

**ISOLATION, IDENTIFICATION AND PATHOGENICITY
OF FUNGAL PATHOGEN(S) CAUSING BLACK
SIGATOKA DISEASE OF LOCAL PLANTAIN AND
BANANA IN GHANA**

BY

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MAJOR - PLANT PATHOLOGY

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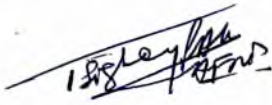
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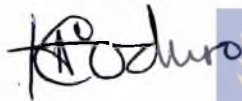


DECLARATION

I hereby declare that, except for references to other people's work which have been duly cited, this work is the result of my own original research and that this Thesis has neither in whole nor part been presented for another degree elsewhere.



FRANCIS KODJO TSIGBEY (STUDENT)



DR. K.A. ODURO (MAJOR SUPERVISOR)



DEDICATIONS

Dedicated to my mother and to my father (Post humous).



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I am grateful to Dr K.A. Oduro, my major supervisor, for his patience, confidence, encouragement and very useful criticisms when things were not going through. In addition for all his provisions throughout this research. To him I owe much.

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To God Belongs all the Glory.

ABSTRACT

A study was conducted at the Crop Science Department, University of Ghana, Legon, to isolate, identify and test pathogenicity of fungal isolates causing the reported black sigatoka disease of plantain in Ghana and compare the isolate with that isolated from local tall banana having leaf spots.

Field symptoms of plantain and banana were compared and found to be similar. Initial specks indicating infection were minute, reddish-brown and were conspicuous only on the abaxial leaf surfaces on both plants. Specks turned dark-brown and later dark-black with adjoining yellow margins. The dark black areas dried out and turned grey on both plants.

The fungi species were isolated from necrosed banana and plantain leaves by Ascospore Discharge Technique. Ascospores from necrosed banana and plantain leaves were hyaline, two-celled, with one cell larger than the other and measured 13.9 by 3-2 μ m and 13.8 μ m by 3.2 μ m respectively. Ascospores of both isolates germinated in Sterile Distilled Water (SDW) after 1 $\frac{1}{2}$ hours and on PDA and Carrot Leaf Decoction Agar (CDA) between 12-18 hours and in all cases by bipolar germ tubes. No sporulation was observed for both isolates in SDW. Colony growth of both isolates on PDA and CDA were extremely slow and mycelia compact, erumpent, appearing dome-shaped from side view, and with a hard rind on the surface.

Sporulation was not observed on PDA for both isolates, but profuse sporulation was observed on CDA six days after inoculation. Conidia in culture was *Cercospora*-type, with thickened hilum. Conidia of the plantain and banana isolates on the average measured 78.1µm by 3.6µm and 78.4µm by 3.6µm, respectively.

Greenhouse inoculations revealed that the plantain isolate on plantain had an incubation period of 18 days, whilst the banana isolate pathogenic on the local tall banana also had an incubation period of 23 days. Symptoms in both cases were typical for black sigatoka and progressed to the mature-spot stage with grey centres.

Cross-infection studies in the greenhouse revealed that plantain isolate of *M. fijiensis* was pathogenic on local tall banana with incubation periods of 29 days, and which produced typical black Sigatoka symptoms until the mature spot stage. Plantain ascospore inoculation on local tall banana also produced black sigatoka symptoms with 21 days of incubation period, after which symptoms progressed to the mature spot stage.

On plantain, the banana isolate of *M. fijiensis* took 26 days for specks to appear and symptoms which were typical for black sigatoka progressed to the mature-spot stage. Control plants in the greenhouse did not develop symptoms. Neither ascospores nor conidia were produced in the greenhouse on inoculated plants due to unfavourable environmental factors thus rendering reisolation of the fungus from diseased leaves impossible.

The study therefore established that black sigatoka disease in Ghana is found on both plantain and the local tall banana, with the causal agent being *Mycosphaerella fijiensis* Morelet.

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LIST OF ABBREVIATION

LTB	LOCAL TALL BANANA
ADT	ASCOSPORE DISCHARGE TECHNIQUE
R.H.	RELATIVE HUMIDITY
BS	BLACK SIGATOKA
YS	YELLOW SIGATOKA
SDW	STERILE DISTILLED WATER
PDA	POTATO DEXTROSE AGAR
CDA	CARROT LEAF DECOCTION AGAR

CHAPTER ONE

INTRODUCTION

Banana (*Musa sapientum* L.) and Plantain (*Musa paradisiaca* L.) are staple foods for millions of people throughout the developing world, with banana especially eaten in the developed world. For others also, they comprise a significant portion of their diet.

In Ghana, plantain is a major source of carbohydrate. Cooked unripe plantain is pounded into "fufu", a local dish, and eaten with soup, whilst both the ripe and unripe forms are cooked and eaten with stew. Ripe plantain is also fried and eaten with cooked bambara beans and cowpea with palm oil as a major delicacy. Other use of plantain in Ghana is shredding unripe plantain and frying into chips for consumption. On the other hand, a greater proportion of banana produced in the country is eaten ripe with roasted groundnut as a meal.

It has been estimated by the Ministry of Agriculture that, national plantain production between 1986 and 1990 was about 5,206,615 metric tonnes (mt) (Ministry of Agriculture, PPMED 1990,) There is however no production estimates of banana in the country.

Besides serving as food for domestic consumption, the two crops can be developed with proper agronomic and disease management practices, to serve as foreign exchange earners for the country. In light of this,

attention has been drawn to the crops for their improvement to increase yields. Areas of improvement

include selection and breeding for high yielding varieties, quick and efficient methods of propagation, selection for dwarf varieties that can resist lodging during strong winds and the efficient control of economically important diseases.

Banana and plantain, like any other biological entities, have several diseases and pests that attack them. Important among these are Bunchy Top, Mosaic, Moko (Bacterial wilt) and Panama diseases (Vascular wilt). Banana and plantain leaf spot disease (Sigatoka) caused by *Mycosphaerella* spp is one of the most important diseases of banana and plantain and causes serious losses in yields in many commercial plantations (Wardlaw, 1961).

The commonest form of the disease (yellow Sigatoka) is caused by *Mycosphaerella musicola* Leach with the conidial state *Pseudocercospora musae* Zimm (Deighton), while the other form and the more devastating, black Sigatoka, is caused by *Mycosphaerella fijiensis*, Morelet with the conidial state *Paracercospora fijiensis*, Morelet (Deighton).

In Ghana, yellow Sigatoka disease on improved commercial banana was reported in 1958 (Wardlaw, 1972) and had since been of epidemic proportions. This resulted in Sigatoka epiphytotics on the Subin banana plantation that led to its total collapse. Until the 1970s, plantain in the West-African sub-region was regarded to be virtually free from economically important diseases including Sigatoka. Later, black sigatoka disease was

reported to have reached Ghana in the early nineteen eighties (Wilson and Buddenhagen, 1986).

An outbreak of a plantain disease in Ghana necessitated a scientific enquiry. Subsequently, Oduro et al. (1991) in a survey reported a 62.5% national incidence and observed that the new plantain disease in Ghana was fairly similar to some aspects of the detailed symptoms of black sigatoka as reported by other workers like Meredith and Lawrence (1970).

On basis of the symptoms alone, Oduro et al. (1991) considered the plantain disease in Ghana to be black sigatoka and therefore recommended further studies to identify the causative organism.

Oduro et al. (1991) also noted and supported by pictures that the leaf spot disease on the local tall banana in Ghana was symptomatologically similar to that of the new plantain disease.

Cultivation of the Local Tall Banana (LTB) in Ghana is not done on any commercial basis, but normally planted to serve as shade crop for newly established cocoa plantations, and the produce eaten ripe as dessert or the unripe fruit eaten cooked. The local tall banana is very hardy and even long after the abandonment of the cocoa farms, they still exist as isolated grooves along roads and in secondary forests.

These grooves of local tall banana severely diseased by sigatoka, could serve as inoculum reservoir for the sigatoka pathogen. In addition, dried diseased leaves could be efficient means of transporting infective

propagules to disease-free areas since such materials are used for packaging and as head pad. With this information about the local tall banana, any attempt in controlling Black Sigatoka of plantain without any scientific study on the relationship between black sigatoka of plantain and the leaf disease of local tall banana could be negated.

The purpose of this study therefore was firstly to isolate, identify and test the pathogenicity of fungal pathogen(s) on plantain in Ghana and compare it or them with the isolate from the local tall banana in the country.

Secondarily to establish the true relationship between the new plantain disease and the neglected disease of local tall banana.

CHAPTER TWO

REVIEW OF THE LITERATURE

2.1. OVERVIEW OF PLANTAIN AND BANANA DISEASES

Banana and plantain (*Musa* spp), like any other plants, have several diseases that attack them. Almost all the pathogens that infect one species also attack the other. The pattern of growth, especially leaf arrangement, which creates dense canopies when the crop is planted over large tracts of land in association with the high rainfall required for growth provide a conducive environment for pathogenic fungus infection.

