub-cylindrical, 3–8 septate, subtruncate ends, no visible scarring, 18–65 x 2–3 µm.

Table 4: Summary of fungi reported from banana leaves (Brown *et al.* 1998, Photita *et al.* 2001, Photita *et al.* 2002).

Phylum	Species	Authority
Ascomycota	*Alternaria alternata*	(Fr.) Keissl.
	Antennularia tenuis	Earle
	Anthostomella moelleriana	G. Winter
	Botryosphaeria musae	
	Cladosporium cladosporioides	(Fresen.) G.A. de Vries
	Cladosporium liukiuensis	
	Colletotrichum gloeosporioides	(Penz.) Penz. & Sacc.
	Colletotrichum musae	(Berk. & M.A. Curtis) Arx
	Cryptosporella musarum	J.N. Kapoor
	Diaporthe musae	Speg.
	Dothidea musae	Klotzsch
	Dothidella musae	Höhn.
	Epicoccum nigrum	Link
	Fusarium lateritium	Nees
	Fusarium solani	(Mart.) Sacc.
	Glomerella musarum	Petch
	Guignardia cocoicola	Punithalingham
	Guignardia musae	F. Stevens
	Lasiodiplodia theobromae	(Pat.) Griffiths & Maubl.
	Leptosphaeria musae	
	Leptosphaeria musigena	
	Leptosphaeria taichungensis	
	Metasphaeria taiwanensis	
	Micronectriella stoveri	C. Booth
	Microsphaeriopsis sp.	
	Mycosphaerella formosana	
	Mycosphaerella liukiuensis	
	Nectria foliicola	Berk. & M.A. Curtis
	Nectria nymaniana	Henn.
	Phacidium musae	Lév.
	Phyllachora musicola	C. Booth & D.E. Shaw
	Physalospora fallaciosa	Sacc.
	Plicaria musicola	Henn.
	Pyriculariopsis parasitica	(Sacc. & Berl.) M.B. Ellis
	Sphaerulina musae	
	Sphaerulina musicola	
	Sphaerulina pulii	J.M. Yen
	Stachybotrys sp.	
	Venturia musae	

	Verticillium sp.	
Basidiomycota	*Cyphella musicola*	Berk. & M.A. Curtis
	Helotium musicola	Speg.
	Psathyra musicola	Henn.
	Uredo musae	Cummins
	Uredo musicola	
	Uromyces musae	Henn.
Mitosporic	*Alternaria musae*	Bouriquet & Bataille
	Cercospora fengshanensis	T.Y. Lin & J.M. Yen
	Cercospora hayi	Calp.
	Cercospora musae	Massee
	Cercospora musae sapienti	A.K. Kar & M. Mandal
	Cercospora musaecola	Sawada
	Cercospora pingtungensis	T.Y. Lin & J.M. Yen
	Chaetophoma musae	Cooke
	Fusidium musae	
	Gliomastix elata	C.H. Dickinson
	Hainesia tellingsii	Koord.
	Haplobasidion musae	M.B. Ellis
	Leptothyrium musae	Cif. & Gonz. Frag.
	Nigrospora musae	McLennan & Hoëtte
	Nigrospora oryzae	(Berk. & Broome) Petch
	Parapyricularia musae	M.B. Ellis & Peregrine
	Pellionella musae	
	Penicillium atrobrunneum	Cooke
	Pestalotiopsis chethallensis	Sohi & O. Prakash
	Pestalotiopsis palmarum	(Cooke) Steyaert
	Phomatospora musae	
	Phyllosticta gastonis	Roum.
	Phyllosticta musae	F. Stevens & E. Young
	Phyllosticta musicola	F. Stevens & E. Young
	Pyricularia angulata	Hashioka
	Sphaeropsis paradisiaca	Mont.
	Veronea musae	

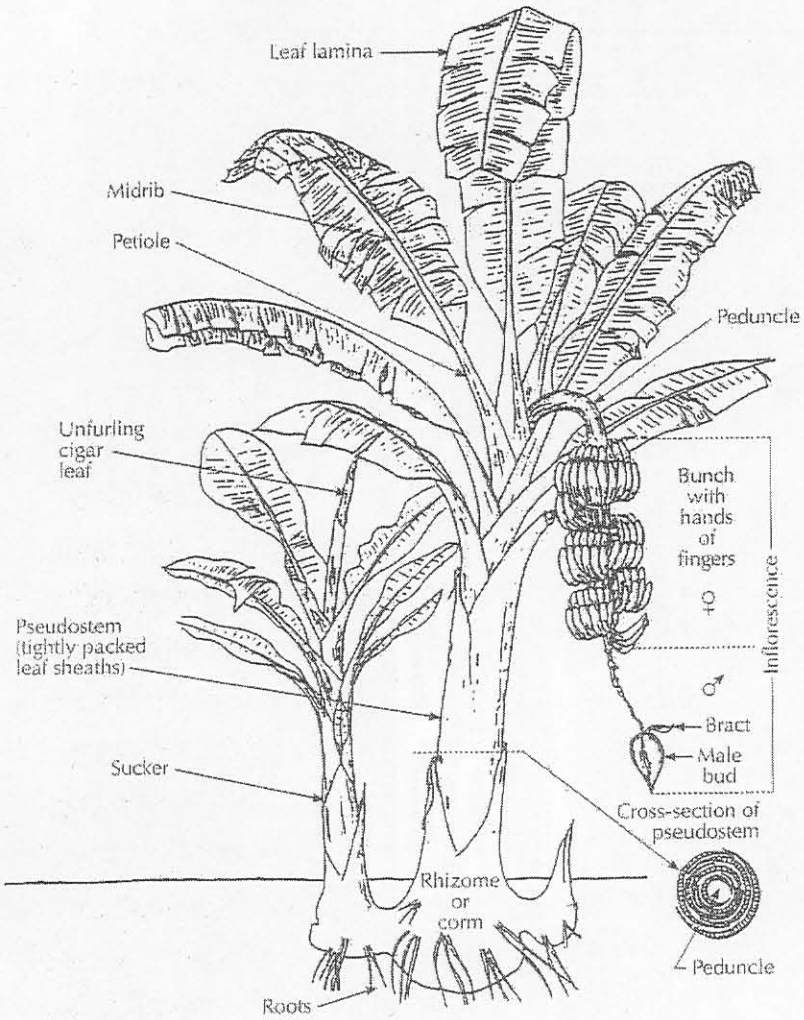

Figure 1: Anatomy of the banana plant (Jones 2000).

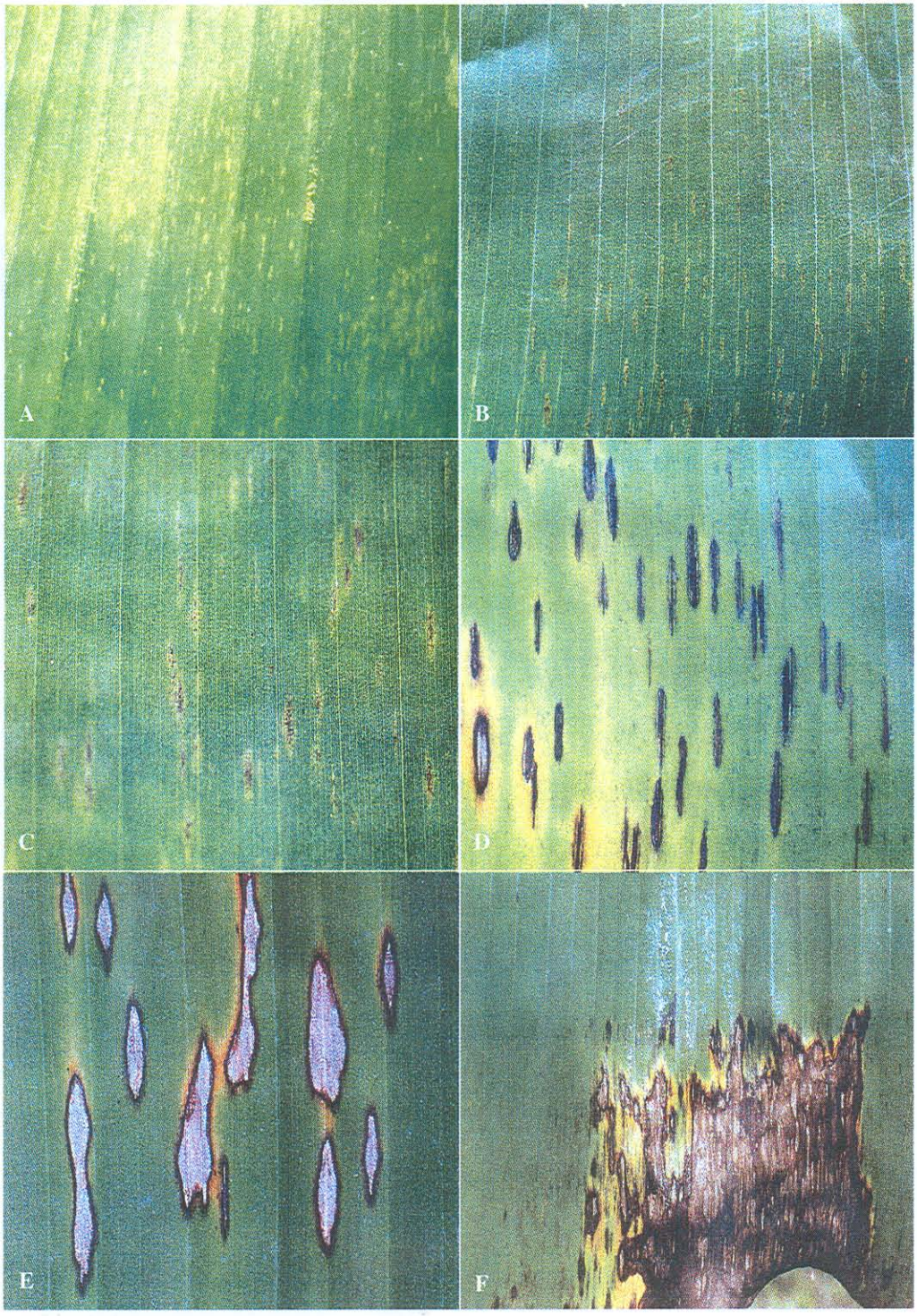

Figure 2: Damage caused by *Mycosphaerella musicola* to the banana plant. A. Stage 1. B. Stage 2. C. Stage 3. D. Stage 4. E. Stage 5. F. Severe leaf infection (photos courtesy of R.A. Peterson).

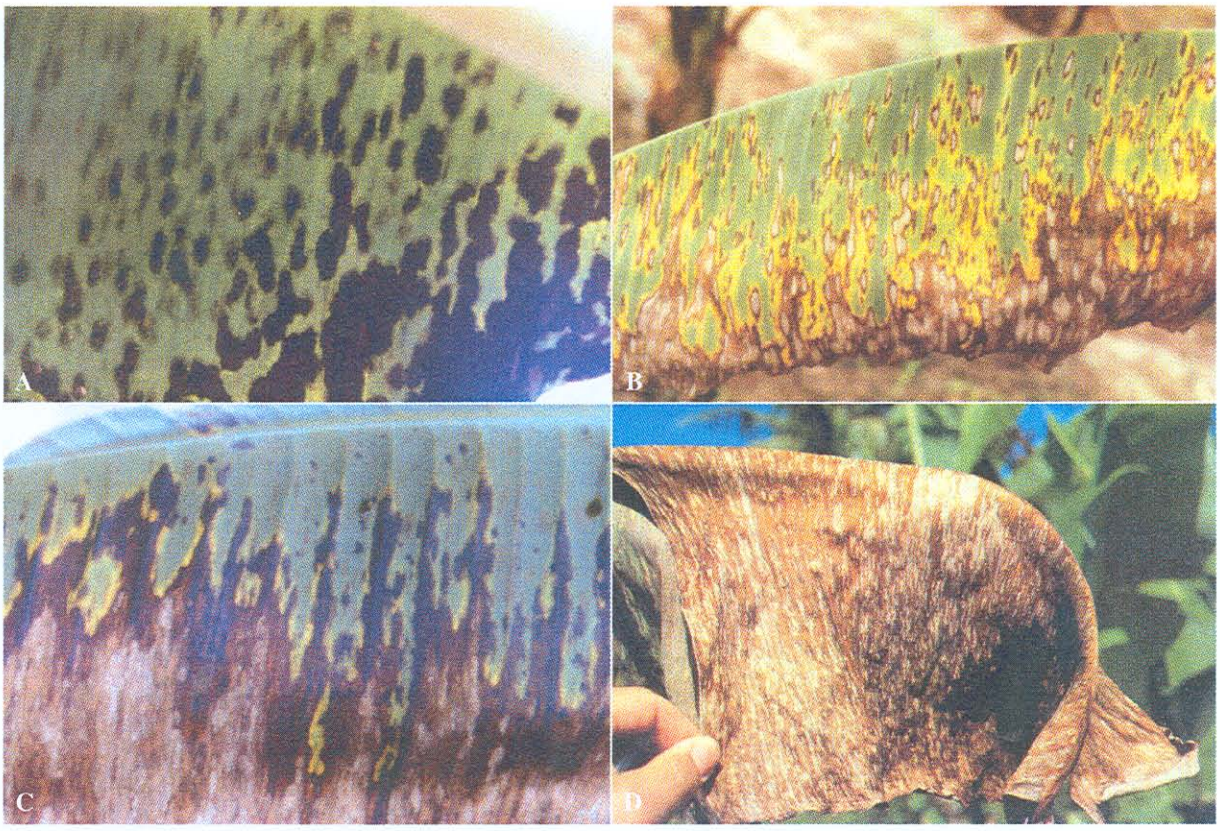

Figure 3: Black Sigatoka symptoms on banana leaves of the Cavendish subgroup. A. Lower surface symptoms between stages 4 and 5. B. Stage 6 symptoms on the upper leaf surface (plantain subgroup). C. and D. Stage 6 symptoms on the upper leaf surface. (Jones 2000).

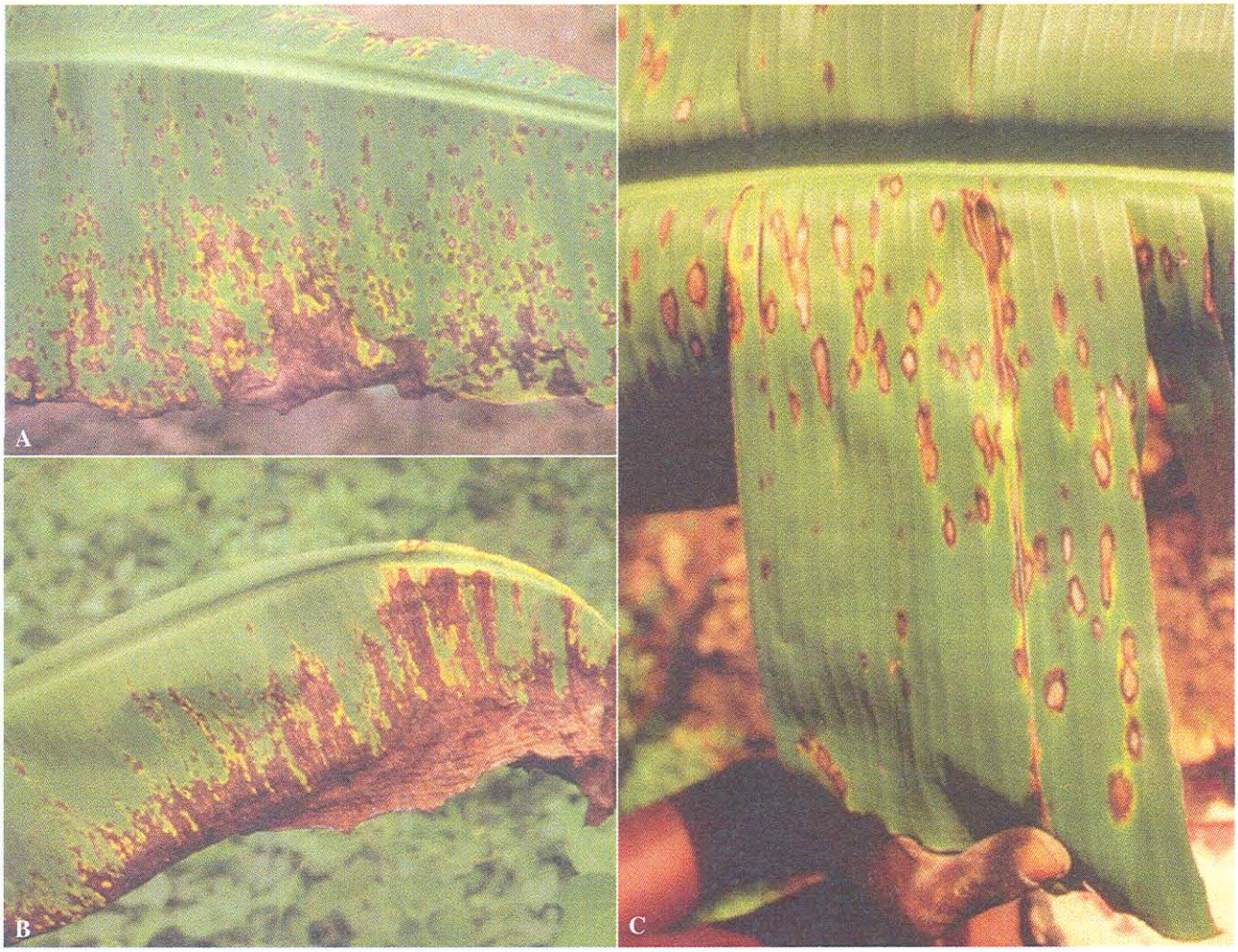

Figure 4: Mature lesions of eumusae leaf spot on A. Grande Naine (Thailand) B. Embul (Sri Lanka) and C. Anamala (Sri Lanka) cultivars (Jones 2000).

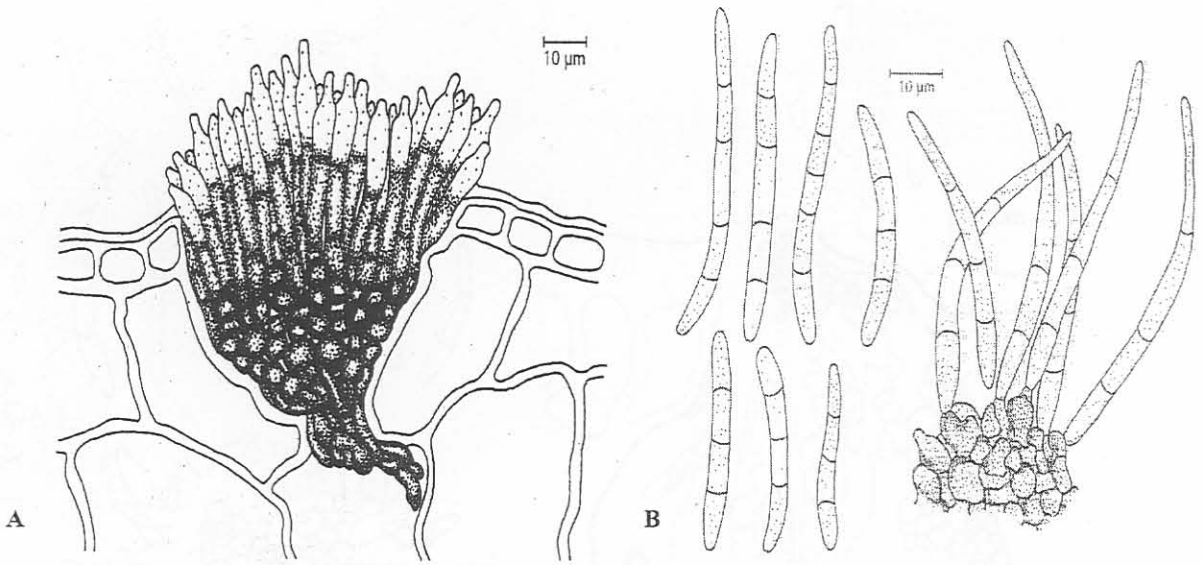

Figure 5: A. Sporodochium of *Mycosphaerella musicola* showing bottle-shaped conidiophores borne terminally on stromatal hyphae. B. Conidia and conidiophores of *M. musicola*. (Jones 2000)

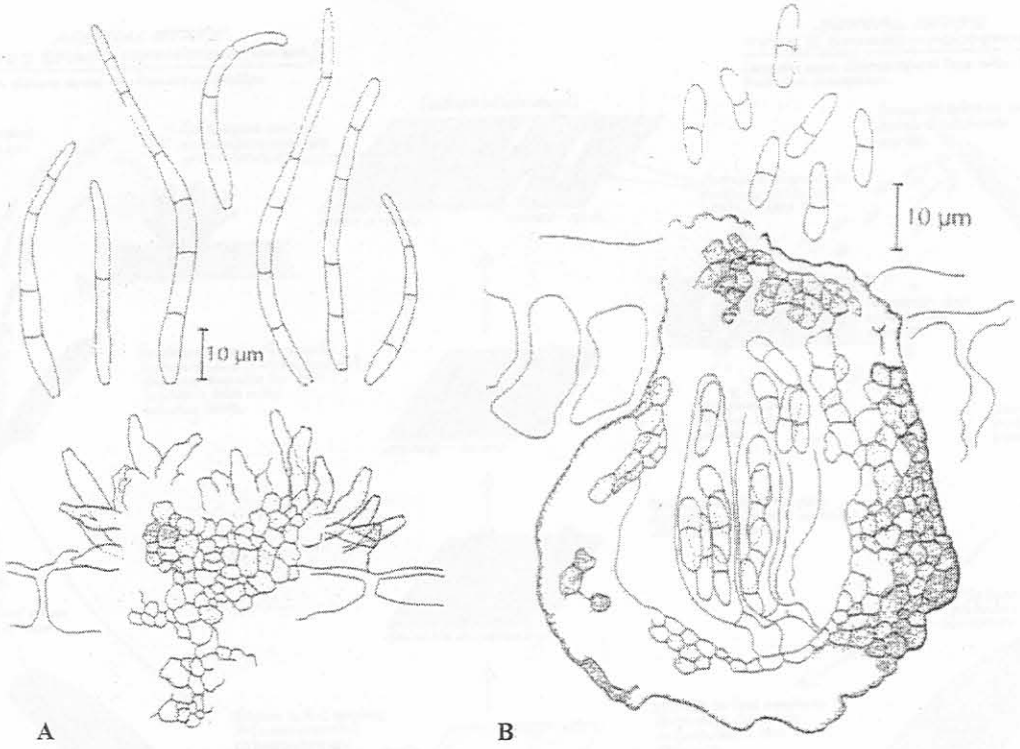

Figure 6: The asexual and sexual stages of *Mycosphaerella fijiensis* on banana leaves. A. Stroma with conidiogenous cells and conidia. B. Ascostroma with asci and ascospores (Jones 2000).

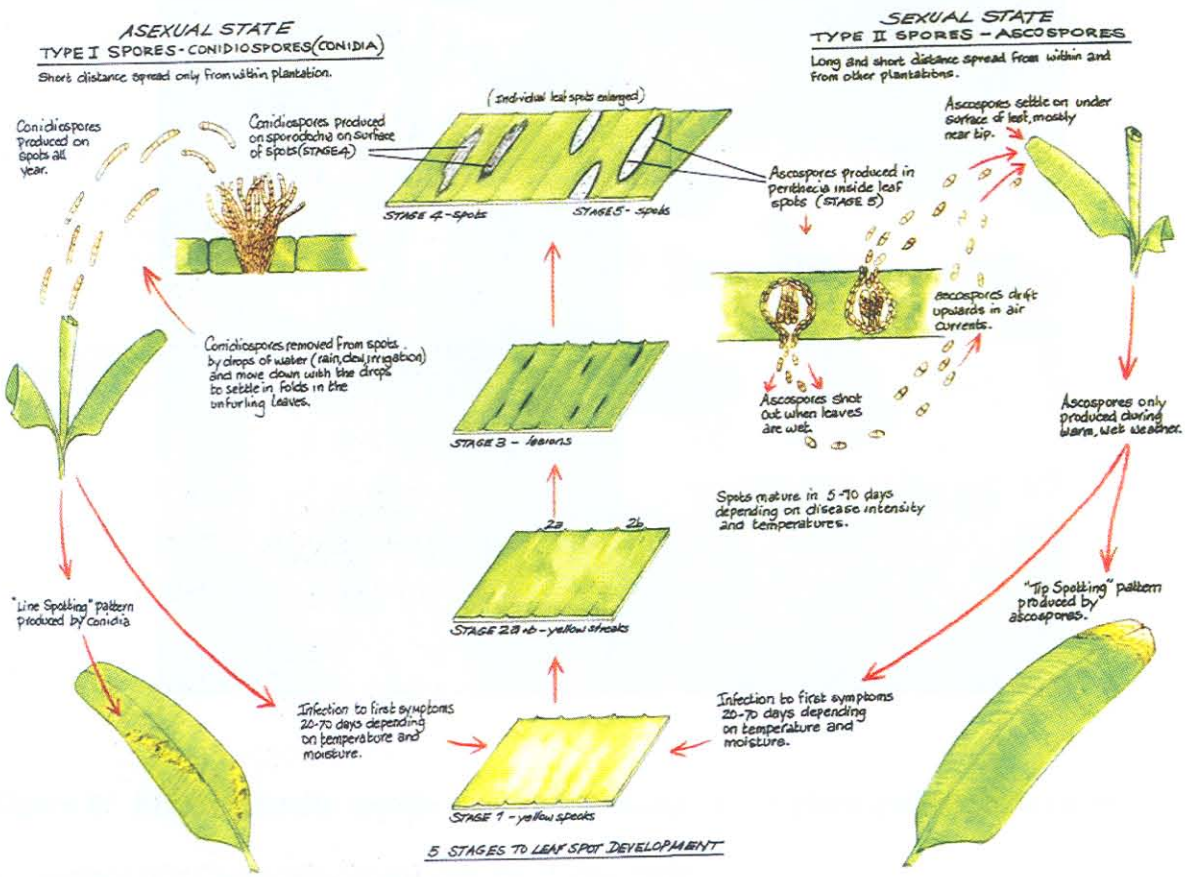

Figure 7: Life cycle of yellow Sigatoka (courtesy of R.A. Peterson)

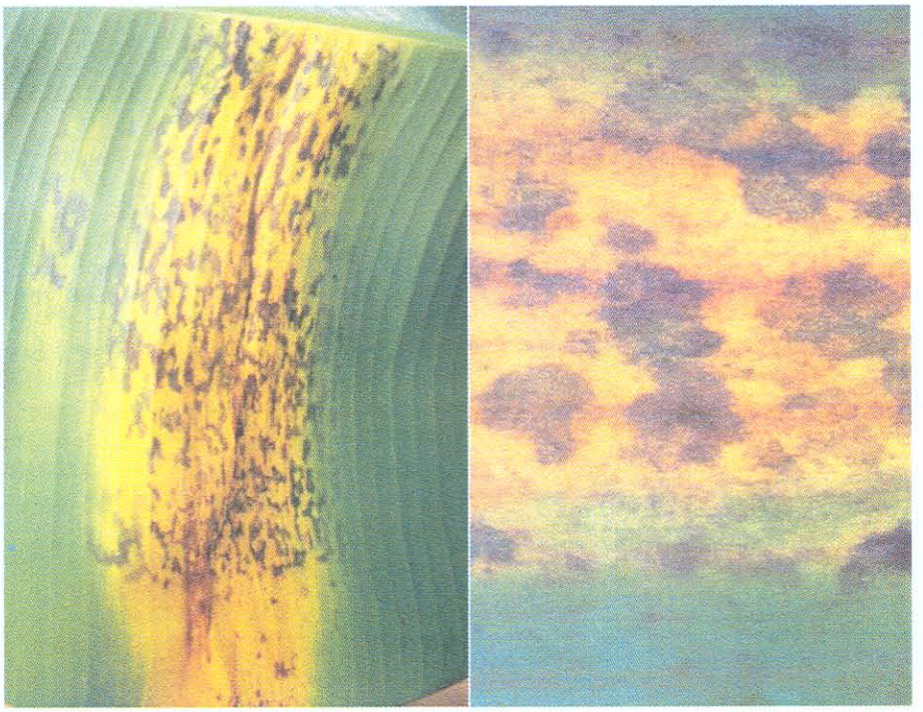

Figure 8: Mycosphaerella speckle causing increasing loss of photosynthetic area on the upper leaf surface of a Cavendish banana cultivar (Jones 2000).

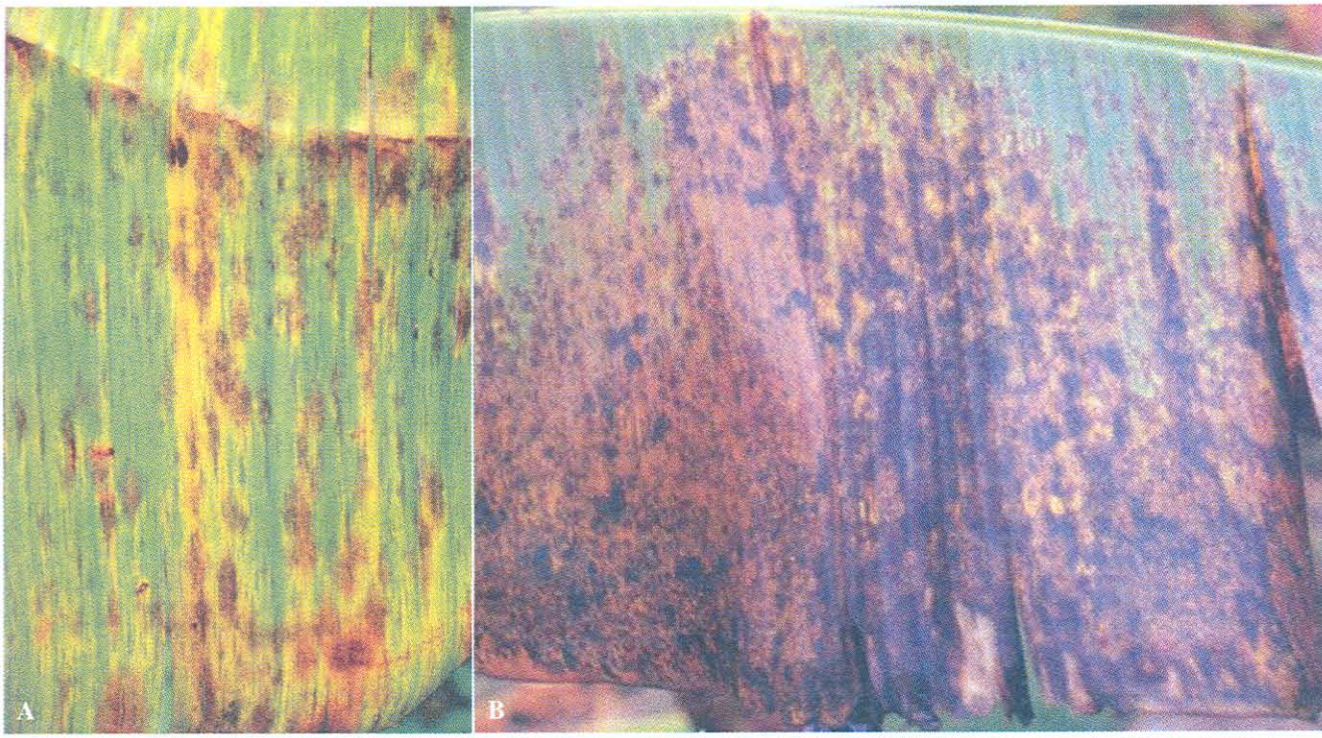

Figure 9: Symptoms of Cladosporium leaf speckle. A. Symptoms on a dessert banana cultivar. B. Leaf necrosis (Jones 2000).

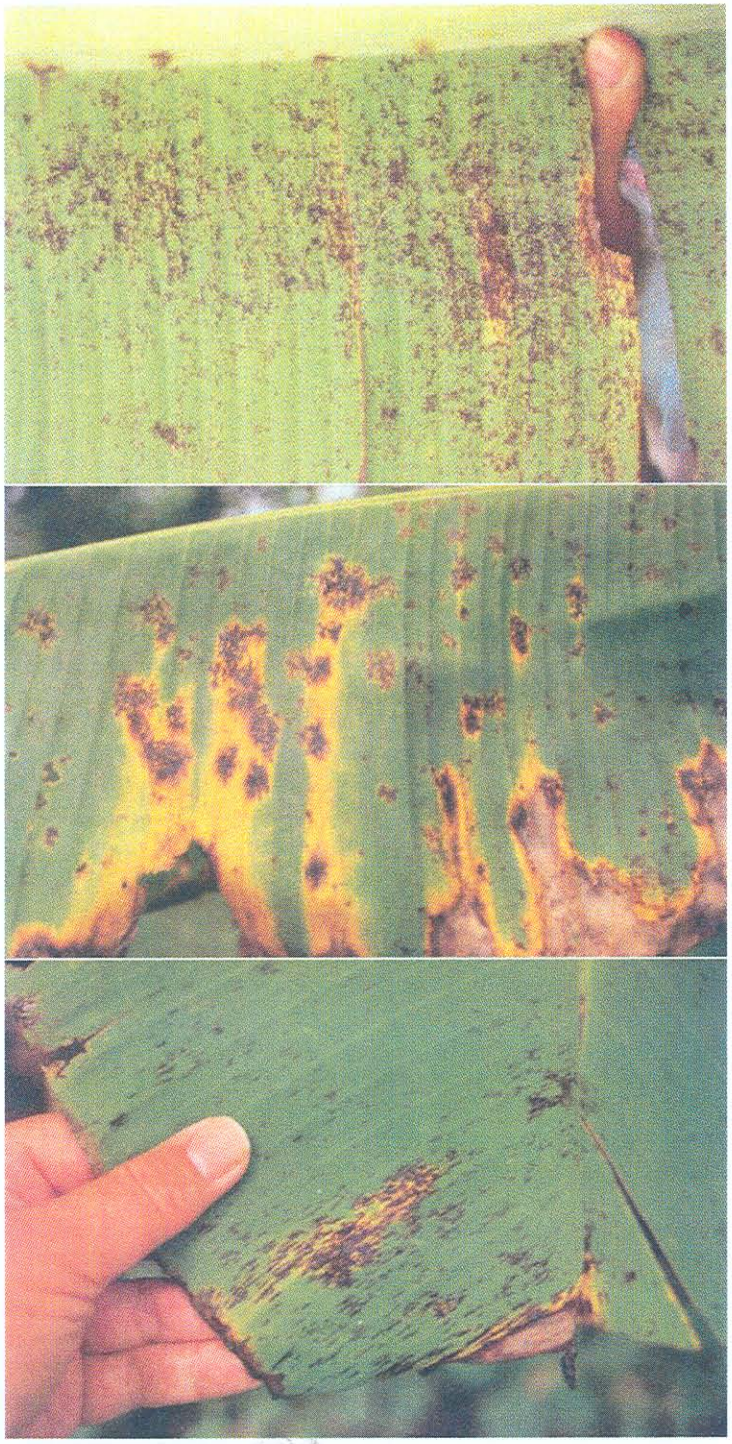

Figure 10: Various symptoms of leaf speckle (Jones 2000).

Figure 11: Symptoms of tropical speckle on the abaxial leaf surface A. Tan blotch speckle. B. Grey to black patch speckle (Jones 2000).