Banana and plantain diseases can be grouped into five major categories thus: leaf, pseudostem, root, rhizome and fruit diseases. All these diseases are of economic importance depending on the causative organism, environmental conditions and adopted control measures.

Some economically important diseases, include viral diseases such as Bunchy Top disease, Roxana disease, infectious chlorosis, and mosaic disease. Bacterial diseases include Moko (Bacterial wilt), Bacterial Head rot, Nematode diseases include the Burrowing nematode infection while Fungal diseases include Panama Disease (Vascular wilt), *Cordana* Leaf spot, Leaf Speckle, Yellow and Black Sigatoka Disease (Wardlaw, 1972).

2.2. MYCOSPHAERELLA DISEASES OF PLANTAIN AND BANANA

Mycosphaerella diseases of plantain and banana are two of the most economically important leaf spot diseases. They are popularly called Yellow Sigatoka and Black leaf streak or black sigatoka depending on which particular *Mycosphaerella* sp is causing the disease. The commonest form in Ghana, Yellow sigatoka, is caused by *Mycosphaerella musicola* Leach. with the conidial state *Pseudocercospora musae* Zimm. (Deighton) (*Cercospora musae* Zimm).

The current predominant form of the disease in Ghana, Black Sigatoka/Black Leaf streak, is caused by *Mycosphaerella fijiensis* (Morelet), with the conidial state *Paracercospora fijiensis* Morelet (Deighton). So far, the two species only attack members of the *Musa* genus.

2.3. ECONOMIC IMPORTANCE OF YELLOW AND BLACK SIGATOKA DISEASE

Combined attack of Yellow and Black Sigatoka diseases have caused great economic losses wherever they had appeared. They have been reported that within a few years, a flourishing banana industry may be destroyed and this led Wardlaw (1939) to describe it as a disease of first class importance. Apart from monetary expenditures, by virtue of the nature of the Sigatoka disease, if judicious control measures are not employed, environmental pollution with sprayed chemicals can result.

The onset of Sigatoka disease has been a tragedy to both individual planters and whole agricultural regions. Motz and Mallory (1944), gave an account of how sigatoka disease led to the collapse of the banana industry in Mexico. They indicated that, the disease was observed first in 1937; by 1941 production had fallen from 525,000 metric tonnes to 240,000 metric tonnes. Similarly, sigatoka outbreaks led to the collapse of the banana plantation at Subri in Ghana (Oduro personal communication). Further, Guyot and Cuille (1958) remarked that, of the 62 million bunches produced in 1954 in Ecuador, only 19 million were exported. This was due to failure to provide protective measures against Sigatoka on the small farms and holdings.

Wardlaw (1972) reported that after the earlier incubation period, the disease could destroy a farm in two years in the absence of adequate control. In Ghana, the outbreak of the black sigatoka disease on plantain had catastrophic effects on plantain production. The major cause is due to the fact that plantain production is done on small scale by peasant farmers, hence control measures are not adequately administered. Consequently output fell with its attendant high cost of the produce on the Ghanaian market. Mbwaga (1990) reported that black sigatoka was first reported in 1989 in Tanzania and between 1989-90, caused almost 100% infection.

Black sigatoka disease inflict both physical and physiological damages on banana and plantain. Physically, the disease is responsible for the

destruction of the photosynthetic tissue of the plant, culminating in the loss of the leaf due to leaf necrosis. Stover (1974) reported that heavy spotting give rise to early leaf senescence and that it is one of the first effects of sigatoka on the plant and can shorten leaf life by as much as 25 days. Heavy spotting resulting in 4-5 viable leaves at bunch initiation give rise to lighter bunches, reduced fruit weight and increase field ripening (Stover 1974, Ramsey et al. 1990). The average age of the youngest leaf spotted is the most sensitive indicator of danger from premature ripening (Stover, 1974). Merchan V (1989) observed that under severe attack of black Sigatoka, bunches mature prematurely with low weights and fingers do not fill out.

2.4. DIFFERENCES AND SIMILARITIES BETWEEN BLACK SIGATOKA (BS) AND YELLOW SIGATOKA (YS) DISEASES

Yellow sigatoka (YS) is caused by *Mycosphaerella musicola* Zimm and is characterised by a general necrosis of the leaves of both plantain and banana. The evident feature of the disease is the presence of a profusion of small discrete spots on the lamina of older leaves, with areas of scorched or brown leaf tissue where the spots are closely grouped together. The first indication of infection of YS is the appearance of light yellow or brownish-green, indistinct linear marking 1-2mm or more in length lying parallel to the veins. These streaks are normally scattered on the upper surface of the leaf. These streaks increase slightly in size, forming dark

muddy-brown to black, linear to oblong, or elliptic areas up to 1cm in length and about one-third in breadth. These spots are usually seen on the fourth to sixth leaves. On younger plants, similar but longer and broader spots are formed which appear dark-brown, oval to almost about 1cm in diameter. The centre of the elliptical brown spots dries out to light greyish-buff colour, with a narrow, well-defined dark-brown margin. Several workers including Stahel (1937) reported that between the dark-brown margin and the normal green areas of the leaf, there is frequently a bright-yellow transitional zone.

Stahel (1937) gave a precise description on the nature and sequence of symptom development. He observed that the first symptoms, 15-17 days after inoculation with conidia are minute yellow-green speckles or streaks. Some 22-24 days after inoculation, these speckles become streaks 8-10mm long with the leaf having a slightly rusty appearance. From here the spots show some enlargement and development of brown necrotic colour. After the brown spot stage, the spots collapse and yield the characteristic pale grey buff sigatoka spot.

Stover and Fulton (1966) reported that the pattern of spots over the surface of the lamina is influenced by the type of spore causing the infection. Conidial infections show linear distributions whereas ascospore infections do not, but the spots are generally distributed over the leaf. Furthermore, typical ascospore symptoms consist of heavy concentration of infection towards the drooping leaf-tip.

The asexual fruiting body of *M. musicola* was described by Leach (1946) as sporodochium which was defined by Ainsworth (1961) as a mass of conidiophores tightly placed together upon a stroma or mass of hyphae. Other terms used include acervulus (Stahel 1937), others are fascicles (for the mass of conidiophores) and Stromata (for the underlying mass of hyphae).

Meredith and Lawrence (1970) observed that sporodochium of *M. musicola* was amphigenous on leaf materials which they examined, with the proportion being higher on the lower than on the upper surface.

Meredith and Lawrence (1970) described the conidia of *M. musicola* as being pale to very pale olivaceous, smooth, straight or variously curved, occasionally undulate and almost perfectly cylindrical to obclavato-cylindrical. The tip of conidia was obtuse or subobtuse and the basal cell shortly attenuate to the unthickened truncate hilum. They further observed that conidia were 1-9 septate, with septa being indistinct and only fairly visible in unstained mounts. There were no constrictions at the points of septation. Conidia in culture were longer (up to 102 μm , average 72 μm) and narrower (2-3.5 μm average 3.0 μm) than those found on leaves (Meredith and Lawrence 1970).

Despite the widespread presence of YS disease, Trujillo and Goto (1963) published an account of the occurrence of a new banana leaf spot disease in the Hawaiian Islands. The symptoms were similar to those of YS except that the individual lesions first appeared as

elongated light brown streaks and not as yellow streaks. Rhodes (1964) first described black sigatoka and considered it similar to Yellow Sigatoka, but stated that it was more severe, as symptoms appear on younger leaves and, subsequently, more damage is done to photosynthetic tissue. Leach (1964 a,b) stated that, the main difference in symptoms between Yellow Sigatoka and black sigatoka occurs in the earlier stages of spot development.

For yellow sigatoka, the streaks that characterise the initial stages of the disease are yellow and clearly visible on the upper leaf surface but not conspicuous on the underside, while the surface of leaves infected with black sigatoka or black leaf streak are clearly distinguishable on the lower leaf surface (Leach 1964). The streaks are light brown to reddish-brown, often with a purplish hue when large numbers of streaks occur close together. The streaks remain inconspicuous on the upper surface until the time when they become water soaked and turn black rapidly (Meredith and Lawrence 1969). The most striking feature of black leaf streak or black sigatoka disease is the ability to form an enormous mass of narrow streaks on banana leaves as soon as infection becomes severe.

For so many years the two pathogens cannot be distinguished in culture if conidia are not produced. However, recently Johanson (1992) developed a technique capable of differentiating the two pathogens in culture by a Polymerase Chain Reaction technique.

Meredith and Lawrence (1969), identified six stages of black sigatoka on mature banana plants as follows:

1. Initial speck stage
- (2) First streak stage
3. Second streak stage
- (4) First spot stage
5. Second spot stage
- (6) Third or mature spot stage

Similar trends were observed for yellow sigatoka on banana and plantain.