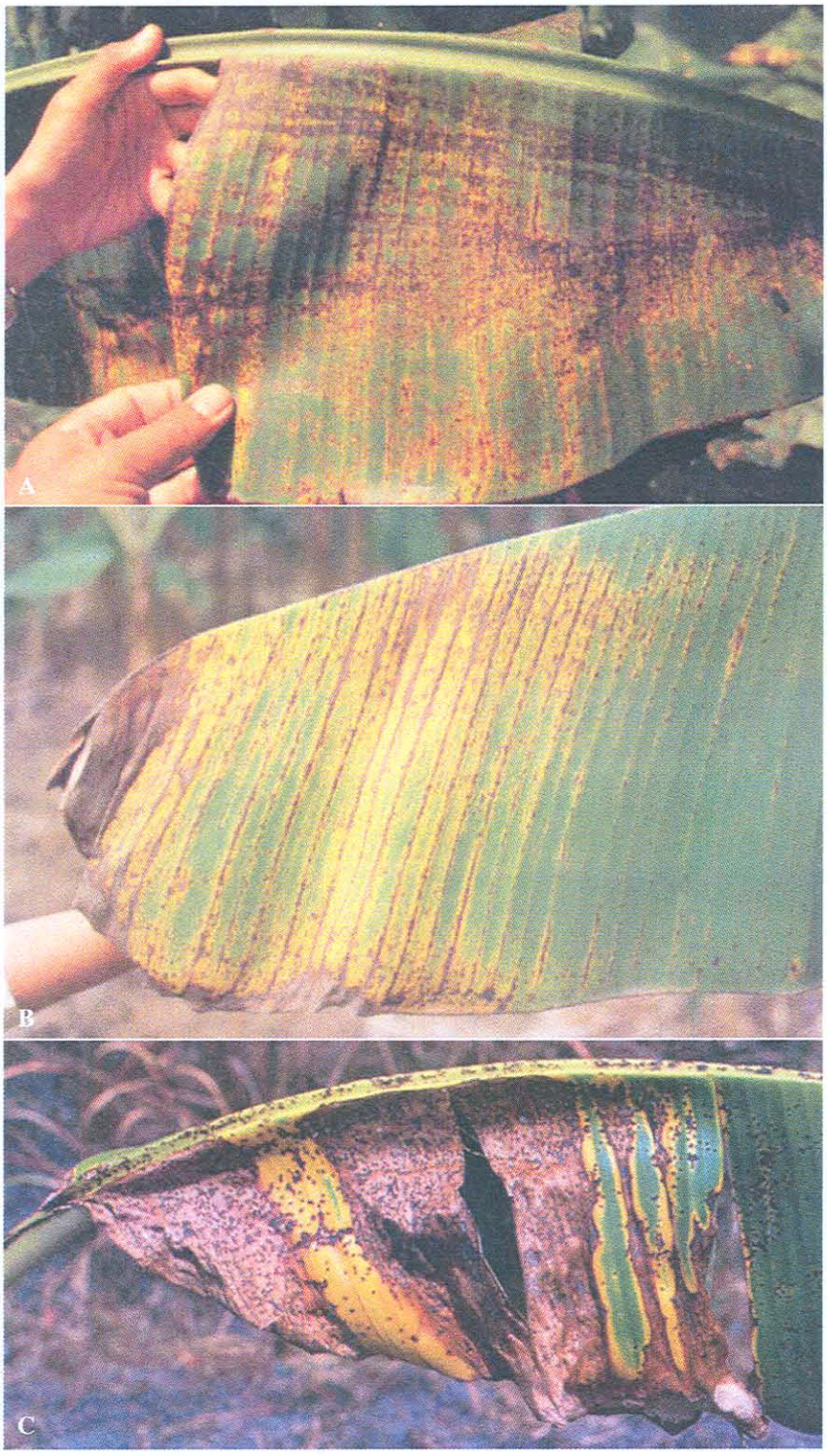

Figure 12: Symptoms of freckle A. Water droplet distribution of symptoms. B. Vein line association of symptoms. C. Larger spots also present on midrib of leaf (Jones 2000).

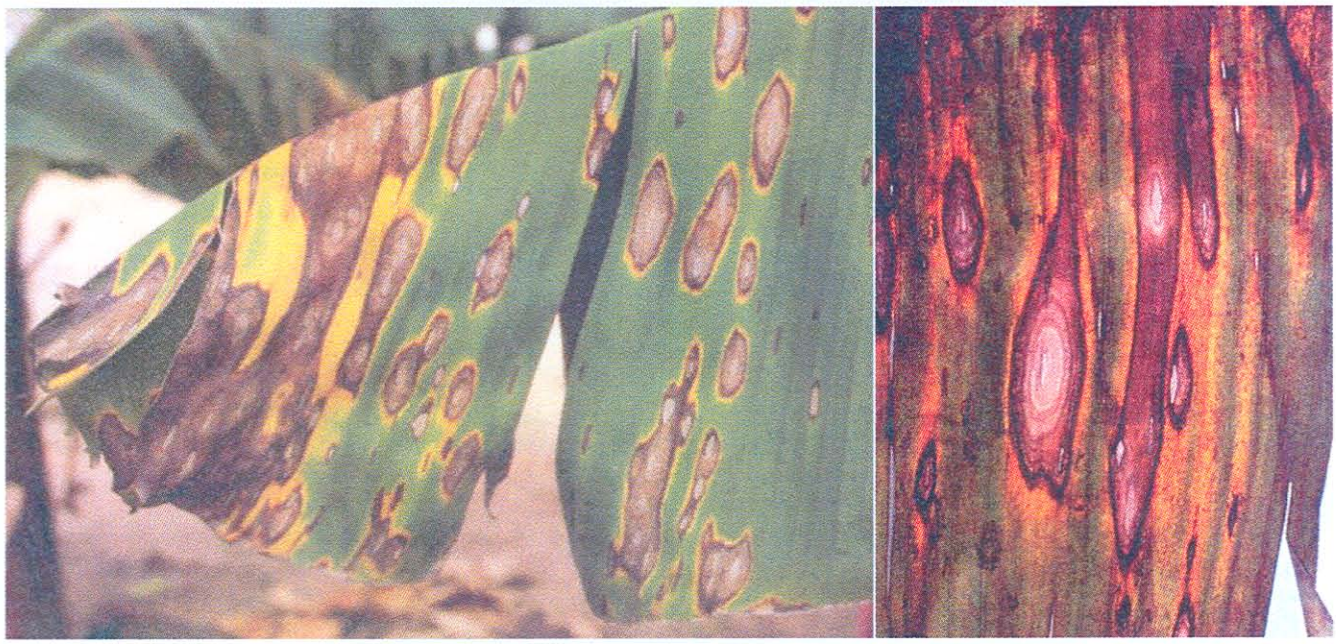

Figure 13: Symptoms of Cordana leaf spot on Cavendish cultivars (Jones 2000).

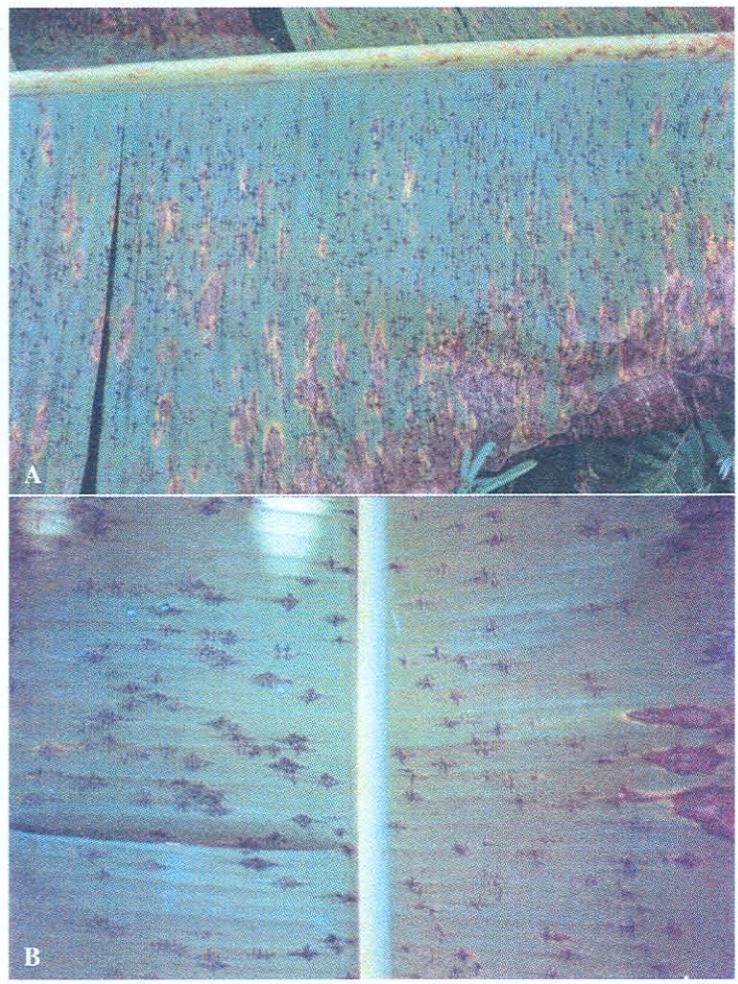

Figure 14: Symptoms of black cross leaf spot on A. Abaxial surface B. Adaxial surface of a banana leaf (Jones 2000).

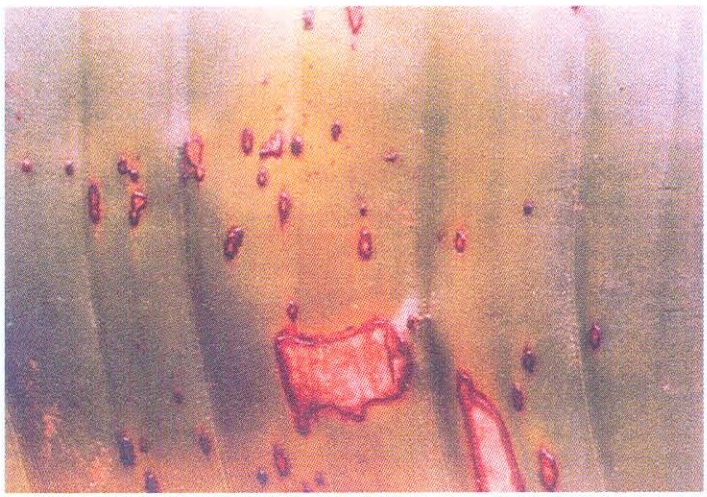

Figure 15: Symptoms of Deightoniella leaf spot on enset (Jones 2000).

Figure 16: Symptoms of Drechslera leaf spot on A. Banana B. Enset (Jones 2000).

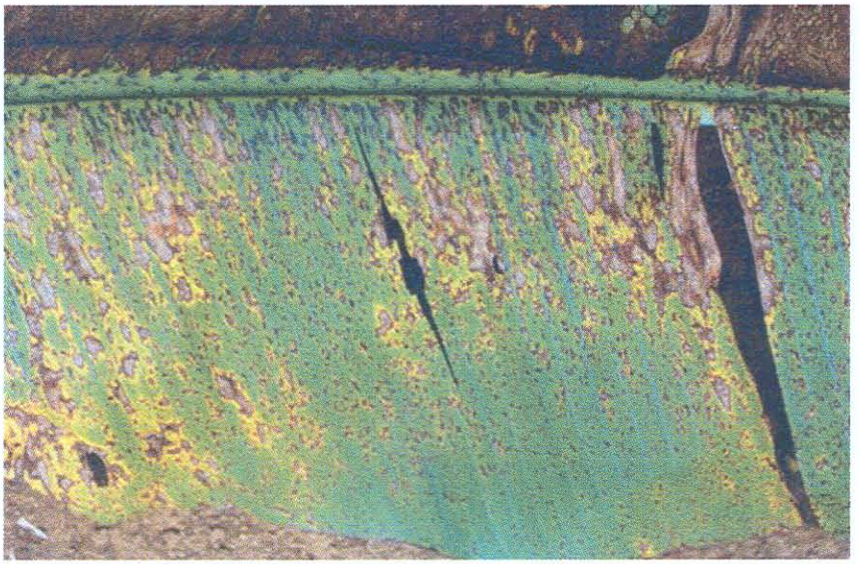

Figure 17: Symptoms of Malayan leaf spot on leaf and midrib (Jones 2000).

Figure 18: Symptoms of Pyricularia leaf spot A. Leaf midrib and lamina of enset. B. Petioles and leaf sheaths of enset (Jones 2000).

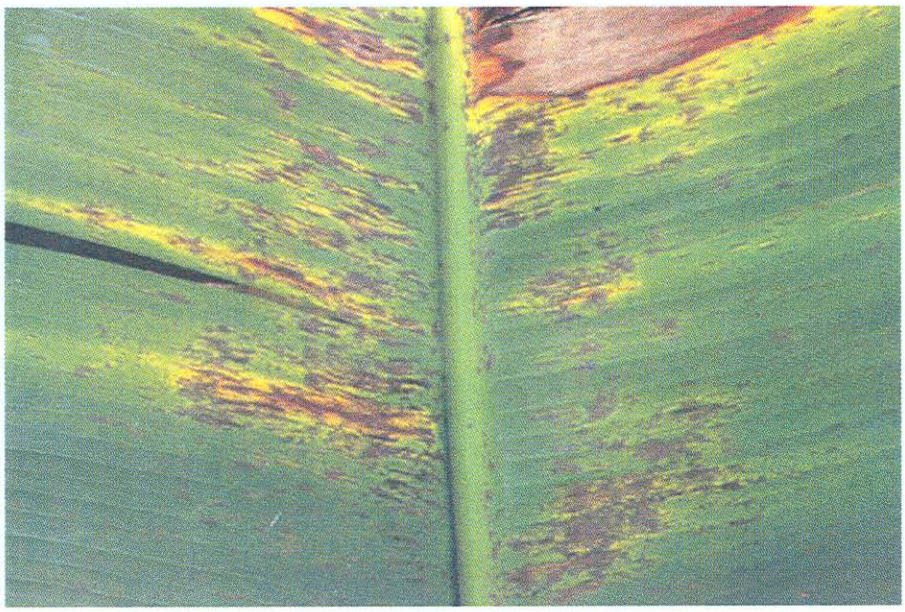

Figure 19: Symptoms of rust on a Williams banana cultivar (Jones 2000).

CHAPTER 2

FUNGI ASSOCIATED WITH LESIONS ON BANANA FOLIAGE IN SOUTH AFRICA.

FUNGI ASSOCIATED WITH LESIONS ON BANANA FOLIAGE IN SOUTH AFRICA

ABSTRACT

A survey was conducted to determine the identity and distribution of fungi associated with banana leaf diseases in South Africa. Banana leaves were randomly collected from the five banana-growing areas in the country. Isolations were made from leaf lesions following surface disinfestation, incubation in moisture chambers, or spores were collected directly from lesions. Single-spore isolates were cultured on half-strength potato-dextrose agar and identified. Four foliar diseases were observed in the different banana-growing areas. Yellow Sigatoka (caused by *Mycosphaerella musicola*) was present in all five areas, Mycosphaerella speckle (caused by *M. musae*) and Cordana leaf spot (caused by *Cordana musae*) in four, and Cladosporium speckle (caused by *Cladosporium musae*) in one. Various other fungi, mostly saprobes, were also isolated. The most common species included (in order of prevalence) *Alternaria alternata, Colletotrichum gloeosporioides, Nigrospora oryzae, N. sacchari, N. sphaerica, Pestalotiopsis* sp., *Phoma glomerata, Selenophoma asterina* and *S. juncea.*

INTRODUCTION

Various fungi are associated with the foliage of banana plants. Diseases caused by foliar pathogens such as *Mycosphaerella musicola* R. Leach ex J.L. Mulder (yellow Sigatoka), *M. fijiensis* M. Morelet (black Sigatoka) and *M. eumusae* P. Crous & X. Mourichon (eumusae leaf spot) are major constraints to production of the crop (Jones 2000). Other leaf pathogens that can be damaging to predisposed plants, or under climatic conditions conducive to disease, include *M. minima* Stahel (leaf speckle), *M. musae* (Speg.) Syd. & P. Syd. (Mycosphaerella speckle), *Acrodontium simplex* Mangenor & de Hoog (leaf speckle), *Cercospora hayi* Calp. (brown or diamond spot), *Chaetothyrina musarum* (Speg.) Theiss. (sooty blotch), *Cladosporium cladosporioides* (Fresen.) G.A. de Vries (sooty mould), *Cladosporium musae* E.W. Mason (Cladosporium speckle), *Colletotrichum musae* (Berk. & M.A. Curtis) Arx (leaf spot, anthracnose, fruit and stem rot), *Cordana musae* (Zimm.) Höhn. (Cordana leaf spot), *Deightoniella torulosa* (Syd.) M.B. Ellis (black leaf spot), *Drechslera gigantea* (Heald & F.A. Wolf) S. Ito (eyespot), *Haplobasidion musae* M.B. Ellis (Malayan or diamond leaf spot), *Hendersonula toruloidea* Nattrass (leaf spot, tip rot), *Periconiella musae* Stahel ex M.B. Ellis and *Veronaea musae* M.B. Ellis (tropical speckle), *Pestalotia leprogena* Speg. (ringspot), *Phyllachora musicola* C. Booth & D.E. Shaw (black-cross leaf spot), *Phyllosticta musae* F. Stevens & E. Young (leaf spot), *Uredo musae* Cummins and *Uromyces musae* Henn. (rust), *Curvularia* sp. (leaf spot), *Helminthosporium* sp. (fine speckle, leaf spot) and *Pyricularia* sp. (pyricularia leaf spot) (Brown *et al.* 1998, Jones 2000).

In addition to the pathogenic associations, numerous fungal taxa have been reported as endo- or epiphytes on *Musa* species, particularly wild banana (*Musa acuminata* Colla). Endophytes isolated most commonly include *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., *Curvularia* spp., *Fusarium* spp., *Nigrospora* spp., *Pestalotiopsis* spp., *Phomopsis* spp., xylariaceous taxa and sterile species (Brown *et al.* 1998, Photita *et al.* 2001). Some of these endophytes, e.g. *Colletotrichum gloeosporioides*, can cause disease in banana, whereas others may become problematic under environmental stress (Brown *et al.* 1998). However, many asymptomatic endophytic colonisers exist mutualistically with their hosts, benefiting the latter by protecting them from attack by pathogens (Petrini 1993, Dorworth & Callan 1996).

Three foliar diseases have been reported in South Africa on banana, viz, yellow Sigatoka (Van den Boom & Kuhne 1969), Mycosphaerella speckle (Brodrick 1973) and a leaf spot (Roth 1965). However, identification of yellow Sigatoka and Mycosphaerella speckle was based only on observation of symptoms and not on isolation of the causal organisms, whereas leaf spot was ascribed to a complex comprising *Glomerella cingulata* (Stoneman) Spauld & H. Schrenk, *Cordana musae, D. torulosa* and *Helminthosporium* sp., together with two bacterial species belonging to the genera *Pseudomonas* and *Xanthomonas*. Roth (1965) also found *Fusarium* sp., *Nigrospora* sp. and *Verticillium* sp., as well as various bacteria, to occur saprophytically in diseased tissue. From the above it is evident that the identity of fungi associated with banana foliar diseases in South Africa is unclear and that very little is known about endo- and epiphytes occurring in and on the crop. The aim of this study therefore was to isolate and identify the fungi associated with banana foliage, particularly lesioned sections, in the various banana-growing areas of South Africa.

MATERIALS AND METHODS

A total of 517 leaf samples displaying disease symptoms were randomly collected in 1999 and 2000 from Williams, Chinese Cavendish and Grande Naine cultivars in various banana plantations in southern KwaZulu-Natal, Komatipoort, Kiepersol, Levubu and Tzaneen, South Africa. Samples were placed in envelopes and stored at 5 °C until primary isolations were made.

Leaf sections with lesions were excised, submerged in a 2 % sodium hypochlorite for 30 sec, transferred to 70 % ethanol for 1 min, and rinsed twice in sterile distilled water (SDW). Aseptically blot-dried segments (ca. 4 mm^2) were dissected from the periphery of each lesion and plated on half-strength potato-dextrose agar (½ PDA) (Merck) (19 g PDA + 10 g agar (Merck) in 1 l water) supplemented with 200 mg/l Novobiocin in 90-mm Petri dishes. Plates were incubated for 3–7 d at 25 °C and fungi that developed were isolated. Excised lesioned leaf sections were sprayed with 70 % ethanol, placed into a 90-mm Petri dish containing sterile filter paper moistened with SDW, and incubated at 20 °C. After 1–2 d, each leaf sample was examined for fruiting structures and a tiny piece of agar touched to the fruiting structure and cultured on ½ PDA with Novobiocin. Isolates were grown at 25 °C for approximately 2–3 weeks before identification. For direct isolation, spores were collected from lesions in 100 µl SDW pipetted onto a lesion, allowed to stand for 30 sec, transferred to 400 µl SDW in an Eppendorf tube and mixed. The total volume was spread onto a 2 % water agar plate (20 g agar (Merck) in 1 l distilled water) and 24–48 hr later, single spores were collected and cultured on ½ PDA with Novobiocin. Fungal isolates were identified according to morphological characteristics.

Fungal structures were also observed *in situ* by scanning electron microscopy. Leaf lesions were prepared by fixing in 3 % glutaraldehyde for a minimum of 1 hr. Three rinse steps of 15 min each in 0.075 M phosphate buffer were carried out, followed by dehydration of the samples in 50, 70 and 90 % ethanol for 15 min at each concentration, and 3 x 15 min in 100 % ethanol. Samples were mounted on stubs, coated with gold in a Polaron sputter coater and viewed with a Jeol JSM scanning electron microscope at 5 kV.

RESULTS

Four leaf pathogens were identified (Table 1). *Mycosphaerella musicola* was the most prevalent, being isolated from 31 % of all the leaf samples and present in all five regions. *Mycosphaerella musae* and *Cordana musae* were isolated from 18 and 6 % of the samples, respectively. *Cladosporium musae* occurred only in Levubu, where it was isolated from 28 % of the samples. The appearance of yellow Sigatoka lesions conformed to literature (Fig. 1A) (Jones 2000). Sporodochia developed in sub-stomatal air chambers and emerged through stomatal pores (Fig. 1B). Cordana leaf spot was characterised by ellipsoid, brown lesions having distinct, concentric zones, surrounded by a yellow halo one to several centimetres in diameter towards the leaf margin (Fig. 1C). Conidiophores of the pathogen were pale brown, 150 µm long and 4–6 µm in diameter (Fig. 1D). Two types of speckle symptoms were observed. The most prevalent comprised light brown to tan coloured irregular blotches on the abaxial surface appearing as smoky, dark grey patches on the adaxial side (Fig. 1E). These lesions yielded *M. musae* but the fungus could not be observed *in situ*. Less common was a diffuse grey-green blotching of the adaxial surface of older leaves (Fig. 1F), which became yellow-orange and then necrotic with age

and was also found along the midrib of leaves. *Cladosporium musae* occurred in the lesions as conidiophores with terminal or intercalary branches of conidiogenous cells at the apex (Fig. 1G).

Various other fungi were also isolated from banana leaves. About 20 % of these isolates remained sterile and could not be identified. A fairly high presence of xylariaceous taxa amongst them was nevertheless evident. In order of prevalence, the identified taxa were *Nigrospora oryzae* (Berk. & Broome) Petch (isolated from 10.1 % of the leaf samples), *N. sphaerica* (Sacc.) E.W. Mason (3.7 %), *Alternaria alternata* (Fr.: Fr.) Keissl. (2.9 %), *Selenophoma asterina* (Berk. & Broome) B. Sutton (2.3 %), *Pestalotiopsis* sp. (2.1 %), *N. sacchari* (Speg.) E.W. Mason (1.4 %), *Phoma glomerata* (Corda) Wollenw. & Hochapfel (1.4 %), *Coll. gloeosporioides* (1.2 %), *S. juncea* (Mont.) Arx (1.0 %), *A. tenuissima* (Kunze: Fr.) Wiltshire (0.8 %), *Bipolaris cynodontis* (Marigoni) Shoemaker (0.8 %), *Diapothe* sp. (0.6 %), *Epicoccum nigrum* Link (0.6 %), *A.* cf. *citri* Ellis & N. Pierce (0.4 %), *Drechslera dematioidea* (Bubák & Wróbl.) Subraman. & P.C. Jain (0.4 %), *Colletotrichum musae* (0.2 %), *Curvularia lunata* (Wakker) Boedijin (0.2 %), *Drechslera* sp. (0.2 %), *Exserohilum rostratum* (Drechsler) K.J. Leonard & Suggs (0.2 %), *Guignardia mangiferae* A.J. Roy (0.2 %), *Harpographium* sp. (0.2 %), *Myrothecium verrucaria* (Alb. & Schw.) Ditmar (0.2 %) and *Pithomyces sacchari* (Speg.) M.B. Ellis (0.2 %).

Discussion

This study confirmed the presence of yellow Sigatoka and Mycosphaerella speckle in South Africa and validated the original diagnoses of the two diseases (Van den Boom & Kuhne 1969, Brodrick 1973) by isolating the causal organisms and identifying them as *M. musicola* and *M.*

musae, respectively. The pathogen was confirmed to be *Cordana musae* and not the more recently described *Cordana johnstonii* M.B. Ellis, which causes smaller leaf spots (Priest 1990) and appears to be adapted to cooler environments (Jones 2000). *Cladosporium musae* is a new recording for South Africa. *Deightoniella torulosa*, previously reported by Roth (1965) from the Mpumalanga and Limpopo lowveld regions, could not be isolated.

Yellow Sigatoka was the most prevalent of the various diseases and is reported here for the first time from KwaZulu-Natal. The second-most prevalent disease, Mycosphaerella speckle, has previously also been described as widespread in South Africa, but not as serious as yellow Sigatoka (Brodrick 1973). In accordance with Jones (2000), infection by *M. musicola* appeared to predispose plants to attack by *Cordana musae* as the latter pathogen was often isolated from Cordana-like lesions surrounding yellow Sigatoka spots. However, the incidence of *M. musicola* and *M. musae* seemed to be inversely related, a phenomenon that has not been reported before. Currently, banana leaf diseases in South Africa are under control as a result of implemented spray and deleafing programmes. However, due to the severe yellow Sigatoka outbreak in 1999 and 2000, banana leaf disease status in South Africa is tentative and regular surveys should continue.

With the exception of *Colletotrichum gloeosporioides*, *Colletotrichum musae* and *N. oryzae*, which were described by Roth (1965), Doidge (1950) and Jacobs (1973), respectively, the fungal species isolated here represent new entries for banana in South Africa. Although most were probably opportunistic secondary invaders, taxa such as *A. alternata*, *Colletotrichum gloeosporioides*, *Colletotrichum musae*, *Curvularia* spp., *Diaporthe* spp. (as *Phomopsis* spp.), *E. nigrum*, *N. oryzae* and *Pestalotiopsis* spp. have previously been reported as endophytes of banana (Brown *et al.* 1998, Photita *et al.* 2001, Photita *et al.* 2002). The presence of an endophyte

component is supported by the relatively high incidence of sterile isolates (Brown *et al.* 1998, Photita *et al.* 2001) and the regular occurrence of xylariaceous taxa, which are particularly well adapted to an endophytic existence (Whalley 1995) and commonly occur as endophytes in virtually all tropical plants (Rodriques & Samuels 1990, Pereira *et al.* 1993, Rodriques 1994, Brown *et al.* 1998, Photita *et al.* 2001). However, other common banana endophytes such as *Fusarium* spp. (Brown *et al.* 1998, Photita *et al.* 2001) could not be isolated.

Alternaria alternata and *Curvularia lunata*, isolated from four and one of the banana-growing regions in South Africa respectively, have been associated with leaf lesions on banana in China (Qi *et al.* unpublished data). *Guignardia mangiferae*, a cosmopolitan endophyte of woody plants isolated from one leaf sample collected in KwaZulu-Natal, has previously also been reported from lesions on banana leaves in New South Wales, Australia (Baayen *et al.* 2002). The commonly encountered *N. oryzae* and *N. sphaerica* are well known as banana fruit pathogens (Jacobs 1973, Brown *et al.* 1998). *Phoma glomerata*, isolated from three of the banana-growing regions, could easily have been misidentified as *P. jolyana* Piroz. & Morgan-Jones, causal agent of black finger disease (Brown *et al.* 1998). Separation of the two species is based on the presence of *Alternaria*-like chlamydospores borne catenately in *P. glomerata* and laterally in *P. jolyana* (Sutton 1980), though chlamydospores initially are borne terminally in *P. jolyana* and do not always remain single. Nevertheless, Koch's postulates could not be confirmed for any of the species isolated when artificially inoculated onto banana leaves in the present study (data not presented).

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Table 1: Fungi isolated from banana leaves in South Africa.

Species	Incidence[a]				
	Kiepersol	Komatipoort	KwaZulu	Levubu	Tzaneen
Alternaria alternata	5	7	1	2	-
Alternaria cf. *citri*	-	2	-	-	-
Alternaria tenuissima	1	2	-	1	-
Bipolaris cynodontis	2	-	1	1	-
Cladosporium musae	-	-	-	33	-
Colletotrichum gloeosporioides	2	1	3	-	-
Colletotrichum musae	-		-	1	-
Cordana musae	10	8	9	6	-
Curvularia lunata	-	1	-	-	-
Curvularia pallescens	1	-	-	-	-
Diaporthe sp.	-	1	2	-	-
Drechslera dematoidea	-	1	1	-	-
Drechslera sp.	-	-	1	-	-
Epicoccum nigrum	1	2	-	-	-
Exserohilum rostratum	-	-	1	-	-
Guignardia mangiferae	-	-	1	-	-
Harpographium sp.	-	1	-	-	-
Mycosphaerella musae	12	10	36	36	-
Mycosphaerella musicola	37	36	23	29	33
Myrothecium verrucaria	-	-	1	-	-
Nigrospora oryzae	11	2	27	1	11
Nigrospora sacchari	3	1	3	-	-
Nigrospora sphaerica	4	4	9	1	1
Pestalotiopsis guepinii	-	2	7	2	-
Phoma glomerata	1	5	-	-	1
Pithomyces sacchari	-	-	1	-	-
Selenophoma asterina	1	7	2	2	-
Selenophoma juncea	1	2	1	1	-
Sterile	12	17	6	3	1
Total leaf samples	104	110	135	119	47
Total fungal isolates	116	120	163	122	61

[a] Percentage leaf samples from which fungus was isolated.

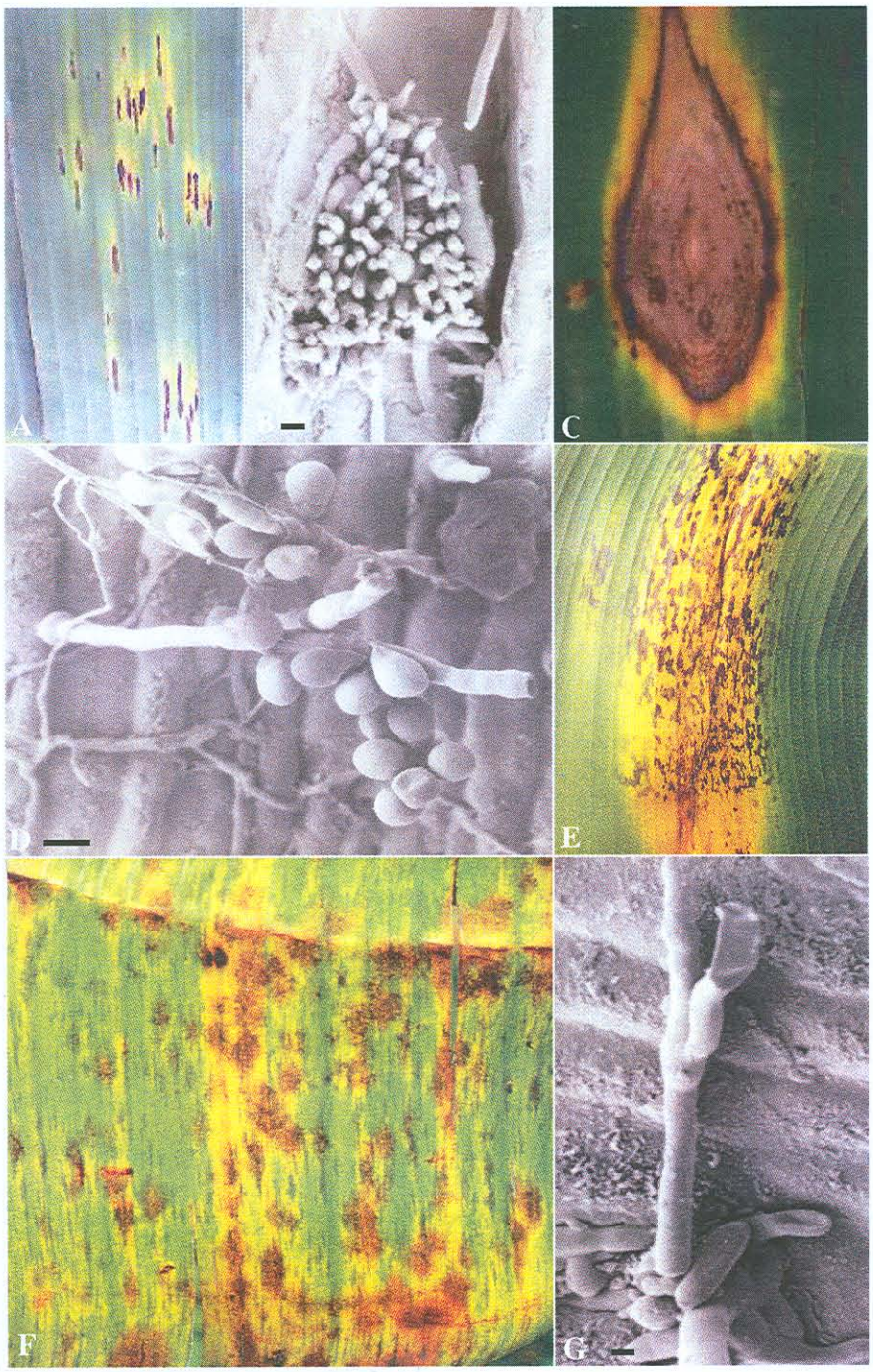

Figure 1: Symptoms and morphology of A. Yellow Sigatoka. B. *Mycosphaerella musicola.* C. Cordana leaf spot. D. *Cordana musae.* E. Mycosphaerella speckle. F. Cladosporium speckle. G. *Cladosporium musae* (scale bars 10 µm).

CHAPTER 3

IDENTIFICATION OF THE PATHOGEN ASSOCIATED WITH SIGATOKA DISEASE OF BANANA IN SOUTH AFRICA

AUSTRALASIAN PLANT PATHOLOGY: IN PRESS

A.K.J. Surridge [A], A. Viljoen [A], P.W. Crous [B] and F.C. Wehner [A]

[A] Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa.

[B] Centraalbureau voor Schimmelcultures, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands.