According to Meredith and Lawrence (1969) both yellow sigatoka and black sigatoka in certain territories are sometimes present together in the same plantation. However, in the Pacific and the lowland areas of Latin America and Africa, yellow sigatoka could no longer be found and may have been completely replaced by Black Sigatoka (Jones and Mourichon, 1993).

Although there are no marked morphological differences between the perithecia and ascospores of *M. musicola* and *M. fijiensis* (Natural, 1988) there are notable differences between the Cercospora-type conidial state. Ascospores of *M. fijiensis* and *M. musicola* can be distinguished on V-8 juice agar. Germ tubes of ascospores of *M. fijiensis* are characteristically curved whereas those of *M. musicola* are straight (Meredith and Lawrence 1969). The number of perithecia per unit area on black sigatoka lesions was considerably greater than those on Yellow Sigatoka (Leach 1964). The conidiophores of *M. fijiensis* emerge singly or in diverging fascicles of 28 stalks from many stomata on the lower surface of the leaf. Few conidiophores emerge from stomata on the upper surface of the lesion. Conidiophores are pale to medium

olivaceous brown, becoming slightly paler towards the tip. They are straight to variously bent, often with several geniculations, very rarely branched, basal swelling up to 8µm diameter. One or more medium sized scars are present near the tip of the conidiophore, either lying flat against the apex or side of the conidiophore, or on a slightly sloping shoulder (Meredith and Lawrence, 1969). Apart from the colour of the conidiophores, none of the above characteristics are applicable to those of *M. musicola*. Conidiophores of *M. musicola* are straight to mildly curved, without septa, only very rarely branched, smooth, not shouldered or geniculate and without conidial scars. Conidiophores are mostly bottle-shaped with rounded or nearly truncate apices (Meredith and Lawrence 1969).

The major comparisons of the conidial states of *M. musicola* and *M. fijiensis* after Meredith and Lawrence (1969) are given below:

	<i>M. musicola</i>	<i>M. fijiensis</i>
<u>Conidiophores</u>		
First appearance	First spot stage	Initial specks or first-streak stage
Habit	In dense fascicles (sporodochia) on a dark brown or black stroma	Emerge singly or in small groups (2-8 stalks), sporodochia and stromata absent.
Distribution	Abundant on both surfaces of lesion sometimes more frequent on the upper surface.	Largely confined to the lower leaf surface of lesion.
Morphology	Almost straight, hyaline, mainly without septation geniculation or branching, spore scars absent.	Straight or sly bent, pale to medium brown, 0-5 septate, often geniculate, rarely branched near base well-defined, slightly thickened spore-scars.
Dimensions (μM)	5-25 x 2-3.5	16.5-62.5 x 4-7
<u>CONIDIA</u>		
Morphology	Cylindric to obclavato-cylindric, straight or curved, pale to very pale olivaceous, 0-6 septate, indistinct basal hilum	Obclavate to cylindro-obclavate, straight or curved, hyaline to very pale olivaceous 1-10 septate, distinct slightly thickened basal hilum.
Dimensions (μM)	10-81 x 2-6 (mean 59 x 3)	30-132 x 2.5-5 (mean 72.5 x 4)

2.5 SOME CHARACTERISTICS OF THE *CERCOSPORA* SPP

Cercospora Fres. is reported to be one of the largest and most heterogeneous genera of Hyphomycetes (Subdivision Deuteromycotina), and nearly 3,000 specific names of *Cercospora* have been mentioned in the world literature (Pons 1989). Sporulation of several *Cercospora* spp in culture is difficult and depends mainly on the type of media used. Nagel (1934) reported that considerable difficulty is encountered in obtaining conidial production in pure culture of *Cercospora beticola* Sacc., and intimated that several workers presented conflicting evidence regarding conidial production of other *Cercospora* spp. Nagel (1939) reported that *Cercospora brunckii* Ell. and Galiv., grew well on several media but that it did not readily produce conidia. Aderhold (1900), dealing with *C. beticola*, observed that conidia was produced somewhat sparingly over the entire mycelial mat but in greater numbers at the borders of the colonies when the organism was grown on sugar-beet-leaf medium. Typical conidia of *C. cerasella* were produced in pure cultures from conidia and ascospores of the fungus, however when conidia were plated on potato-dextrose agar, corn-meal agar, bean-dextrose agar, synthetic nutrient agar or cherry-leaf-decoction agar to which were added fragments of diseased leaves as additional source of nutrients, no fruiting was observed although the fungus made a good vegetative growth (Jenkins, 1930). However, if tubes of these media on which the organism had been growing for a time were

again sterilised and plated with conidia, conidia was produced in culture.

Nagel (1934) stated that certain of the *Cercospora* spp he studied demonstrated some specificity of medium as determined by the amount of sporulation. There was greater vegetative growth and less sporulation in the case of *Cercospora beticola* on potato-dextrose agar, while the same organism on beet-leaf agar produced little vegetative growth and greater numbers of conidia, whereas *C. cruenta*, *C. davisii*, *C. dubia*, *C. physalidis* and *C. setariae* fruited abundantly on ordinary potato-dextrose agar (Nagel 1934). Kilpatrick and Johnson (1956) reported that previous attempts by some other workers to obtain sporulation of *Cercospora kikuchii* (T. Matsu and Tomoyasu) Gardner in artificial culture have been unsuccessful and Murakishi (1951) tried 15 different types of media but failed to obtain sporulation. Nagel (1934), as well as Nagel and Dietz (1932), *C. beticola* reported that mycelial transfer of *C. beticola* resulted in sterile hyphae, whereas spore transfer yielded colonies that produced abundant sporulation. Sporulation of *C. nicotianae* Ell and EV. was obtained on steamed tobacco leaf decoction mixed with 1.2 percent agar (Diachum and Valteau, 1941).

Kilpatrick and Johnson (1956) obtained abundant sporulation of twelve *Cercospora* spp__ including *C. kikuchii* grown on agar medium prepared from carrot leaves steamed without pressure for one hour. They observed that sporulation did not occur on the medium prepared from

carrot leaves sterilized under pressure, and cultures exposed to daylight also sporulated more abundantly than those in dishes incubated in darkness. Darkness however did not suppress sporulation completely. Calpouzoz (1954) reported that Meredith and Butler (1939), were the first to observe sporulation of *C. musae* in pure culture. These observations were repeated by Leach (1941), Dantas (1948), and Fernandez (1953). However in all these cases the production of conidia was either not abundant or not consistent enough to offer a reliable supply of spores.

The critical factors controlling sporulation in culture by *C. musae* are genetic and not environmental (Calpouzoz 1954). Calpouzoz (1954) further reported that the slow growing colonies of *C. musae* show a heterogenous morphology, as they consist of patchwork of sectors differing in colour and texture, with some sectors sporulating.

2.6. SOME FEATURES OF *M. FIJIENSIS* CULTURES

Natural (1988) reported that the size of *M.fijiensis* conidia isolated varied with the cultivar from which they were obtained. Colonies derived from single spores became visible 4-6 days after incubation on PDA under continuous light, with the colonies of *M. fijiensis* compact, raised, hemispherical with irregular zonation, non-circular margin and a velvety surface (Natural, 1988). Natural (1988) also reported that ascospores were hyaline, 2-celled with one cell larger than the other, with a slight constriction at the septum and that

ascospore germination which was bipolar occurred 24 hours after discharge on PDA.

Meredith and Lawrence (1969) attributed the dark centres of *M. fijiensis* colonies to the presence of dumb-bell-shaped hyphal cells. While Stover (1976) indicated that the presence of these cells is considered as an indication of pathogenic *Cercospora*. Several workers have reported conidial production of *M. fijiensis* on PDA (Meredith and Lawrence 1969, Natural 1988). Natural (1988) reported that sporulation of *M. fijiensis* occurred on PDA under continuous light after 14-21 days of incubation at 22-25°C, though some isolates failed to produce conidia.

Pigment production was reported on several media such as PDA, Coconut Glucose Yeast Extract agar (CGYEa), Czapek's Yeast Extract Agar (CzYEA) and Synthetic medium Glucose Yeast Extract agar (SymGYEA), but this disappeared with age (Natural 1988). Subsequently, Natural (1988) reported that pigment production was highly correlated with toxin production in vitro. Though Asante et al (1977) and Fajola (1978) reported non-pigment producing isolates of *Cercospora* spp., Upadhyay et al. (1991) isolated Fijiensin, a phytotoxic metabolite from a culture of *M. fijiensis* and found it to be phytotoxic to various banana cultivars. However it was observed that fijiensin production could only be detected in liquid cultures after 21-24 days though symptoms appeared after 10-15 days, on field plants.