IDENTIFICATION OF THE PATHOGEN ASSOCIATED WITH SIGATOKA DISEASE OF BANANA IN SOUTH AFRICA

ABSTRACT

Three species of *Mycosphaerella* are known to cause Sigatoka leaf diseases in banana, namely *Mycosphaerella musicola* (yellow Sigatoka), *M. fijiensis* (black Sigatoka), and *M. eumusae* (eumusae leaf spot). Although yellow Sigatoka has previously been reported from South Africa, the identity of the causal organism has never been confirmed. Little is known about the presence of Sigatoka leaf diseases of banana in South Africa. Extensive surveys of the five banana growing areas in South Africa were conducted between 1999 and 2001, which resulted in the collection of various Sigatoka-like leaf spot samples. After morphological examination of the infected material, monoconidial isolates of the causal organism were established from each sample. A molecular confirmation of identity was conducted using species-specific primers for *M. musicola* and *M. fijiensis*. Sequence data of the ITS region was further obtained to compare the South African *Mycosphaerella* isolates from banana with *M. musicola*, *M. fijiensis* and *M. eumusae*. These results confirm that only *M. musicola* is presently associated with yellow Sigatoka symptoms in South Africa.

INTRODUCTION

Three Sigatoka leaf diseases have been described on banana (*Musa* spp.) viz. yellow Sigatoka, black Sigatoka and eumusae leaf spot, caused by *Mycosphaerella musicola* R. Leach ex. J.L. Mulder & R.H. Stover, *M. fijiensis* M. Morelet and *M. eumusae* P. Crous & X. Mourichon respectively (Carlier *et al.* 2000; Jones 2000; Crous and Mourichon 2002). Yellow Sigatoka has the widest distribution though it is rapidly being replaced by black Sigatoka in many tropical coastal regions (Stover 1972; Carlier *et al.* 1994; Jones 2000). Currently, *M. eumusae* has been detected only in Nigeria, Mauritius and South-East Asia (Carlier *et al.* 2000), thoughits similarity to *M. musicola* and *M. fijiensis* suggests that it could be more widely spread (Carlier *et al.* 2000).

In South Africa, only yellow Sigatoka has thus far been reported (Van den Boom & Kuhne 1969). However, the disease was identified using phenotypic symptoms only, without any attempt to isolate or identify the causal organism. Primary lesions caused by *M. musicola*, *M. fijiensis* and *M. eumusae* are very similar in appearance, consisting of dark-brown to black specks that develop into streaks several millimetres long, with a grey centre surrounded by a yellow halo (Jones 2000). Differences in symptomology are mostly related to the presence, nature and position of fruiting structures, but these are not readily discernible macroscopically. Positive identification of the disease therefore depends on morphological or molecular confirmation of the causal organism.

Considering the rapid rate at which black Sigatoka is spreading (Jones, 2000) and the possibility that eumusae leaf spot may previously have been overlooked in some countries (Carlier *et al.* 2000), a reappraisal of the pathogen status of Sigatoka disease in South Africa was indicated. Severe outbreaks of the disease during the 1999/2000 growing season provided further motivation for such an endeavour. This report provides evidence, based on morphological and molecular studies, that the causal organism of Sigatoka disease in South Africa is indeed *M. musicola*.

Methods and Materials

Isolation of *Mycosphaerella*

Between 1999 and 2001, 163 leaf samples showing disease symptoms of Sigatoka were randomly collected from Cavendish cultivars in the five banana growing areas of South Africa: Levubu (31), Tzaneen (34), Kiepersol (41), Komatipoort (34) and southern KwaZulu-Natal (23). Samples were taken to the laboratory in envelopes and were maintained at 5 °C until being processed (2–3 d).

Single-conidial isolations were made from stage 5 leaf spots (Jones 2000) on the respective samples. Leaf spots were ellipsoidal in shape with a yellow halo surrounding a dark outlined area of sunken white necrotic tissue in which the sporodochia were found. Fifty microlitres of sterile distilled water was pipetted onto a mature lesion, left for 10 sec and then transferred to 50 µl sterile distilled water in an Eppendorf tube. The suspension was mixed well

and spread over the surface of a 20 g/l water agar (Merck) plate. Plates were incubated at 25 °C for 1–2 d to induce conidial germination. Germinating conidia were transferred to half-strength potato-dextrose agar (PDA) (Merck) (19 g/l PDA + 10 g/l agar) supplemented with 0.2 g/l Novobiocin. All cultures are maintained on PDA slants and under mineral oil at 4 °C at the Forestry and Agricultural Biotechnology Institute (FABI) in Pretoria, South Africa.

Morphological identification

Conidia were collected from stage 5 lesions as described above, suspended in lactophenol, and studied under the light microscope. Fresh leaf lesions were also excised and fixed in 3 % glutaraldehyde for a minimum of 1 hr, followed by three rinses of 15 min each in 0.075 M phosphate buffer. Samples were dehydrated for 15 min in 50, 70 and 90 % ethanol, respectively, and three times in 100 % ethanol. Critical point drying was performed in liquid carbon dioxide before mounting the samples on stubs, sputtering them with gold and examining them under a scanning electron microscope.

Artificial inoculation

Selected cultures (CMW 6346, CMW 6347, CMW 6365, CMW 6368, CMW 6373, CMW 6375) were inoculated onto young banana plants (cv. Cavendish) in a greenhouse at 27 °C (day/night) and monitored over a 4-month-period. Plants chosen for inoculation were approximately 1 m high, having 3–4 leaves, and growing in 5 l bags. Plants were moved into the greenhouse 30 d prior to inoculation to allow for acclimatisation. Plants were watered every third day and exposed to normal sunlight hours. The adaxial surface of the leaves was lightly abraded with a syringe needle to remove a portion of the waxy cuticle. An agar plug covered in mycelium

was then placed onto the epidermis, covered with clear strip of laboratory film, and marked with the isolate number. Two leaves per plant and three plants per isolate were inoculated.

DNA isolation

Molecular diagnosis of the *Mycosphaerella* species associated with Sigatoka disease in South Africa was achieved by PCR and sequencing of the ITS region of the DNA from lesions on all leaf samples and from selected *Mycosphaerella* isolates, respectively. Initially, DNA was extracted using the method described by Raeder and Broda (1985). DNA extraction from a final selection of leaf samples, and all fungal isolates for sequencing, was achieved using a second method which yielded cleaner DNA in larger quantities. Approximately 1 g of fresh leaf tissue or fungal mycelium was placed in an Eppendorf tube and ground with ca. 10 µg sterile river sand in 500 µl of DNA Extraction Buffer (DEB: 200 mM Tris-HCl (pH 8), 150 mM NaCl, 25 mM EDTA (pH 8), 0.5 % SDS). A further 200 µl DEB was added to the suspension with 500 µl of phenol and 300 µl chloroform, vortexed, and centrifuged for 60 min at 10 000 rpm. The supernatant was transferred to a new tube, 500 µl of phenol and 500 µl chloroform added, and centrifuged for 5–10 min at 10 000 rpm. The phenol/chloroform step was repeated until the interphase was clean. A further 500 µl chloroform was added and the tubes centrifuged for 5 min at 11 000 rpm. The supernatant was transferred to a new tube, double the volume of 100 % ethanol was added, and the suspension was mixed. The DNA was allowed to precipitate at 4 °C overnight, and was pelleted by centrifugation for 30 min at 11 000 rpm. Pellets were washed with 500 µl 70 % ethanol, dried and resuspended in 100 µl sterile distilled water and 3 µl RNAse (2.5 µM).

Polymerase chain reaction

Species-specific primers described by Johanson and Jeger (1993) were used to distinguish between *M. fijiensis* and *M. musicola*. Primer MM137 was specific for *M. musicola*, and MF137 for *M. fijiensis*. R635 served as a reverse primer for both species. Authentic *M. fijiensis* DNA (courtesy Dr H. Hayden, University of Queensland) and DNA isolated from a morphologically confirmed South African *M. musicola* isolate were included as positive controls. A modification of the Johanson and Jeger (1993) method of PCR was used to amplify the DNA. Each PCR tube contained a total volume of 25 µl: 18.7 µl sterile distilled Sabax water, 2.5 µl PCR buffer with $MgCl_2$ (10x), 2 µl dNTPs (2.5 µM), 0.5 µl primer 1 (50 µM), 0.5 µl primer 2 (50 µM), 0.5 µl DNA (27 ng/µl), and 0.3 µl Expand Taq (5U/µl). DNA amplification was performed in a PCR thermal cycler using the following programme: 3 min at 94 °C, 40 cycles of 1 min at 94 °C, 1 min at 60 °C and 3 min at 72 °C, followed by 5 min at 72 °C, and then held at 4 °C. The PCR product was analysed on a 1 % agarose gel.

Sequencing of the ITS region

Eight randomly selected *Mycosphaerella* isolates were subjected to an ITS PCR with primers ITS1 and ITS4 (White *et al.* 1990). The ITS PCR products were purified with a "High pure PCR product purification kit" (Roche). A sequencing reaction was performed, with each tube containing 1 µl clean PCR product, 2 µl "Big Dye" (Roche) sequence mix, 0.32 µl primer and 1.68 µl sterile Sabax water. The PCR product was cleaned by adding 15 µl sterile Sabax water, transferring the entire volume to a sequencing tube, adding 2 µl of 3 M sodium acetate and 50 µl 95 % ethanol, and allowing it to stand on ice for 10 min. Sequencing tubes were then centrifuged at 10 000 rpm for 30 min. The ethanol solution was removed, the pellet rinsed in 150

µl 70 % ethanol, and the tubes centrifuged at 10 000 rpm for 5 min. The ethanol was aspirated and the pellet dried under vacuum for approximately 10 min. Tubes were transferred on ice to the sequencer. DNA sequences were determined using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase (Applied Biosystems, UK). ITS2 and ITS3 were included as internal primers to confirm the sequence data obtained (White *et al.* 1990).

Sequence analysis

ITS sequences for *M. musicola, M. fijiensis* and *M. eumusae* were obtained from GenBank (Table 1). DNA sequences were manually aligned, and inserted gaps treated as missing data. Ambiguously aligned regions were excluded from the data set before analysis. Phylogenetic analysis was based on parsimony using PAUP 4.0b8 (Phylogenetic Analysis Using Parsimony, Version 4.0b8; Swofford, 2000). Heuristic searches were done with random addition of sequences (1000 replicates), tree bisection-reconnection (TBR), branch swapping and MULPAR effective and MaxTrees set to auto-increase. Phylogenetic signal in the data sets was accessed by evaluating tree length distributions over 100 randomly generated trees. The consistency (CI) and retention (RI) indices were determined for all data sets. Phylogenetic trees were rooted with *Mycocentrospora acerina* (R. Hartig) Deighton (Stewart *et al.* 1999) as a monophyletic sister outgroup to the remaining taxa. Bootstrap analyses were conducted to determine confidence in branching points (1000 replicates) for the most parsimonious trees generated.

RESULTS

Morphological characterisation

The morphology of all isolates from banana leaves in South Africa appeared to be consistent with that of *Pseudocercospora musae* (Zimm.) Deighton anamorph of *M. musicola* (Jones 2000). Masses of conidia were produced in sporodochia on both sides of the leaf, though more abundantly on the upper surface. Some sporodochia developed in sub-stomatal chambers and emerged through the stomatal pore (Fig. 1A). Conidiophores were straight, sometimes slightly curved and bottle-shaped. They lacked septa and were unbranched. The conidiophore apex was rounded and lacked significant scarring (Fig. 1A). Conidia were pale brown, smooth, and varied in shape from straight to curved, cylindrical, ranging from 50–120 µm in length and 2–6 µm in width (Fig. 1B). Conidial apices were obtuse, and the base lacked a thickened hilum. No conidia were produced in culture.

Artificial inoculation

Inoculated banana plants exhibited typical yellow Sigatoka symptoms for all isolates tested, within 3–4 months of inoculation, from which the fungus could readily be re-isolated.

PCR with species-specific primers

DNA from Sigatoka lesions on South African leaf samples produced a 1018 bp band on a 1 % agarose gel with the *M. musicola* primer MM 137 (Fig. 2), in accordance with Johanson and Jeger (1993). The samples produced banding patterns corresponding with the positive control sample of DNA of *M. musicola*. No bands were produced when leaf DNA samples were tested

with the *M. fijiensis* primer, MF 137. When the *M. fijiensis* DNA was tested with MF137, a 1018 bp band was produced, confirming the validity and accuracy of the PCR reactions.

Sequencing of the ITS region

Comparison of the ITS region of the South African isolates with those of *M. musicola*, *M. fijiensis* and *M. eumusae* indicated that the local isolates are phylogenetically similar to *M. musicola* (Fig. 3). However, the South African isolates appeared to have greater similarity with each other than with isolates of *M. musicola* from other countries. The clade containing isolates of *M. fijiensis* and *M. eumusae* could be differentiated from the *M. musicola* clade by at least eight base pairs, and the two former species from each other by 11 base pairs. Base pair differences were also evident between isolates of the same species from different geographic regions. The high CI and RI values of 0.926 and 0.904, respectively, support the validity of this tree.

DISCUSSION

The morphological, molecular and phylogenetic studies reported here confirmed that the Sigatoka disease on banana in South Africa is caused by *M. musicola*, thereby verifying the diagnosis by Van den Boom and Kuhne (1969) of the disease as yellow Sigatoka. Although the teleomorph was not present, conidium and conidiophore morphology of the fungus present in the Sigatoka lesions conformed to the description of the anamorph of the yellow Sigatoka pathogen, *M. musicola*. The identity of the fungus was further confirmed by PCR with species-specific primers for the identification of *M. musicola* (Johanson and Jeger 1993), and by proving that the

ITS sequences of local isolates were similar to *M. musicola,* rather than *M. fijiensis* or *M. eumusae*. Isolates of *M. musicola* from South Africa appear to be closely related, and sequencing of the ITS region revealed a 100 % homology. The South African isolates, however, differ from the isolates from Southeast Asia and Central America with at least four base pairs.

The occurrence of *M. musicola* in South Africa is consistent with the belief that yellow Sigatoka is more prevalent in subtropical areas (Jones 2000), and the high incidence of the disease in the 1999/2000 season could probably be ascribed to the above-average rainfall and warm winters that prevailed during this period. The results furthermore indicate that black Sigatoka and eumusae leaf spot have not yet reached South Africa, despite the ability of *M. fijiensis* to adapt to cooler climates (Jones 2000), and the presence of *M. eumusae* in Mauritius (Carlier *et al.* 2000), which is situated at the same latitude as the banana growing areas of South Africa.

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Table 1: Collection and sequence details of the fungi included in the phylogenetic analysis.

Fungus	Culture number	Location	Date	Collector	Host Cultivar	GenBank accession number
M. musicola	CMW 6375	Komatipoort	19-May-00	A. Viljoen	Williams	AF509728
M. musicola	CMW 6373	Kiepersol	17-Mar-00	A. Viljoen	Williams	AF509729
M. musicola	CMW 6314	Kiepersol	24-Jun-00	A. Viljoen	Grand Naine	AF509730
M. musicola	CMW 6325	Komatipoort	16-Mar-00	A. Viljoen	Grand Naine	AF509731
M. musicola	CMW 6368	KwaZulu Natal	24-Jun-00	A. Viljoen	Grand Naine	AF509732
M. musicola	CMW 6340	Kiepersol	14-Jun-00	A. Viljoen	Williams	AF509733
M. musicola	CMW 6346	Kiepersol	14-Jun-00	A. Viljoen	Williams	AF509734
M. musicola	CMW 6365	Tzaneen	24-Jun-00	A. Viljoen	Chinese Cavendish	AF509735
M. fijiensis	ATCC 36054	Honduras	-	R.H. Stover	Musa AAA	AF297225
M. fijiensis	ATCC 22117	Hawaii	-	D.S. Meredith	Gros Michel	AF297234
M. fijiensis	ATCC 22116	Philippines	-	D.S. Meredith	Giant Cavendish	AF181705
M. musicola	ATCC 22115	Philippines	-	D.S. Meredith	Lacatan bananas	AF181706

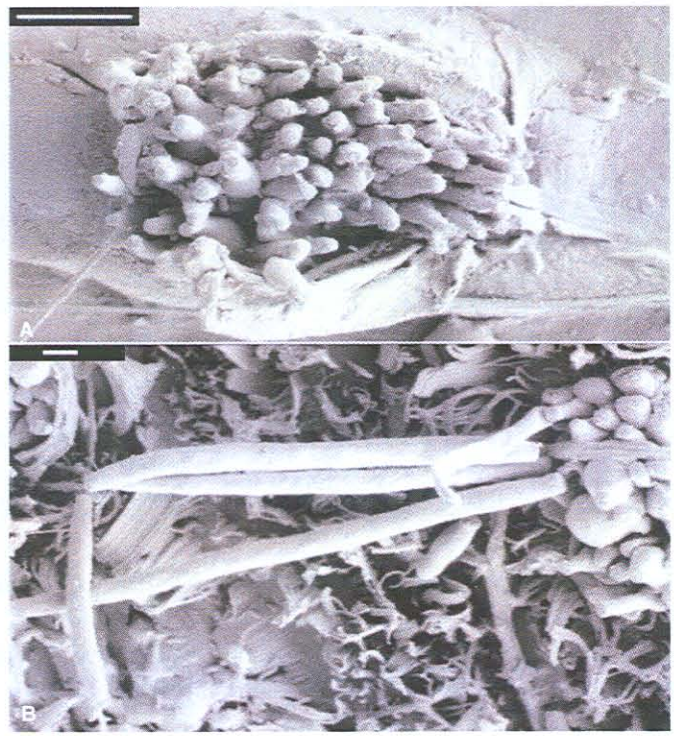

Figure 1: Electron micro-graphs of *Mycosphaerella musicola*. **A.** Pustule on the banana leaf surface formed by conidiophores in a sporodochium, emerging through a stomatal pore. **B.** Conidia (Bar = 10 µm).