2.7. EPIDEMIOLOGY OF BLACK SIGATOKA DISEASE

The infecting propagules for black sigatoka disease are conidia and ascospores. Conidia production is at its maximum when the brown lesions begin to turn into a dark-brown or black spot and decreased when the center of the spot begin to turn light gray (Stover 1970). Sporulation occurred on successive nights in the absence of rain provided dew was present (Stover, 1970), whilst conidia production was abundant as a result of long hours of leaf wetness. Stover (1970) reported no sporulation on leaves during nights when dew was not formed. Conidia are released by rain and dew and then disseminated by water (Stover, 1970), and in the absence of spray during the dry months, spotting can gradually build up as a result of conidial inoculum. The emergence of streaks at these times is associated with longer incubation periods.

Ascospores produced by perithecia are rain-dependent and are disseminated by wind (Stover, 1970) and rapidly move disease outward from primary foci of infection, whereas conidial infection is localised. Frequent rains produce both conidia and ascospores which contribute to a rapidly increasing epidemic disease that is difficult to control (Stover, 1970). Conidia survive 3-4 weeks on leaf surface whereas ascospores take 8-14 weeks in perithecia.

Stover (1968) reported a seasonal trend in the production of perithecia and sporodochia in different countries and climates. Perithecia and sporodochia production is high during wet seasons and declines in the

dry seasons. Preponderance of perithecia or sporodochia varied from country to country and depended on the rainfall pattern (Stover, 1968). Consequently, when the rainfall is high throughout the year, perithecia production predominates and when the rainfall is lower, sporodochium production is high. Stover (1964) indicated that since perithecia produces only one major crop of ascospores, as opposed to sporodochium which produces conidia intermittently, the contribution of conidia to total inoculum is greater than that of ascospores during most time of the year. However perithecia are five to ten times more numerous in spots developed from mass infections than in scattered single spots (Stover, 1964).

Merchan V (1989) reported that in Columbia the highest concentration of ascospores was registered in the rainy week, with 62,700 ascospores/m³ of air. Stover and Fulton (1966) confirmed the findings of Brun (1963) that ascospores were dispersed long distance in horizontally moving air currents and deposited on the furled heart leaf giving rise to a typical left laminal infection. The unfurling heart leaf, is an ideal target for ascospores and conidia and this make adequate protection by fungicide spray very difficult (Stover and Fulton, 1966). Inadequate protection is due to the almost perpendicular heart leaf, coupled with a constantly expanding cylinder exposing new susceptible tissue.

The critical time for banana to suffer repeated attacks of sigatoka was during the three to four weeks prior to shooting (Stover, 1974) and a physiologically

active substance is believed to be produced and translocated from the diseased leaf tissue (Stover 1974). Stover (1970) reported the production of ethylene by sigatoka pathogen in diseased tissue, which might be responsible for the premature fruit ripening associated with sigatoka attacks.

Artificial infection of banana and plantain by *Mycosphaerella* spp was achieved by spraying conidial suspension in water on plant leaves (Stahel, 1937; Goos and Tschirch, 1963) and typical sigatoka disease symptoms were produced.

The presence of other fungus fructifications including *Leptosphaeria musarum* Sacc., *Hendersonia* spp. at the dry centres of cercospora spots on banana was also reported (Simmonds 1933, Parham 1934, Stahel 1937).

2.8 HOST-PARASITE INTERACTION OF BLACK SIGATOKA DISEASE

The infecting propagules of *M. fijiensis* are ascospores and conidia, and the courts of infection on plantain and banana are the adaxial and abaxial leaf surfaces. By microscopic examination, it was observed that the abaxial leaf surface might be more susceptible to infection than the adaxial leaf surface due to the abundance of stomata and the lesser amount of wax on this surface. Conidia and ascospores germinate by germ tubes in a film of water on the leaf surface and penetration into the leaf tissue is by way of the stomata.

Goos and Tschirch (1963) reported that conidia germinated within 24-48 hours on inoculated leaves

enclosed in plastic bags in the greenhouse. They further reported that germ tube development was more extensive after a further 24 hours exposure to mist, whilst no conidia germinated on inoculated leaves left in the open green house. Stomatal penetration was reported to begin 4-6 days after inoculation on leaves covered with plastic bags and it was suggested that fluctuating humidities were more favourable for this process (Goos and Tscirch, 1963). Goos and Tschirch (1963) reported that in the greenhouse spot developed within 28-35 days after inoculation on banana, and speculated that, the rate of disease development appeared to be related to the moisture relations of the plant and was affected by watering regimes.

Salle' et al. (1989) reported that after penetration via stomata, hyphae run parallel to the leaf surface in the large open areas of the spongy mesophyll and branches form which make contact with host cell walls without the development of haustoria. Twenty-eight days after inoculating a susceptible cultivar Grande Naine (AAA genotype), there were intravacuolar accumulations of tiny spherical globules in parenchyma cells which stained heavily black with paragon (Salle' et al. 1989).

Vasquez et al. (1988) reported that ascospores germinated on water-agar with three and four germ tubes and that ascospores germinated on both adaxial and abaxial surfaces of leaves. Under greenhouse conditions, Vasquez et al (1988) reported that the first symptoms appeared 22 days after inoculation. Scanning Electron

microscope (SEM) studies by Vasquez et al. (1988) revealed bacteria in association with ascospores coupled with an apparent maceration of ascospores and speculated a possible biological control mechanism for *M. fijiensis*.

The pattern of symptom development in greenhouse-grown plants differ somewhat from that in the field, in that, infection occurs on all leaves in the greenhouse as greenhouse grown plants develop thinner wax deposits than field grown plants (Freeman and Turner, 1985).

Differences exist in the susceptibility of different *Musa* spp to both *M. fijiensis* and *M. musicola*. Plant characteristics which give them resistance to diseases may be morphological or biochemical, acting directly or indirectly on the parasite (Goodman et al. 1986). The components of resistance may be present in the host before it comes into contact with the pathogenic agent (toxic compounds present before infection). In many cases however resistance mechanisms are induced in the host cell at contact with the pathogen (elicitation of phytoalexins) (Goodman et al. 1986).

Mourichon et al. (1989) reported that no correlation existed between duration of incubation and cultivar susceptibility to black sigatoka, but that the duration of the period of development of the symptoms is a perfect reflection of the degree of susceptibility. The ratio of necrotic guard cells to the number of penetration sites is a good indicator of the rate at which the host cells react in response to direct contact with the parasite (Mourichon et al. 1989). Mourichon et al (1989) further

reported that preformed toxic substances exist in the leaves of banana plants to *M. fijiensis*. Salle et al (1989) speculated that resistance in some highly resistant *Musa* might be due to a hypersensitive reaction resulting in the death of the fungus and reported that in a highly resistant cultivar "Yangambi" (AAA genotype), very small necrotic zones were formed with hyphae being very scarce in the tissues. Salle et al. (1989) published the findings of Jayapal and Mahadevan (1968) that a correlation exist between the polyphenolic content of healthy tissues and their resistance level against *M. musicola* and *Helminthosporium gibberosporium* on *Musa*.

Vasquez et al. (1988) suggested a relationship between the stomatic density of a particular cultivar and its degree of resistance to *M. fijiensis*. The number of stomata per unit area decrease with an increase degree of ploidy and the decrease seems to be a function of the B-genome. (Vasquez et al. 1988). Laville (1983) observed that the presence of a single B genome rarely gives varieties higher resistance to *M. fijiensis* and that resistance seems to intensify only with genome ABB.

2.9 CONTROL OF YELLOW AND BLACK SIGATOKA DISEASE

In general, disease control is of utmost importance if crop productivity is to be increased and this can be achieved by any of the following methods: Cultural, chemical, genetic or resistance control, or the combination of any of these methods depending on the crop, the environment and the availability of other

logistics. For black and yellow sigatoka diseases, control attempts make use of any of the methods above and their combinations.

Chemical control by use of fungicides seems more effective. However, it is associated with certain potential environmental hazards due to spray chemicals. By the nature of the crop growth pattern and disease cycle, sigatoka disease control calls for frequent spraying of fungicides and most often on large plantations, by the use of helicopters which will subsequently increase spray drift. By any means, the early detection of the initial infection symptom of the disease is of utmost importance in disease control. Wioldmaker (1988) suggested the use of four strategies in control as follows:

1. monitoring the epidemiology of the disease through adapted early warning disease survey,
2. the use of the systemic fungicide propiconazole integrated with other mode-of-action fungicides,
3. low volume application techniques, and
4. change from overhead to under-canopy irrigation.

Stover (1989) gave a good account of how petroleum oil controls banana/plantain leaf spots. He stated that oil improves the spreading and sticking properties of all fungicides as well as enhancing the penetration of systemic fungicides and it has a fungistatic effect on

the pathogen inside the leaf. Oil is effective whether applied as an oil in water emulsion or alone. However, a problem associated with oil sprays is their phytotoxicity effect which is characterised by extensive leaf bronzing.

Fungicidal control of banana and plantain leaf spot disease is currently the most widespread form of control. Protectant fungicides, such as Bordeaux mixture, were mostly used between 1936-1962 (Stover 1989), and they were found to be incapable of providing effective coverage for the ever increasing leaf surface against ascospores and conidia impingement. Control was basically by reducing conidial production in the leaf spot. Other protectant fungicides used include Dithane M-22 (maneb, Bravo 500, and Dithane flowable (Stover 1989). The systemic fungicides that were used first in Black Sigatoka control included Benomyl (Benlate) and Tridemorph (Calixin). The systemics were found to be superior because they penetrated the leaf and they acted on the pathogen long before it could sporulate (Stover 1989). Other systemic fungicides in use include the triazoles - Propiconazole (Tilt), and Flusilazole (Punch), Stover (1989).

Eswaramurthy et al. (1989) reported that insertion of Bavistin (Carbendazim) 60-70mg, or aureofungin (90mg) capsules into the rhizome of banana gave good control of both *Fusarium oxysporum f.sp. cubense* and *Mycosphaerella musicola*. However, one major setback in the use of systemic fungicides is the appearance of resistant strains of the pathogen. Perez-Vicente et al. (1985)

reported the appearance of benomyl-resistant race of M. musicola in Cuba. Furthermore, there was cross-tolerance to thiophanate-methyl and carbendazim.

Fungicide resistance was manifest by normal ascospore discharge from leaves treated with benomyl and normal germination of conidia from benomyl treated areas, with subsequent colonies sporulating.

Genetic/resistance control of Black and Yellow Sigatoka diseases include the selection of resistant varieties through tissue culture and subsequent in vitro testing, using toxins or culture filtrates of *M. fijiensis* and breeding bananas and plantains resistant to black sigatoka. Maize tissues were found to be resistant to culture filtrate of *Helminthosporium maydis* race T. (Gengenback et al. (1977) quoted by Leopivre and Acuna (1988). In a similar vein, Leopivre and Acuna (1988) found a correlation between in vivo and in vitro phytoalexin production for *M. fijiensis*.

One major setback in banana/plantain breeding is the production of infertile seeds. Rowe and Rosales (1989) reported that several sub-species of wild *Musa acuminata* have high levels of resistance to black sigatoka and have been used in crosses. They found that the diploid hybrid, SH-3437 has an outstanding resistance to black sigatoka and can ,therefore, be used as a parental line in various phases of breeding.

Cultural methods of sigatoka disease control can be achieved by crop hygiene. Basically, it involves

removal and burning of infected leaves before they show mature spot symptoms.

Emebiri and Obiefuna (1992) reported that leaf removal and/or intercropping with a fast growing multi-branched cassava cultivar reduced disease incidence by 16% and severity by 10%. Total crop destruction and strict quarantine restrictions can also be used to control black sigatoka disease (Vidal, 1992).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Field Symptomatology and Collection of Diseased Leaves of Plantain and Banana

Symptoms of black sigatoka on both plantain and banana in the field were observed anytime samples were being taken. Both adaxial (upper surface) and abaxial (under surface) leaf surfaces were observed and disease stages noted. Photographs were then taken of infected leaves of both plantain and banana.

Diseased leaves collected for fungus isolation were those showing specks, streaks and scorching with grey centres, which were found at the leaf tip and margins. The scorched margin was cut-off from the other infected portions and placed in damp polythene bags to hasten perithecia maturation for ascospore discharge.

3.2 Isolation Techniques to Obtain the Causal Agents

Three methods were attempted in order to obtain the suspected pathogen *Mycosphaerella fijiensis* Morelet (Deighton), from diseased leaves of both banana and plantain:

- (i) Single conidium isolation by Spore Pick Technique from diseased leaf surface.
- (ii) Induced mycelial growth on media from first-streak stage diseased leaf discs.

(iii) Ascospore Discharge Technique (ADT) from diseased leaves.

3.2.1 Single Conidium Isolation

Plantain and banana leaves showing various stages of black Sigatoka disease symptoms collected from the field were mounted under stereoscopic microscope and observed. With the aid of sterile mounting pins dipped in Sterile Distilled Water (SDW), spore picking was done.

In another instance, diseased leaves were washed in SDW to rid off surface contaminants and cut into small discs, which were then mounted on the inside of bell-jars containing a beaker of water to induce humid conditions (Fig. 1). Microscopic examinations were made on the leaf discs after four days to scan for conidia.



Fig.1 Incubation of diseased plantain (A) and banana (B) leaf discs under bell-jar to induce conidia production.

3.2.2 Induced Mycelial Growth

Fresh leaves of plantain and Local Tall Banana (LTB), showing first streak symptoms (reddish-brown streaks appearing on abaxial leaf surface) of black sigatoka were cut into small discs (5mm²), surface sterilised in 1% sodium hypochlorite (NAOCL) solution for one minute and then rinsed in SDW. The sterile leaf discs were then blotted dry on sterile Whatman No. 1 filter paper. The leaf discs were then plated on the surface of Carrot Leaf Decoction Agar (CDA) and Potato Dextrose Agar (PDA).

The CDA was prepared from 300g of finely chopped fresh carrot leaves, 12g agar and 1 litre distilled water. 300g of fresh carrot leaves were washed under tap water and then finely blended in a good chopper. The blended leaves was then mixed with 500ml distilled water in a conical flask. The material was then steamed for one hour without pressure and then strained through two layers of calico and cotton wool. The strained decoction was then sterilised by filtering through sterile sintered glass filter (Gallen Kamp Sintered Glass 3,24/29). The sterile decoction was then added to 500ml distilled water containing 12g of dissolved agar autoclaved at 121°C at 15lb/in pressure for 15 minutes. The medium was thoroughly mixed and poured into sterile petri dishes under aseptic conditions. The pH of the medium was found to be 7.3. This method of medium preparation was adopted after Kilpatrick and Howard (1956).

The Potato Dextrose Agar was prepared by weighing 39g of dehydrated PDA (Manufactured by MERCK, Germany) and dissolving it in 1 litre of distilled water by gently warming and constantly stirring with a magnetic rod. When the media was fully dissolved, the Stirrer was removed and the media autoclaved for 15 minutes at 121°C at 15 lb/in pressure, under aseptic conditions. The pH of the media was found to be 6.5.

The plated leaves on media (Fig.2) were incubated under room temperature. Plates were observed under the microscope for the growth of intercellular hyphae from the cut edges of the plated leaf discs.



Fig.2. Infected leaves plated on media, to induce mycelial growth.

3.2.3 Isolation From Plantain (Brodi Yuo) By Ascospore Discharge Technique (ADT)

Diseased plantain leaves showing stage six or the mature spot stage of black Sigatoka disease, which was evident as necrosed leaf sections showing grey centres, were taken from "Brodi Yuo" in the field (A.R.S. Kade). The diseased leaves were incubated in polythene bags under moist conditions for three (3) days to hasten the maturation of perithecia. The diseased leaves were cut into 4cm² discs and dried under the sun for two (2) days, and later taken to the laboratory to be washed under several changes of tap water in order to get rid of surface contaminants. The washed leaves were then further sterilised in 10% NaOCl solution for 3 minutes and rinsed in Sterile Distilled Water (SDW). The disinfected leaves were then immersed in SDW for 20 minutes, and then stapled onto a Whatman No.1 filter paper.

The stapled leaf discs were placed in the lids of Petri dishes with the base dishes containing SDW, PDA and CDA. Fig.3. The Petri dishes were then covered with the upper surface of the diseased leaf facing the media in the dish. This isolation method was adopted after Meredith and Lawrence (1969).

The plates were observed after 15 minutes under the microscope for the discharge of ascospores. It was found that a lot of ascospores were ejected onto the media. Ascospore discharge increased with time and after an hour the filter paper with the stapled leaf disc was removed and the plate incubated at room temperature (26°C) under

continuous light. The light source was provided by 40watts incandescent bulb, placed 32.5cm above the plates.

3.2.4. Ascospore Discharge Technique For Isolation From Local Tall Banana (LTB)

Since the Ascospore Discharge technique was found to be more reliable than the other methods described earlier, it was adopted for the isolation from the Local Tall Banana. Diseased banana leaves used were those showing stage six or the mature spot stage of black Sigatoka, which is evident as necrosed leaf portions with grey centres. The method of isolation and subsequent treatments were the same as those described for plantain above Fig. 3.