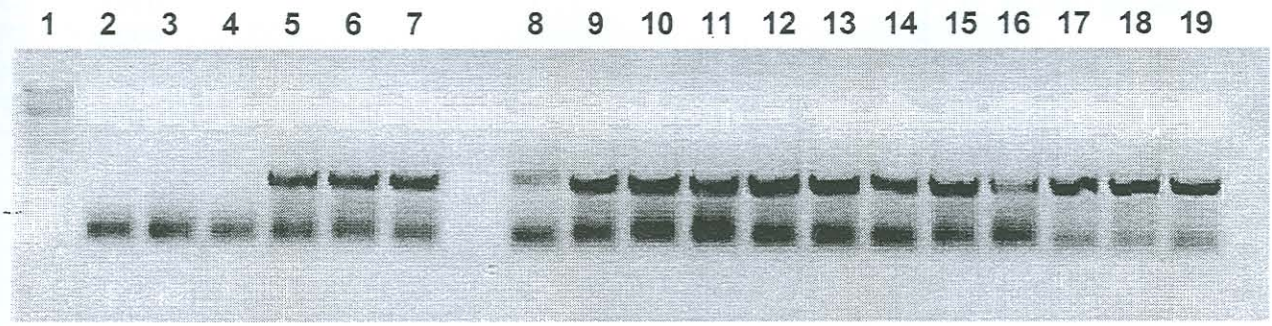

Figure 2: 1% Agarose Gel of PCR products from amplification of infected leaf and fungal DNA with primers MM137 and R635 stained with ethidium bromide. Lanes **1**, λ Marker; **2–4**, Water Controls; **5–7**, Positive Control *Mycosphaerella musicola* DNA (from South African fungal cultures); **8–19**, Positive test for *Mycosphaerella musicola* (infected leaf DNA).

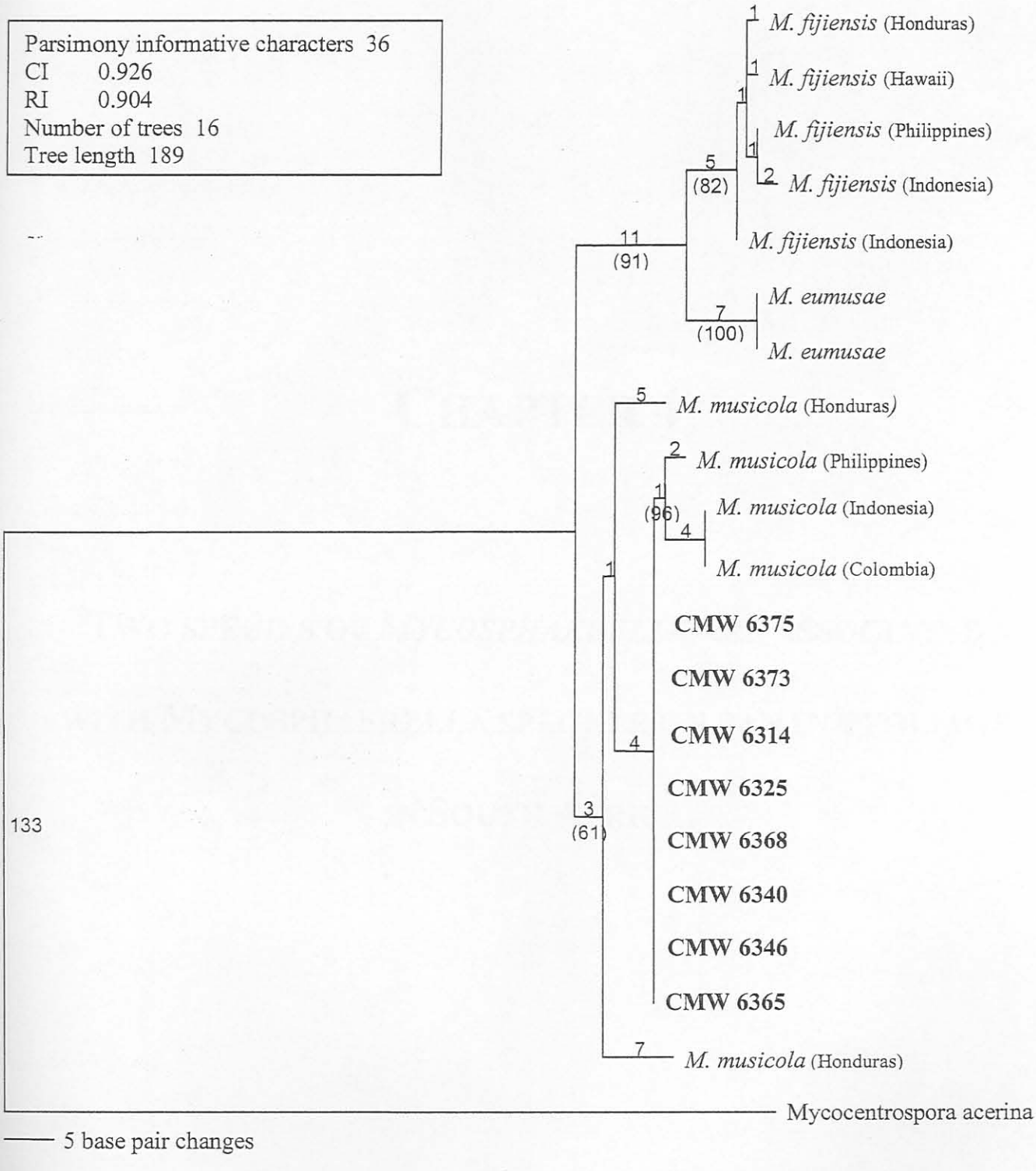

Figure 3: Phylogeny of *Mycosphaerella fijiensis*, *M. eumusae*, *M. musicola* and South African isolates of *Mycosphaerella* causing a Sigatoka-like leaf disease of banana.

CHAPTER 4

TWO SPECIES OF *MYCOSPHAERELLA* ARE ASSOCIATED WITH MYCOSPHAERELLA SPECKLE ON BANANA FOLIAGE IN SOUTH AFRICA

TWO SPECIES OF *MYCOSPHAERELLA* ARE ASSOCIATED WITH MYCOSPHAERELLA SPECKLE ON BANANA FOLIAGE IN SOUTH AFRICA

ABSTRACT

Mycosphaerella musae causes Mycosphaerella leaf speckle on banana leaves. Surveys conducted in the five banana growing areas of South Africa during 1999–2001 resulted in the collection of various leaf specimens exhibiting speckle symptoms. Following morphological examination of the infected material, monoconidial and hyphal tip isolates of the causal organisms were established from each sample. Sequence data of the ITS region was obtained to compare the South African *Mycosphaerella* isolates from banana leaves with *M. musicola*, *M. fijiensis*, *M. musae* and *M. eumusae*. These results confirm that *M. musae* and a species closely related to *M. colombiensis*, a *Eucalyptus* leaf blotch pathogen reported from Colombia, are causing speckle disease. The latter two are responsible for speckle symptoms on banana leaves in South Africa.

Introduction

Four of the various fungal leaf diseases described on banana (*Musa* spp.) involve species in the genus *Mycosphaerella*, namely *Mycosphaerella musicola* R. Leach (yellow Sigatoka), *M. fijiensis* M. Morelet (black Sigatoka), *M. musae* (Speg.) Syd. & P. Syd. (Mycosphaerella speckle), and *M. eumusae* Crous & Mourichon (eumusae leaf spot) (Jones 2000, Crous & Mourichon 2002). *Mycosphaerella fijiensis* is considered to be the most severe pathogen of banana foliage, causing serious losses in tropical regions (Jones 2000). Since 1962, *M. musicola* has acquired a worldwide distribution, and now occurs in every banana growing country except Egypt, Israel and the Canary Islands (Carlier *et al.* 1994, Jones 2000). Although it is a less virulent pathogen than *M. fijiensis,* it is considered to be important in sub-tropical countries. *Mycosphaerella musae* has a worldwide distribution, but is only considered important in areas with sub-tropical climates (Jones 2000).

Very little research has been conducted on foliar diseases of banana in South Africa, with only two *Mycosphaerella* diseases being recorded. Van den Boom and Kuhne (1969) reported the presence of *M. musicola* in South Africa. This report, however, was based on field symptoms, and the local presence of this pathogen has only recently been confirmed via morphology, pathogenicity and DNA sequence data (Surridge *et al.* 2003a). Brodrick (1973) reported the presence of *M. musae* in South Africa, though this report was erroneously quoted as a first report of *Cladosporium musae* E.W. Mason in subsequent literature (Gorter 1977, Crous *et al.* 2000). Although this situation has been resolved by the confirmation of *C. musae* causing Cladosporium speckle on banana in South Africa (Surridge *et al.* 2003b), the etiology of *M. musae* remains unresolved. To elucidate the disease status, surveys of the five banana growing

areas in South Africa were conducted during 1999–2001, which resulted in the collection of various specimens exhibiting symptoms of Mycosphaerella speckle. The purpose of the present study was to establish the identity of the organism(s) responsible for causing Mycosphaerella speckle of banana in South Africa.

MATERIALS AND METHODS

Isolates

Between 1999 and 2001, 147 leaf samples exhibiting symptoms of Mycosphaerella speckle were randomly collected from Cavendish cultivars in the five banana growing areas of South Africa: Levubu, Tzaneen, Kiepersol, Komatipoort and southern KwaZulu-Natal. Samples were transported to the laboratory in paper envelopes and maintained at 5 °C until being processed (2–3 d).

A portion of a lesion on each leaf was excised and submersed in 2 % sodium hypochlorite for 30 sec., transferred to 70 % ethanol for 1 min, and rinsed twice in sterile distilled water. Sections (2 mm x 2 mm) were dissected from the lesion and plated on half-strength potato-dextrose agar (PDA) (Merck, Germiston, South Africa) supplemented with 0.2 g/l Novobiocin in 90-mm Petri-dishes. Plates were incubated for 3–7 d at 25 °C and fungi that developed were isolated by hyphal tip excision and cultured on PDA plates at 25 °C for 60 d until identification.

All cultures are maintained on PDA slants and under mineral oil at 4 °C at the Forestry and Agricultural Biotechnology Institute (FABI) in Pretoria, South Africa. Representative strains have been deposited at the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, The Netherlands.

DNA amplification and phylogeny

Molecular diagnosis of the *Mycosphaerella* species associated with symptoms on banana foliage in South Africa was achieved by PCR and sequencing of the ITS region of the DNA isolated from cultures obtained above. DNA was isolated using the method of Surridge *et al.* (2003a).

Randomly selected Mycosphaerella speckle isolates and *M. musicola* isolates were subjected to an ITS-PCR with primers ITS1 and ITS4 (White *et al.,* 1990). Each PCR tube contained a total volume of 25 µl: 18.7 µl sterile distilled Sabax water, 2.5 µl PCR buffer with $MgCl_2$ (10x), 2 µl dNTPs (2.5 µM), 0.5 µl primer 1 (50 µM), 0.5 µl primer 2 (50 µM), 0.5 µl DNA (27 ng/µl), and 0.3 µl Expand Taq (5U/µl). DNA amplification was performed in a PCR thermal cycler using the following programme: 10 min at 95 °C, 35 cycles of 30 sec at 95 °C, 45 sec at 55 °C and 2 min at 72 °C, followed by 7 min at 72 °C, and then held at 4 °C. The PCR product was analysed on a 1 % agarose gel. The ITS PCR products were purified with a "High pure PCR product purification kit" (Roche, Germany). DNA sequences were determined using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase (Applied Biosystems, UK). ITS2 and ITS3 (White *et al.* 1990) were included as internal primers to confirm the sequence data obtained.

ITS sequences for *M. musicola, M. fijiensis, M. musae* (Table 1) and *M. eumusae* were obtained from GenBank. Representative DNA sequences were manually aligned by inserting gaps. Ambiguously aligned regions were excluded from the data set before analysis and gaps treated as missing data. Phylogenetic analysis was based on parsimony using PAUP 4.0b8 (Phylogenetic Analysis Using Parsimony) (Swofford, 2000). Heuristic searches were done with random addition of sequences (1000 replicates), tree bisection-reconnection (TBR), branch swapping and MULPAR effective and MaxTrees set to auto-increase. Phylogenetic signal in the data sets was accessed by evaluating tree length distributions over 100 randomly generated trees. The consistency (CI) and retention (RI) indices were determined for all data sets. Phylogenetic trees were rooted with *Mycocentrospora acerina* (R. Hartig) Deighton (Stewart *et al.* 1999) and *Fusarium circinatum* Nirenberg & O'Donnell as a monophyletic sister outgroups to the remaining taxa. Bootstrap analyses were conducted to determine confidence in branching points (1000 replicates) for the most parsimonious trees generated.

Discovery of ITS sequence homology between several Mycosphaerella speckle isolates and *M. colombiensis* Crous & M.J. Wingf., using GenBank nucleotide blast searching, lead to the sequencing of the α-elongation factor region of both *M. colombiensis* and the Mycosphaerella speckle isolates. Sequencing was performed using primers EF1 and EF2 (Glass & Donaldson, 1995). Sequences were analysed as above.

Pathogenicity trials

Selected cultures of *M. colombiensis* (STE-U 1104–1106, ex-type) and Mycosphaerella speckle strains (CBS 110967, CBS 110968, CBS 110969 and CMW 10901, CMW 10902, CMW

10903, CMW 10904, CMW 10905, CMW 10906, CMW 10907, CMW 10908) were inoculated onto young banana (cv. Cavendish) and young *Eucalyptus urophylla* S.T. Blake plants in a greenhouse maintained at 27 °C, and monitored over a 4-month-period. Plants used for inoculation were approximately 1 m high and growing in 5 l bags filled with potting soil. Plants were moved into the greenhouse 30 d prior to inoculation to allow for acclimatisation. Plants were watered every third day and exposed to normal sunlight hours. The adaxial surface of the leaves was lightly abraised with a syringe needle to remove a portion of the waxy cuticle. An agar plug overgrown with mycelium was then placed onto the epidermis, covered with clear strip of laboratory film, and marked with the isolate number. Each isolate was inoculated onto two leaves on each of three plants. Sterile agar plugs served as controls. Plants were inspected regularly for symptom development and isolations were made from lesions as described above after 90–120 d.

RESULTS

DNA amplification and phylogeny

Parsimony analysis of the ITS-1 and ITS-2 regions of the rDNA operon was conducted to determine the phylogenetic placement of South African Mycosphaerella speckle isolates in relation to other *Mycosphaerella* species known from banana. Alignment by inserting gaps resulted in a total of 555 characters used in the comparison of the different species. A total of 359 constant characters, 113 parsimony-uninformative characters and 83 parsimony-informative characters were obtained. Heuristic searches on the data generated 100 most parsimonious trees, of which tree number 1 is presented (Fig. 1).

Phylogenetic analysis of the sequence data delineated two species of *Mycosphaerella* to be associated with Mycosphaerella speckle symptoms. One species conformed to *M. musae*, while the other proved to be similar to *M. colombiensis*. Based on ITS sequence data, only one base pair difference (AF309612) was observed between the latter isolates and *M. colombiensis*. The clade containing these isolates and *M. colombiensis* could be differentiated by 33 steps from the clades containing *M. fijiensis, M. musicola, M. eumusae* and *M. musae*. Parsimony analysis of the α-elongation factor region of the genome was conducted to determine the phylogenetic placement of the unknown Mycosphaerella speckle isolates in relation to *M. colombiensis*, and other *Cercospora* and *Mycosphaerella* species. Alignment by inserting gaps resulted in a total of 350 characters. Inserted gaps were treated as "missing" data. A total of 43 constant characters, 37 parsimony-uninformative characters and 270 parsimony-informative characters were obtained. Heuristic searches on the data generated 100 most parsimonious trees, of which tree number 1 is presented (Fig. 2). The unknown Mycosphaerella speckle isolates differed from *M. colombiensis* by 6 base pairs according to α-elongation factor, which may suggest that the banana isolates represent a sister taxon to *M. colombiensis*. Sequence data were deposited in GenBank (Table 1).

Pathogenicity tests

Banana plants inoculated with the South African isolate of *M. musae*, the verified isolate of *M. colombiensis*, as well as the unknown Mycosphaerella speckle isolates, exhibited typical speckle symptoms (Fig. 3A) within 90–120 d of inoculation, from which the fungus could be re-isolated. Known symptoms of *M. colombiensis* on *E. urophylla* in Colombia include amphigenous, light brown leaf spots. *Eucalyptus urophylla* leaves inoculated with the South African isolate of *M. musae*, the verified isolate of *M. colombiensis*, as well as the unknown

Mycosphaerella speckle isolates developed leaf blotch symptoms (Fig. 3B) from which the respective fungi could be re-isolated, confirming Koch's postulates.

Discussion

Comparison of the ITS region of the South African Mycosphaerella speckle isolates delineated them in two clearly differentiated clades, supported by a 100 % bootstrap value. From the sequence data, it appears that the unknown Mycosphaerella speckle isolates could represent *M. colombiensis*. This is further supported by results of the pathogenicity study, where isolates of the unknown speckle pathogen incited spots on leaves of *E. urophylla*, which were indistinguishable from those caused by *M. colombiensis*. Likewise, *M. colombiensis* isolates from *E. urophylla* caused lesions similar to those of *M. musae* and the unknown speckle pathogen on banana leaves. Results also indicated that *M. musae* is capable of inducing leaf spots on *E. urophylla*. Besides endorsing the similarity between the unknown speckle pathogen and *M. colombiensis,* cross-pathogenicity of the two taxa, and of *M. musae*, obviously holds implications for the epidemiology of Mycosphaerella speckle on banana and *M. colombiensis* leaf spot on *E. urophylla*.