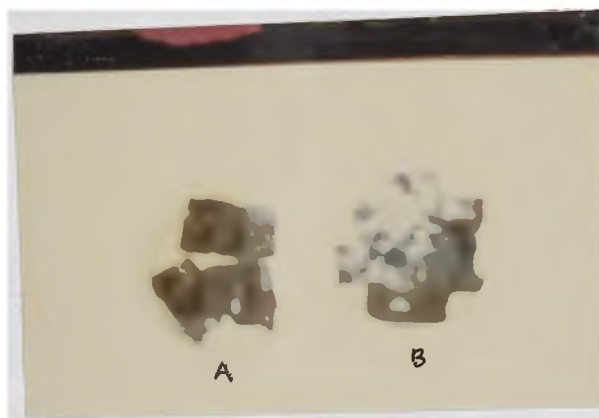


Fig.3. Infected leaves stuck on the lid of Petri dish for ascospore ejection onto media.

A - Infected plantain leaves

B - Infected banana leaves

Note: Viewing from above.

3.3. Micrometry

Length and breadth of ascospores, conidia, and conidiophores were measured by means of an ocular micrometer mounted on the eyepiece of a binocular compound microscope which was standardized by using a substage micrometer. Assistance was received from Dr. K.A. Oduro during the process of standardization.

3.4 Photomicrography

Photomicrographs of fungal spores and other structures were taken by means of a camera mounted above a compound microscope (Fig.4). Help was sought from the technical staff during the loading of the film and the initial stages of the photography.

3.5 Inoculations and Incubation Techniques in The Greenhouse

Plantain and banana suckers were thoroughly washed in water and the old roots peeled off, in order to get rid of other root pathogens and surface contaminants. The suckers were planted in garden soils in plastic buckets. Watering was immediately done and subsequently three (3) times per week. However, during the dry season, watering was done on every other day.



Fig.4. Method used in taking photomicrographs.

Except in one instance where ascospores ejected from diseased plantain leaves were sprayed on leaves of LTB plants, all other inoculations were done with conidial inoculum produced from cultures of *Mycosphaerella fijiensis* grown on CDA produced from ADT as described earlier.

Inoculum was produced by macerating sporulating cultures of *M. fijiensis* grown on CDA, in SDW on a microscope slide.

Inoculation onto plantain and banana leaves was done by first rubbing the abaxial surface of the first fully expanded leaf of the plant at three-leaf stage with a dry sterile cotton wool to remove wax from the leaf surface.

Subsequently, the conidial suspension with mycelial fragments on the slide was gently pressed on the abaxial right-half of the leaf surface.

The inoculum was further evenly spread on the leaf with the fore-finger. The whole plant was then immediately enclosed in a transparent polythene bag as shown in Fig.5, containing a beaker of water to induce humid conditions. The humid condition was maintained continuously for the first eight (8) days and then removed in order to ensure conidia germination and penetration into leaf. Subsequently the polythene bag was replaced every other day until initial specks (first disease symptoms) were observed on the inoculated leaves.

Inoculations of the plants were done on leaf number one when the plants produced three (3) leaves on the

average. This incubation method was adopted after Goos and Tschirch (1963).



Fig. 5. L: Method used for the incubation of inoculated plants in the greenhouse.

R: Uninoculated plants to detect presence of air spora.

The inoculations were done to determine the following:

- (a) pathogenicity of the plantain isolate from "Brodi Yuo" cultivar on plantain,
- (b) pathogenicity of Local Tall Banana isolate on banana of the same variety,
- (c) the effect of the plantain isolate on LTB.
- (d) the effect of the banana isolate on plantain.

In one case, ascospores (instead of conidia) from diseased plantain were used to inoculate local tall banana. Diseased plantain leaves were wetted and plated over SDW for ascospores ejection into the liquid. When the presence of ascospores in SDW was confirmed under the microscope, the ascospore suspension was then sprayed on the banana leaves and incubated as described above.

Other experiments were set up to determine the effect of humidity on inoculation and disease expression, and they consisted inoculated but unincubated plantains and bananas that were not incubated under polythene bags (Fig.6).

In addition, uninoculated plantain and bananas were left in the open greenhouse in order to investigate the presence or absence of inoculum in the open greenhouse (Fig.5,R).

Control treatments consisted of all uninoculated but incubated leaves. These were the other halves of all inoculated leaves that were incubated.

Data collected during the greenhouse studies were as follows:

(i) Disease progression on plants:

(a) Time taken for initial specks to appear (incubation period).

(b) Time take for specks to reach the mature spot stage (stage six), from the date of appearance of initial specks.

(c) Disease cycle (i.e. time taken from the day of inoculation until mature spots developed with grey centres).

(ii) Visual recording of disease symptoms on plants by means of photographs.

Environmental conditions in the greenhouse were as follows:

Temperature, 24°C - 36.5°C.

R.H. in open greenhouse, 35%-87%

R.H in incubation chamber 94%-100%.



Fig.6. Inoculated plantain left in the open greenhouse to investigate the effect of humidity on disease expression. Note the absence of disease symptoms on leaves.

CHAPTER FOUR

RESULTS

4.1 FIELD SYMPTOMS

4.1.1 Field Symptoms on Plantain

From a distance, leaves of plantain appeared scorched as if fire had razed through the field (Fig. 7). On close observation it was found that symptoms were mostly concentrated along leaf margins and were first found on the mature leaves (Figs. 7 and 8). Other leaves showed reddish-brown streaks that were longer and broader with some water-soaked early in the mornings. Mature lesions which were constantly found at leaf margins appeared dark black with yellow halo-margins (Fig. 8 and 9). Similar symptoms were found on young suckers (Fig. 7). Heavily spotted plants with very few or no green leaf tissues showed very poor bunch development (Fig. 10), whereas those with some green leaf tissues had some appreciable bunch development (Fig. 11).

4.1.2 Field Symptoms on Local Tall Banana (LTB).

Patterns of field symptoms followed essentially those on plantain. Infected grooves of LTB appeared blighted mainly at the leaf margins (Fig. 12) from afar. Closer observation revealed several stages of disease development on infected leaf strips (Fig. 13). The spots are broad especially at the leaf margins due to coalescence and appeared grey, surrounded by yellow halo-margins (Fig. 12). Heavily spotted plants with fewer green leaf tissues produced very poor bunches (fig. 14). Young suckers were similarly diseased as was found on plantain.

4.2 Isolation and Cultural Characteristics of the Plantain isolate

4.2.1 Single Conidium Isolation

Microscopic examination of plantain leaves from the field showing typical black Sigatoka symptoms also revealed abundant conidiophores and conidia of other fungi such as *Cordana musae* (Fig. 15,) and *Chloridium* spp on the leaf surface.

Typical conidiophores and conidia of the suspected pathogen *Mycosphaerella fijiensis* were observed. Conidia of *M. fijiensis* were intermingled among the abundant spores of *Cordana* and *Chloridium*, thus making spore picking very impossible.

Whereas *Cordana* was mostly found at the centres and margins of necrosed leaf tissues, *Chloridium* was commonly found on portions of healthy green tissues showing reddish-brown speckles.

Isolation from leaf discs incubated under bell jars was not possible due to the slow growth of the pathogen from the leaf, which was outgrown by other contaminating fungi.

4.2.2 Induced Mycelial Growth on Media

No growth of mycelia of *M. fijiensis* was recorded on PDA and CDA from the cut edges of leaf discs. Though growth of other contaminants believed to be present on the leaf surface were observed. Attempt to increase the period of disinfection of leaf discs in NaOCl resulted in the scorching of the cut edges of the leaf discs.

4.2.3 Isolation by Ascospore Discharge

Ascospores were discharged into SDW, onto CDA and PDA fifteen (15) minutes after plating infected leaf.

Ascospores appeared hyaline to olivaceous green, two-celled, one cell of which is larger than the other with a Septum dividing both cells, but not well-defined and only clearly visible in stain (Fig. 16a,b). Ascospores stained deep blue in lactophenol cotton blue and measured 10.7-16.6 μm (Avg. 13.8 μm) by 2.4-3.8 μm diam. (Avg. 3.2 μm).

In addition to the ascospores of *M. fijiensis*, spores of another fungus believed to be those of *Leptosphaeria* spp were also ejected (Fig. 17). These spores germinated faster, after 30 minutes, with multiple hyphae than those of *M. fijiensis*.

4.2.4 Germination of Plantain Isolate from Spore Discharge in SDW

Ascospore germination began after 1½ hours in SDW and after 2 hours, germ tubes measured 0.7-9.0 µm (avg. 4.3µm). Germination was bipolar but frequently arising first from the top of the smaller cell of the ascospore.

No germ tubes arose from the sides of the cells of the ascospore and not more than two germ tubes were produced by an ascospore. Branching from the germ tubes were very seldom even after a week of growth. Septation of germ tubes began over a period of 12 hours. Germ tubes were very slender, less than 3.0µm in diameter (Fig. 16b) as compared to those on CDA measuring 3.45µm.



Fig. 7: Heavily scorched leaf margins showing mature spots, with a young sucker(s) showing similar symptoms.



Fig. 8: Black spots with halo-margins at leaf tips arrowed.