Notwithstanding their relatedness at molecular level, the unknown speckle isolates and *M. colombiensis* differ morphologically. Ascospores of the latter are obovoid, 1-septate, not constricted at the septum, 11–15 x 3–4 µm. It has a *Pseudocercospora* anamorph with narrowly obclavate or subcylindrical conidia, 1–5 septate, 25–60 x 2.5–3.5 µm, and olivaceous black

colonies (Crous 1998). Colonies of the unknown speckle isolates were olivaceous grey in colour, and did not produce an anamorph. When sporulating on 2 % water agar, the isolates produced obovoid, unconstricted, 1 septate ascospores, 6–10 x 2–2.5 µm, notably smaller than those of *M. colombiensis* and also somewhat shorter than those of *M. musae*. The disinclination of the unknown speckle pathogen to produce an anamorph is reminiscent of *M. musae*, though anamorphs of the latter species have been observed. Stover (1994) reported that *M. musae* existed endophytically, producing brown, verruculose conidia, 55–200 x 2.5–3 µm, with a prominent basal scar. Based on current concepts of cercosporoid fungi (Crous *et al.* 2001), this conforms to the mitosporic genus *Stenella*. Some isolates, however (Stover 1994), also produced conidia that were smooth-walled with faint (*Passalora*) or no (*Pseudocercospora*) scars. These findings suggest that, besides *M. musae* and *M.* cf. *colombiensis*, more *Mycosphaerella* species may be associated with Mycosphaerella speckle on banana. For the time being, however, it is concluded that *Mycosphaerella* causing speckle symptoms on banana in South Africa is paraphyletic.

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M. musicola	CMW 6373	Kiepersol, SA	17-Mar-00	A. Viljoen	Williams	AF509729
M. musicola	CMW 6314	Kiepersol, SA	24-Jun-00	A. Viljoen	Grande Nain	AF509730
M. musicola	CMW 6325	Komatipoort, SA	16-Mar-00	A. Viljoen	Grande Nain	AF509731
M. musicola	CMW 6368	KwaZulu Natal, SA	24-Jun-00	A. Viljoen	Grande Nain	AF509732
M. musicola	CMW 6340	Kiepersol, SA	14-Jun-00	A. Viljoen	Williams	AF509733
M. musicola	CMW 6346	Kiepersol, SA	14-Jun-00	A. Viljoen	Williams	AF509734
M. musicola	CMW 6365	Tzaneen, SA	24-Jun-00	A. Viljoen	Chinese Cavendish	AF509735
M. musicola	ATCC 22115	Philippines	-	D.S. Meredith	Lacatan	AF181706
M. colombiensis	CBS 110967	-	-	P.W. Crous	-	AF309612
M. colombiensis	CBS 110968	-	-	P.W. Crous	-	AF222838
M. cf. *colombiensis*	CMW 10901	Natal, SA	5-Jun-00	A.K.J. Surridge	Williams	ITS: AY217105
M. cf. *colombiensis*	CMW 10902	Mpumalanga, SA	25-Jul-00	A.K.J. Surridge	Williams	ITS: AY217106 EF: AY217112
M. cf. *colombiensis*	CMW 10903	Mpumalanga, SA	25-Jul-00	A.K.J. Surridge	Grande Naine	ITS: AY217107 EF: AY217113
M. cf. *colombiensis*	CMW 10904	Mpumalanga, SA	25-Jul-00	A.K.J. Surridge	Grande Naine	ITS: AY217108 EF: AY217114

M. musae	CMW 10905	Mpumalanga, SA	25-Jul-00	A.K.J. Surridge	Grande Naine	ITS: AY217104
M. fijiensis	ATCC 36054	Honduras	-	R.H. Stover	*Musa* AAA	AF297225
M. fijiensis	ATCC 22117	Hawaii	-	D.S. Meredith	Gros Michel	AF297234
M. fijiensis	ATCC 22116	Philippines	-	D.S. Meredith	Giant Cavendish	AF181705
Mycocentrospora acerina	ATCC 34539	Norway	-	K. Arsvol	Carrot	-
M. colombiensis	CBS 110967	-	-	P.W. Crous	-	EF: AY217109
M. colombiensis	CBS 110968	-	-	P.W. Crous	-	EF: AY217110
M. colombiensis	CBS 110969	-	-	P.W. Crous	-	EF: AY217111
Cercospora piaropi	-	-	-	-	-	EF: AF146149
Cercospora piaropi	-	-	-	-	-	EF: AF146146
Cercospora beticola	-	-	-	-	-	EF: AF146140
M. graminicola	-	-	-	-	-	EF: AW180959
M. graminicola	-	-	-	-	-	EF: AW181005
Fusarium circinatum	MRC 7541	USA	-	T. Gordon	*Pinus radiata*	EF: AF160295

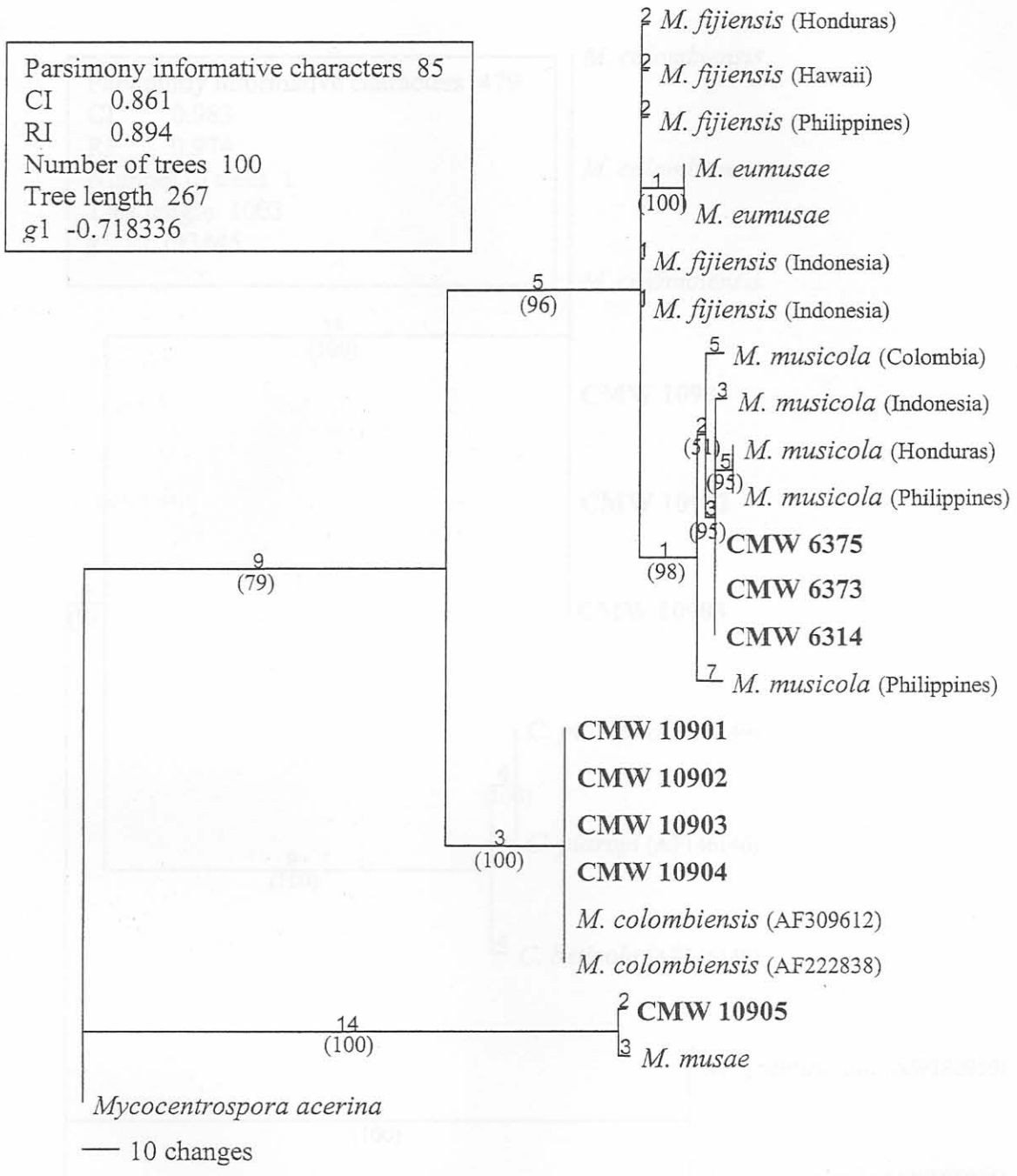

Figure 1: Phylogeny of the internal transcribed spacer sequence of *Mycosphaerella fijiensis*, *M. eumusae*, *M. musicola*, *M. musae*, *M. colombiensis* and South African isolates of *Mycosphaerella* causing Sigatoka and speckle leaf disease of bananas (tree number 1 of 100 trees is presented).

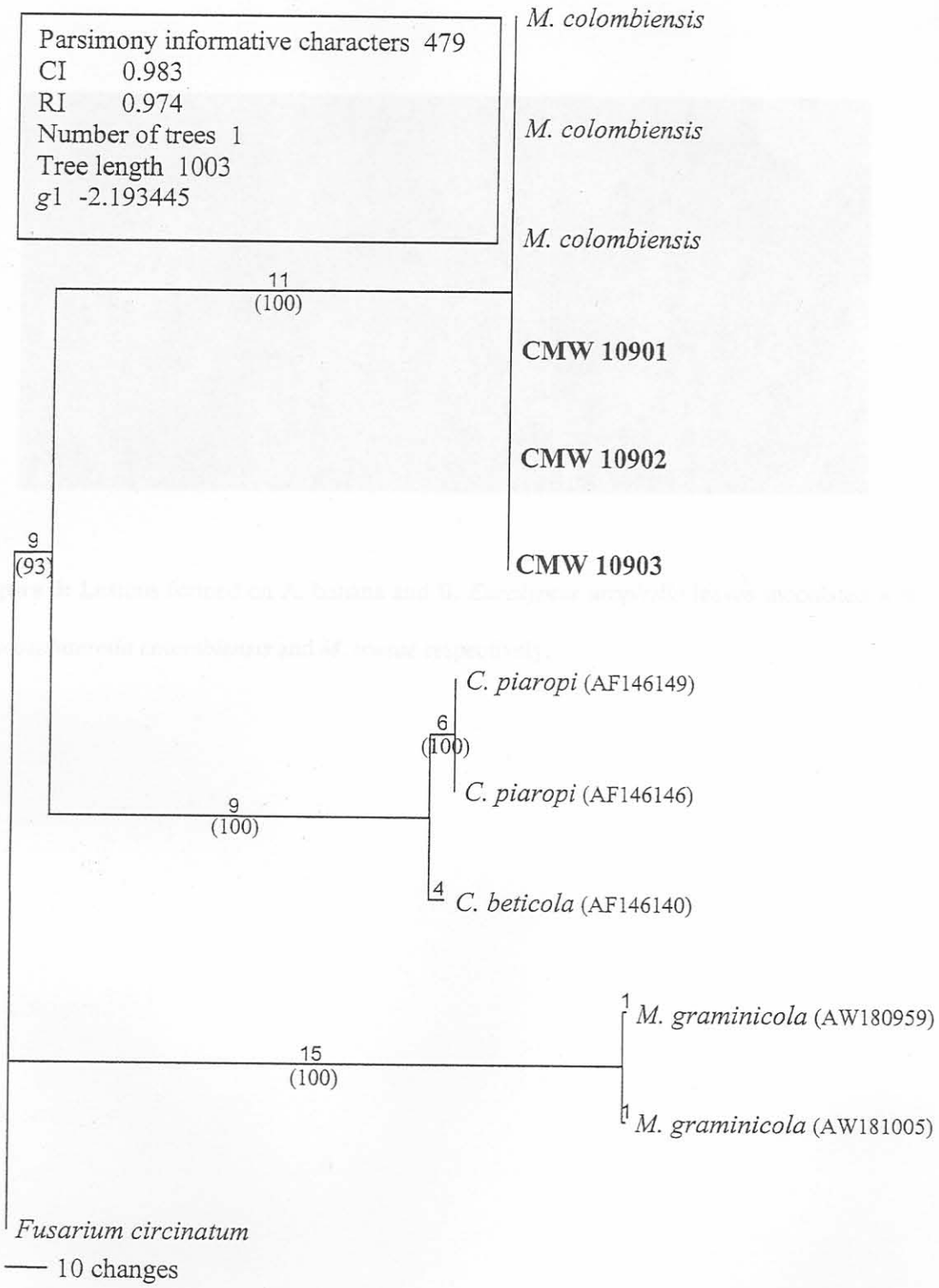

Figure 2: Phylogenetic tree of the alpha-elongation factor gene of *Mycosphaerella colombiensis* and South African isolates of *Mycosphaerella* causing speckle leaf disease of bananas (tree number 1 of 100 trees is presented).

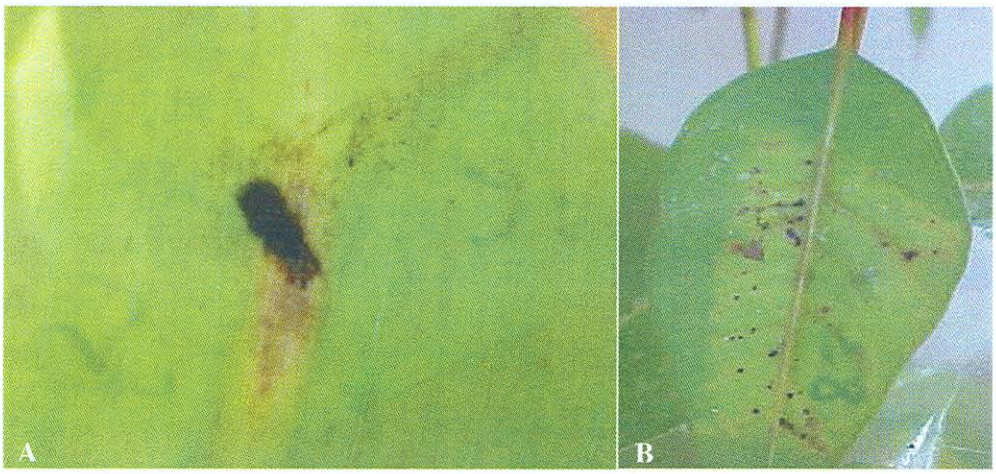

Figure 3: Lesions formed on A. banana and B. *Eucalyptus urophylla* leaves inoculated with *Mycosphaerella colombiensis* and *M. musae* respectively.

CHAPTER 5

FIRST REPORT OF *CLADOSPORIUM MUSAE* ON BANANA IN SOUTH AFRICA

AUSTRALASIAN PLANT PATHOLOGY: SUBMITTED

A.K.J. Surridge [A], F.C. Wehner [A], A. Viljoen [A] and P.W. Crous [B]

[A] Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa.

[B] Centraalbureau voor Schimmelcultures, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands.

FIRST REPORT OF *CLADOSPORIUM MUSAE* ON BANANA IN SOUTH AFRICA

ABSTRACT

An unknown speckle disease was recently observed on Cavendish banana leaves in Levubu, the most northern of the five banana growing regions of South Africa. Morphological examination of infected material and single conidial isolates of the causal organism revealed that it was *Cladosporium musae*. Isolates of the fungus were subjected to pathogenicity testing and sequencing of the ITS region (ITS-1 and ITS-2) and the 5.8S gene of the rDNA operon, and compared with an authentic strain of *C. musae*. These results verified the identity of the fungus as *C. musae*, and constitute the first confirmed report of Cladosporium speckle on banana leaves in South Africa.

INTRODUCTION

Various fungi are known to cause speckle symptoms on banana (*Musa* spp.) leaves, e.g. *Acrodontium simplex* Mangenor & de Hoog (leaf speckle), *Cladosporium musae* E.W. Mason (Cladosporium speckle), *Mycosphaerella musae* (Spreg.) Syd. & P. Syd (Mycosphaerella speckle), *Veronaea musae* Stahel & M.B. Ellis and *Periconiella musae* Stahel & M.B. Ellis (tropical speckle) (Jones 2000). In South Africa, speckle caused by *M. musae* was reported by Brodrick (1973). However, identification was based on symptomology only and no attempt was made to verify the identity of the causal organism. To compound matters further, Brodrick (1973) was misquoted as ascribing the disease to infection by *C. musae* in a subsequent publication (Gorter 1977), which in turn served as reference for the presence of *C. musae* in South Africa (CMI 1988).

In 2000, symptoms resembling those of Cladosporium speckle were observed on Cavendish banana plants in the Levubu area, the most northern of the five banana growing regions in South Africa (surrounding 23.1° S 30.3° E). Symptoms initially appeared as pale-green flecks on the leaf surface that elongated into brown streaks of about 2 cm and longer. With age these lesions characteristically turned orange in colour, with sparse grey-green blotching becoming evident on the adaxial surface of older leaves. Eventually the orange lesions became dark brown, coalesced and occupied large areas of the photosynthetic leaf surface. Invariably associated with the leaf blade symptoms were dark, sunken, water-soaked lesions, 10–20 mm in diameter, along the midrib of the leaves. Severe infection of older leaves occasionally resulted in death of the entire leaf. This report describes the isolation and identification of the causal organism, and confirmation of its pathogenicity.

MATERIALS AND METHODS

Isolation and conventional identification

Thirty-three samples of Cavendish banana leaves displaying speckle symptoms were randomly collected from eight plantations in Levubu. Samples were placed in envelopes and stored at 5 °C until primary isolations were made. A section containing a lesion was excised from each leaf and immersed in 2 % sodium hypochlorite for 30 sec followed by 1 min in 70 % ethanol, and then rinsed twice in sterile distilled water (SDW). Segments (2 mm x 2 mm) were dissected from the lesion margins, and plated on half-strength potato-dextrose agar (½ PDA) (19 g PDA (Merck) + 10 g agar (Biolab, Midrand, Johannesburg) in 1 l deionized water) supplemented with 0.2 g/l Novobiocin to suppress bacterial growth. Plates were incubated for 3–7 d at 25 °C and hyphal tip isolations were plated on ½ PDA. Isolations were also made by inducing sporulation in moist chambers. A leaf section containing a lesion was excised and sprayed with 70 % ethanol until run-off. It was then placed into a 90–mm Petri dish containing a sterile filter paper disc moistened with SDW. After 1–2 d at 20 °C the leaf section was examined for conidiophores under a dissection microscope. Cultures were obtained by touching a small piece of agar to the conidiogenous apparatus and transferring it to ½ PDA supplemented with Novobiocin. Resultant cultures were identified morphologically. Cultures of representative isolates are maintained at the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, The Netherlands.