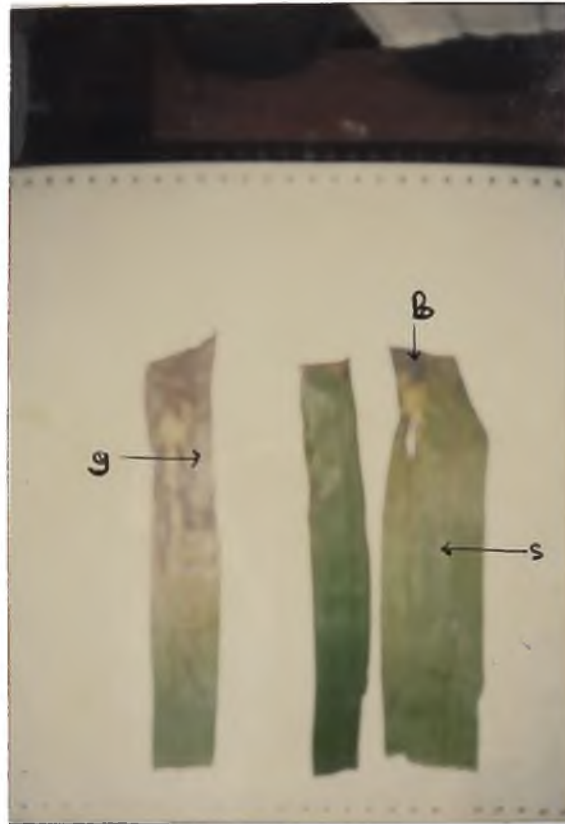


Fig. 9: A closer view of black sigatoka symptoms on infected leaf strips. Note the very dark black spot (B) and grey-centred spot (G) and streaks (s).



Fig. 10: Heavily spotted plant (arrowed s) with poorly developed bunch (arrowed b). Note the absence of healthy green leaves.



Fig. 11: Infected plantain with scorched leaves showing appreciable bunch development. Note the presence of healthy green leaves.



Fig. 12: Diseased banana grooves with heavily spotted leaf margins arrowed.

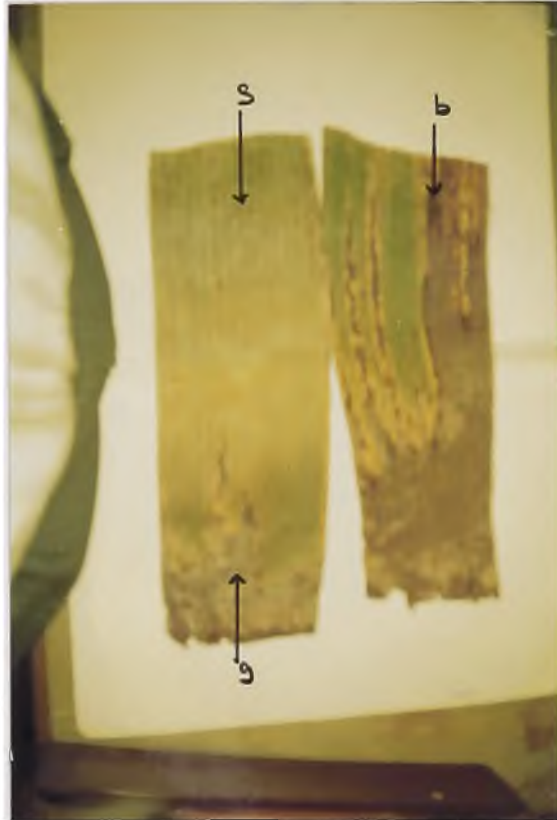


Fig. 13: Closer view of disease on infected leaf strips.

Note the streak(s), black spots (b) and mature spots with grey centres (g).



Fig. 14: Heavily spotted banana without viable leaves with a poorly developed bunch (arrows).

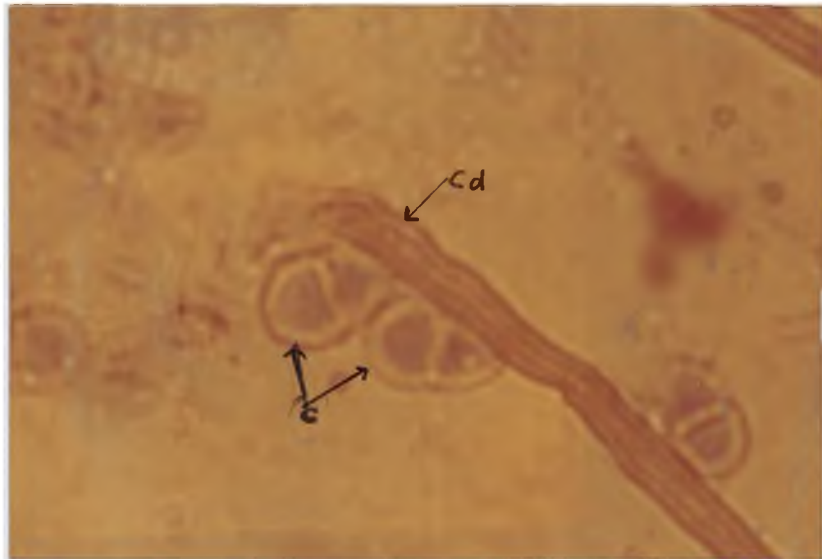


Fig. 15. Conidiophore (Cd) and detached conidia (c) of *Cordana musae* on plantain and banana leaves infected with black Sigatoka in the field. (x 1000).

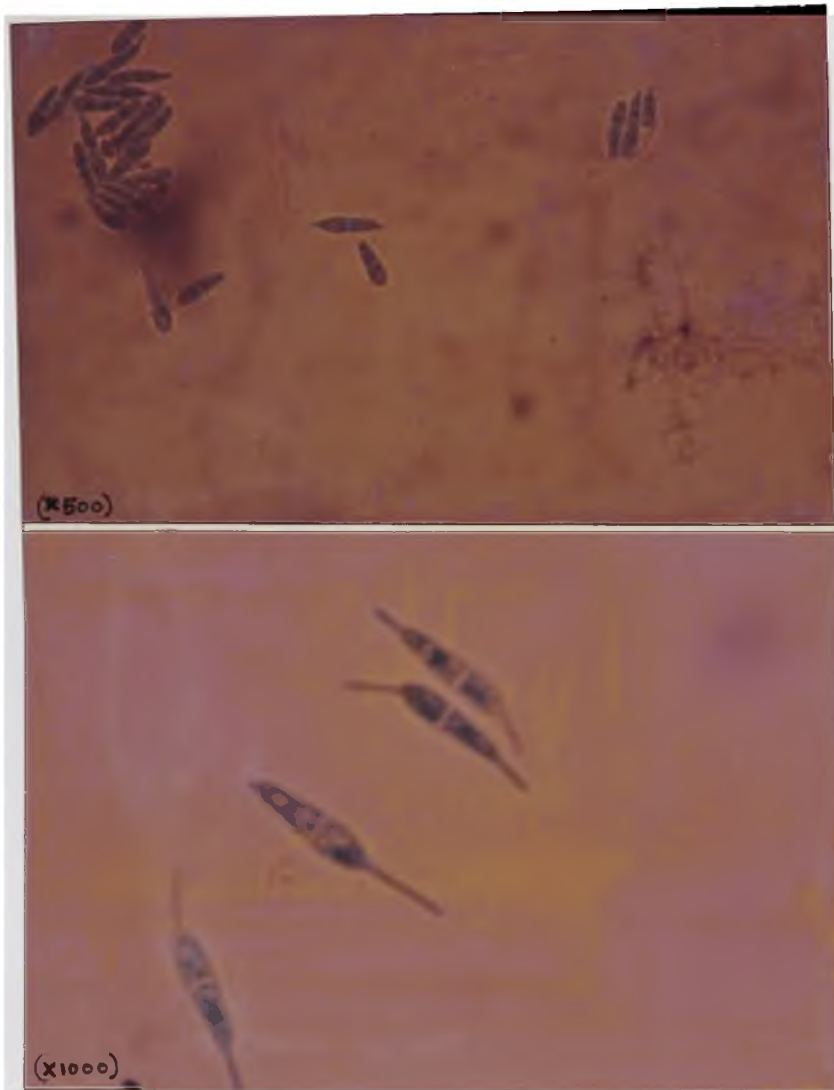


FIG. 16A: Ascospores of *M. fijiensis* from plantain (x 500, x 1000)



FIG. 16B: Germinating ascospores of *M. fijiensis* from Plantain in SDW after 12 hours. (x 500)