The morphology of fungal fruiting structures present on banana leaves was studied using both light and electron microscopy. Conidia and conidiophores were collected from sporulating lesions, suspended in lactophenol, and observed under the light microscope. For scanning electron microscopy, fresh leaf lesions were excised and fixed in 3 % glutaraldehyde

for a minimum of 1 hr, followed by three rinses of 15 min each in 0.075 M phosphate buffer. The samples were then dehydrated for 15 min in 50 %, 70 %, 90 % and 3 x 100 % ethanol, respectively. A critical point drying step followed in liquid carbon dioxide, before mounting the sample on a stub and sputtering it with gold.

DNA isolation

DNA was extracted from three South African isolates (CBS 110961, CBS 110962 and CBS 110965), as well as a verified strain of *C. musae* (CBS 161.74), as described by Surridge *et al.* (2003).

Polymerase chain reaction

DNA from each isolate was subjected to an ITS-PCR using primers ITS1 and ITS4 (White *et al.* 1990). The PCR product resulting from this was purified with a "High pure PCR product purification kit" (Roche). DNA sequences were determined using the ABI PRISM ™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase (Applied Biosystems, UK).

Sequence analysis

Sequences of the ITS region (ITS-1 and ITS-2) and the 5.8S gene of the rDNA operon were manually aligned with inserted gaps treated as missing data. Ambiguously aligned regions were excluded from the data set before analysis. Phylogenetic analysis was based on parsimony using PAUP 4.0b8 (Phylogenetic Analysis Using Parsimony) (Swofford 2000). Heuristic searches were done with random addition of sequences (1000 replicates), tree bisection-reconnection (TBR), branch swapping and MULPAR effective and MaxTrees set to auto-increase. Phylogenetic signal in the data sets was assessed by evaluating tree length

distributions over 100 randomly generated trees. The consistency (CI) and retention indices were determined for all data sets. Other *Cladosporium* species (*C. cladosporioides* (Fresen.) G.A. de Vries, *C. herbarum* (Pers.) Link and *C. sphaerospermum* Penz.) were included for comparison and resolution purposes (Table 1). Phylogenetic trees were rooted with *Mycocentrospora acerina* (R. Hartig) Deighton as an outgroup to the remaining taxa. Bootstrap analyses were conducted to determine confidence in branching points (1000 replicates) for the most parsimonious trees generated.

Pathogenicity

Pathogenicity of eight isolates (CBS 110958, CBS 110960, CBS 110961, CBS 110962, CBS 110963, CBS 110964, CBS 110965, CBS 110966) was confirmed by inoculating leaves of potted Cavendish banana plants approximately one year old and 1 m tall. Plants were transferred to a greenhouse and maintained at 27 °C for 30 d prior to inoculation to allow them to acclimatise. The adaxial surface of the leaves was lightly abraised with a hypodermic needle to remove a portion of the waxy cuticle. An agar plug punched from the periphery of a two-week-old culture on ½ PDA was placed onto the abraded epidermis and secured with clear 50-mm-wide adhesive tape. Each isolate was inoculated onto two leaves on each of three plants. Symptom development was observed for 90 d.

RESULTS

Morphology

The fungus associated with leaf speckle of banana in Levubu conformed to the description of *C. musae* (David 1988). It produced erect colourless to brown conidiophores, 4–6 µm in diameter and up to 500 µm long that were readily visible under a hand lens (x10). The basal cell of the conidiophore had a conspicuously thickened wall (Fig. 1A). Conidiophores occurred either singly or in groups of four to six. Terminal or intercalary conidiogenous cells were produced on branches (3–4 x 50 µm) at the apex of the conidiophore (Figs. 1A, B). Conidia were borne singly or in chains of up to three. They were 3–5 µm wide and 6–22 µm long, smooth, thin-walled, 0–1-septate, subhyaline, and ellipsoidal or fusiform in shape, with a protuberant scar often visible at each end (Fig. 1C).

On ½PDA, colonies were white at first and then turned olivaceous and sometimes rosy buff in colour. The superficial mycelium comprised thin-walled and hyaline hyphae. When viewed with the electron microscope, superficial constrictions around hyphal septa could be observed. Fructifications in culture corresponded with those observed on infected plant material.

Molecular identification

Parsimony analysis of the ITS-1 and ITS-2 regions and the 5.8S gene of the rDNA operon determined the phylogenetic placement of South African *Cladosporium* isolates from banana leaves in relation to other *Cladosporium* species isolated from different hosts. Alignment by inserting gaps resulted in a total of 551 characters used in the comparison of the different species. Inserted gaps were treated as missing data. A total of 258 constant

characters, 62 parsimony-uninformative characters and 231 parsimony-informative characters were obtained. Heuristic searches on the data generated eight most parsimonious trees. The consensus tree presented in Fig. 2 indicated that the South African *C. musae* isolates were the same as the reference strain obtained from CBS. The clade containing South African isolates of *C. musae* and the reference strain showed differences of one to three base pairs. The high CI and RI values of 0.978 and 0.989, respectively, support the validity of this tree.

Pathogenicity

All isolates inoculated onto banana leaves produced symptoms similar to those observed in the field (Figs 3A, B). Typical orange speckling was observed around the point of inoculation (Fig. 3A). Necrosis occurred at the site of inoculation from where symptoms radiated outwards. Lesion size varied between approximately 20 mm in diameter and, occasionally, entire leaf death.

DISCUSSION

This study is the first to confirm the presence of Cladosporium speckle caused by *C. musae* on banana in South Africa. Siboe (1994) recently transferred *C. musae* to the genus *Periconiella* as *P. sapientumicola* G. Siboe on the basis of its short conidial chains and complex conidiophore branching pattern. Although the present phylogenetic analysis supports the removal of the speckle pathogen from *Cladosporium* s.str., its placement in *Periconiella* remains unclear. The established name, *C. musae*, is therefore retained in this report. Morphological and sequence data of the ITS region of the rDNA operon, indicate that South African isolates are similar to the verified isolate of *C. musae* isolated from Honduras

(CBS 161.74). Symptoms in the field also corresponded with those described by Jones (2000), particularly for *C. musae* on AAA cultivars in East Africa.

Cladosporium musae is regarded as a minor pathogen causing loss of photosynthetic area mainly on mature banana leaves in humid climates (Stover 1972), and is not considered to affect yield and fruit quality significantly. Banana cultivars in the Cavendish group do, however, seem to be relatively susceptible (Frossard 1963). Older leaves develop symptoms first, transferring inoculum to younger ones via aerially dispersed conidia, which germinate under high humidity conditions (Jones 2000; Jones 1994).

Cladosporium musae has been reported from Australasia-Oceania, Asia, the Latin-American Caribbean region, and in Africa as far South as Zimbabwe (Jones 2000). In South Africa, it appears to be confined to the Levubu area as it has not been isolated from any other of the banana growing regions (Chapter 3). The occurrence of *C. musae* in Levubu, which is situated just North of the Tropic of Capricorn, is in accordance with the pathogen's preference for tropical climates (Wardlaw 1961, CMI 1988). While conditions were conducive to disease development and spread during the past growing seasons, Cladosporium speckle remained confined to the Levubu area. The most probable explanation for the presence of *C. musae* in South Africa is that diseased vegetative material may have been introduced from neighbouring countries. As *C. musae* is more adapted to a tropical climate, this pathogen may prove to be climatically contained within this region.

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Table 1: Collection and sequence details of the fungi included in the phylogenetic analysis.

Fungus	Culture number	Location	Date	Collector	Host Cultivar	GenBank accession number
C. musae	CBS 161.74	Honduras	Feb 1974	R.H. Stover	Musa sp.	AY186199
C. musae	CBS 110962	Levubu	17-Mar-00	A. Viljoen	Williams	AY186200
C. musae	CBS 110965	Levubu	24-Jun-00	A. Viljoen	Grand Nain	AY186201
C. musae	CBS 110961	Levubu	16-Mar-00	A. Viljoen	Grand Naine	AY186202
C. cladosporioides	-	-	-	-	-	AF455535
C. cladosporioides	-	-	-	-	-	AF455442
C. cladosporioides	-	-	-	-	-	AF455472
C. cladosporioides	-	-	-	-	-	AF455525
C. cladosporioides	-	-	-	-	-	AF455519
C. herbarum	-	-	-	-	-	AF455517
C. herbarum	-	-	-	-	-	AF455479
C. herbarum	-	-	-	-	-	AF455404
C. sphaerospermum	-	-	-	-	-	AF455481
M. acerina	ATCC 34539	Norway	-	K. Arsvol	Carrot	-

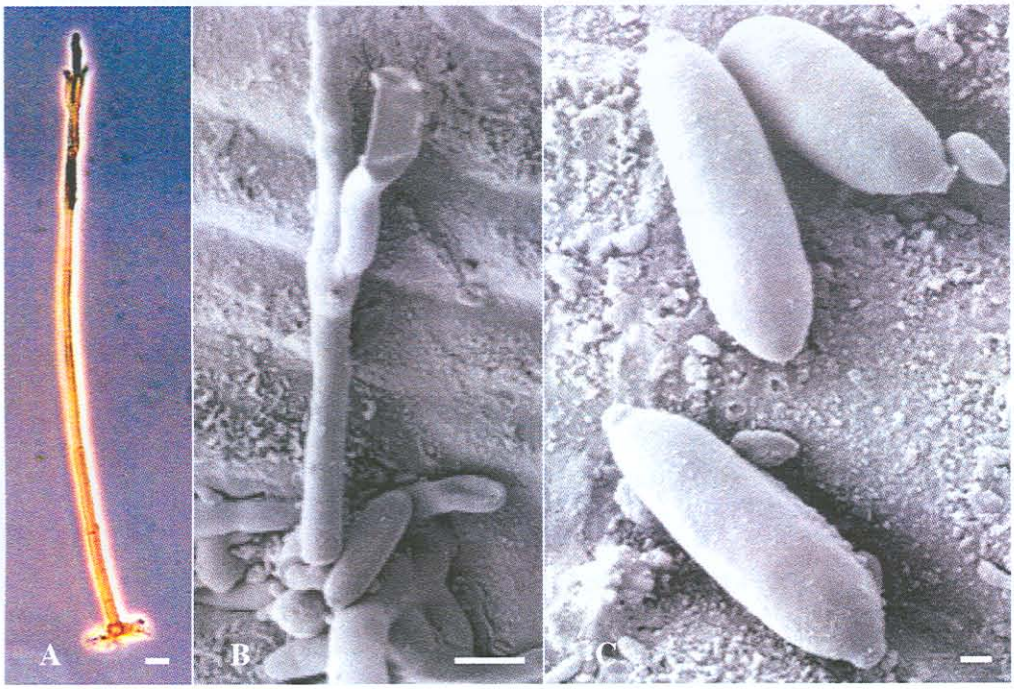

Figure 1: Light and electron micrographs of *Cladosporium musae*. A. Excised conidiophore showing thickened basal cell (Bar = 10 µm). B. Conidiophore on the banana leaf surface (Bar = 10 µm). C. Conidia (Bar = 1 µm).

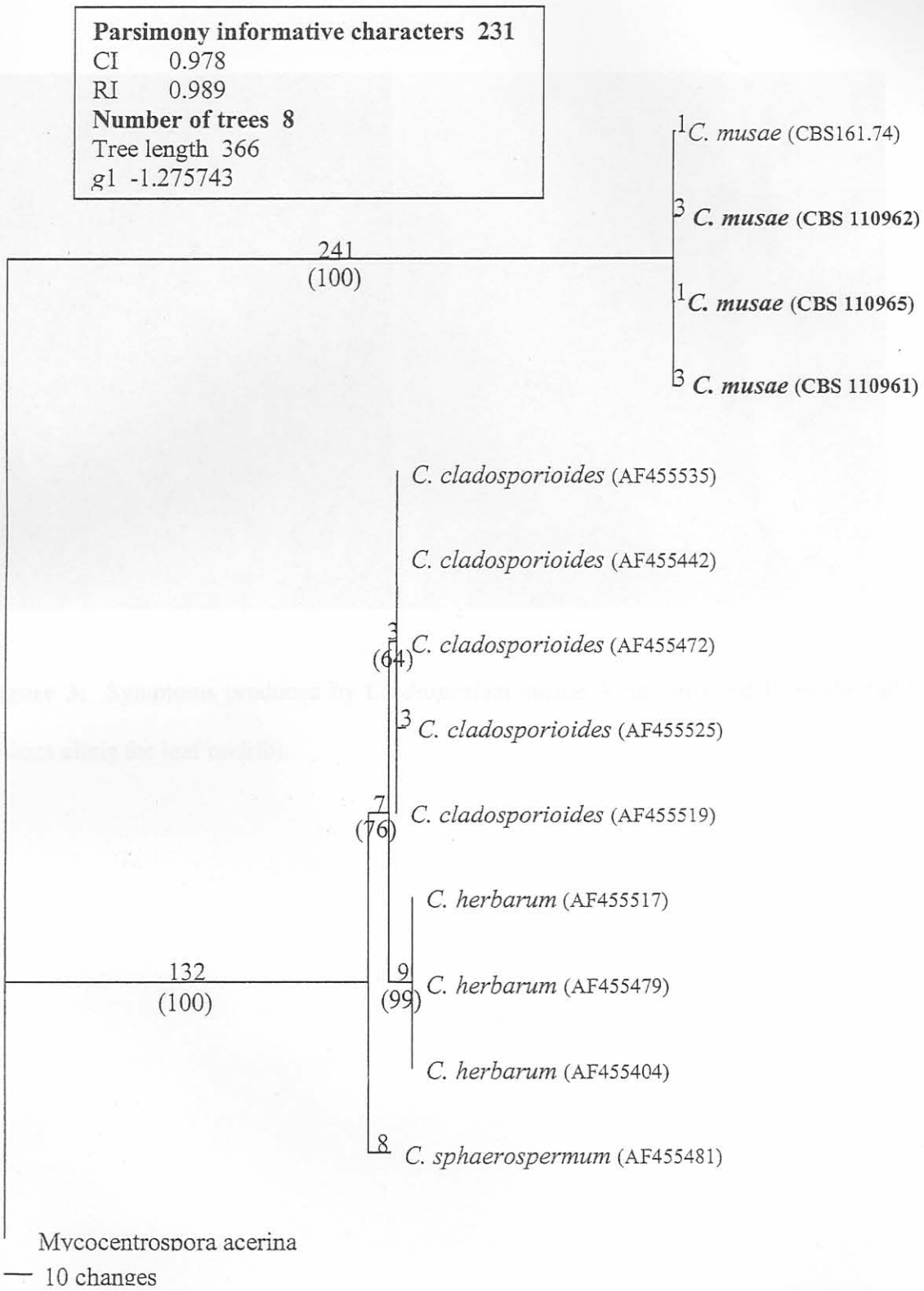

Figure 2: Phylogeny of the internal transcribed spacer sequences of *Cladosporium musae* (CBS 161.74) and South African isolates causing speckle disease on banana leaves.

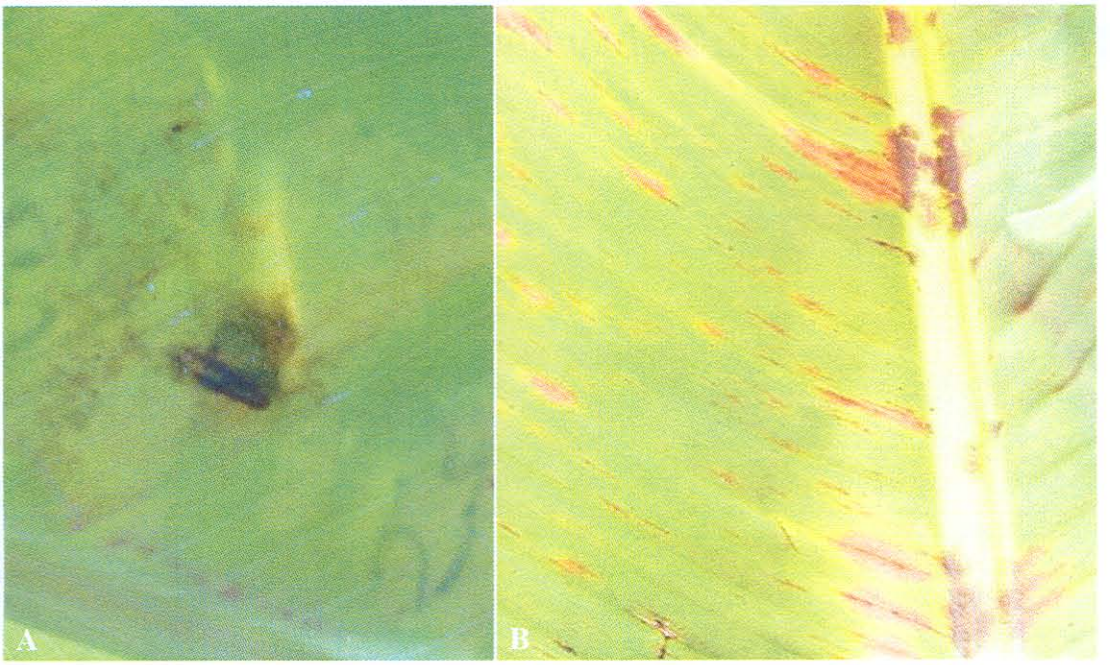

Figure 3: Symptoms produced by *Cladosporium musae* A. in vitro and B. in the field, (note lesions along the leaf midrib).