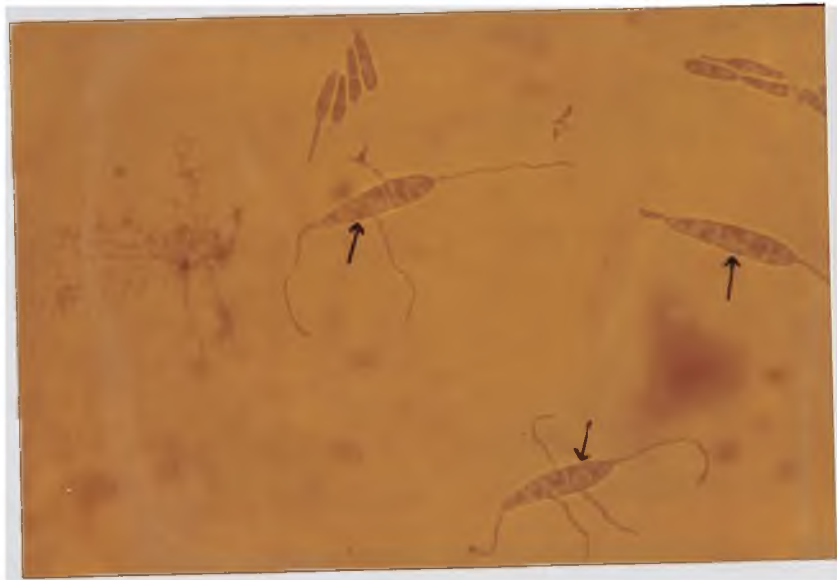


FIG. 17: Ascospores of *Leptosphaeria* spp (arrowed L), ejected together with ascospores of *M. fijiensis* (arrowed M) both germinating in SDW after two hours of ejection.

Note the longer and multiple germ tubes arising from *Leptosphaeria* spores untypical of *M. fijiensis* (x 500)

4.2.5. GERMINATION OF PLANTAIN ISOLATE FROM ASCOSPORE DISCHARGE ON PDA, PH 6.5

Ascospore germination occurred after 15 hours on media and was bipolar. Whitish fluffy colonies were rarely visible on the fourth day. After six days, colonies appeared whitish on the surface with the undersurface dark. Immediately below the superficial white hyphae was a hard rind. Colonies measured 1.5mm in diameter after ten days and still appeared whitish (Fig. 18a). Sporulation was not observed on PDA after four weeks and thereafter on cultures incubated under continuous light at 26°C. However, all other structures found on CDA were present on PDA. Non-sporulating cultures transferred from CDA onto PDA did not sporulate but transfer of colonies from PDA to CDA sporulated.

4.2.6. CULTURAL CHARACTERISTICS OF PLANTAIN ISOLATE ON CDA, PH 7.3, TEMPERATURE OF INCUBATION 26°C.

Ascospore germination began after 12 hours on CDA and became pronounced after 18 hours, with germ tubes measuring 8.6-38.0µm (avg. 25.6 µm) by 3.5 µm diam. Germ tubes were robust and the tips began swelling after 23 hours, a sign of dumb-bell-shaped cell development. Occasionally, additional germ tubes arose from either or both cells of the ascospore, frequently from the tips of the cells after 18 hours. A maximum of four germ tubes as opposed to two in SDW, emerged from an ascospore on CDA. After 48 hours, the germinated ascospores developed dumb-bell-shaped cells with numerous hyphal branches (Fig. 20).

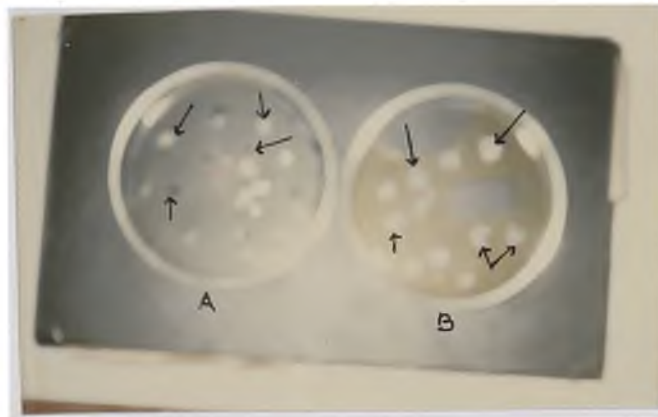


FIG. 18: 14 Days old colonies (non-sporulating) of plantain isolate of *M. fijiensis* on

A - PDA

B - CDA

Note whitish nature of colonies (arrowed)



FIG. 19: Sporulating (s) and non-sporulating (NS) 12-days old Colonies of plantain isolate of *M. fijiensis*.

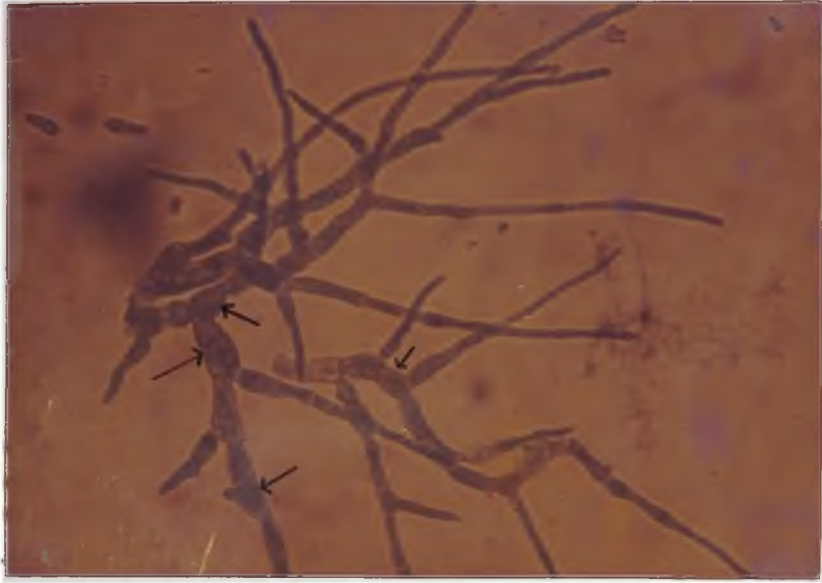


FIG. 20: Hyphae of *M. fijiensis* from plantain on CDA after 48 hours. Culture from ascospores (x 500). Note the beginning of dumb-bell-shaped cells development (arrows).

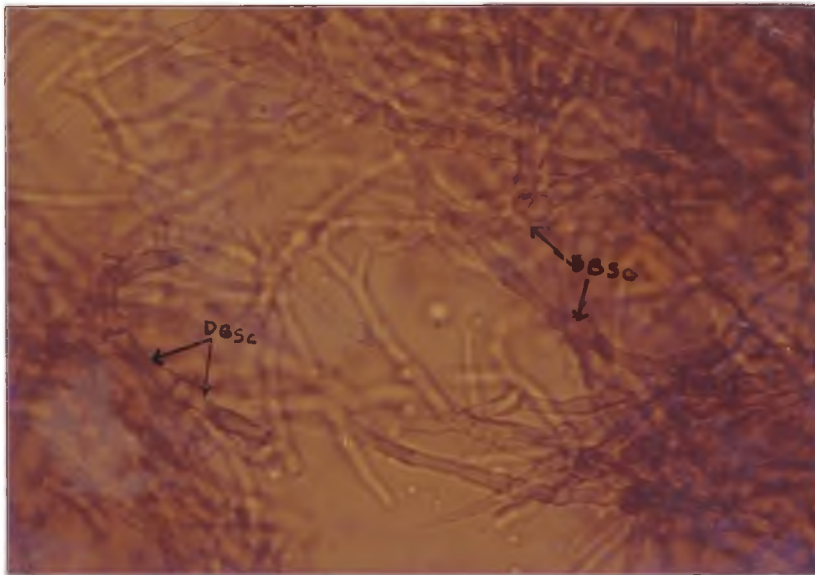


FIG. 21: Hyphae of *M. fijiensis* from plantain showing dumb-bell-shaped cells (DBSC), (x 500).

Colonies resulting from ascospores germination of the plantain isolate on CDA were first rarely visible to the naked eye on media only after four days of incubation under continuous light. Colonies appeared whitish and fluffy on the surface of the media. The superficial fluffy hyphae appeared hyaline under the microscope and stained deep blue in lactophenol cotton blue and were highly branched (Fig. 21). After five days of incubation, the undersurface of the colonies became black, and hyphae in this portion appeared dark brown, with the cells dumb-bell-shaped, (Fig. 21). Dumb-bell-shaped cells measured 3.45 μm diam. Both hyaline and dumb-bell-shaped cells are septate and frequently branched (Fig. 20, 21). The initially hyaline hyphae gradually changed to olivaceous brown with time and it was such light brown hyphae that gave rise to the conidiophores. Conidiophores generally arose from the very end of a hyphal cell segment as if from the septum separating the adjoining hyphal cells (Fig. 23a,b). Conidiophores were light brown but became paler towards the tip and showing conidial scars, (Fig. 23b). Conidiophores are septate (1-13) but frequently 3 and measured 24.2-163.9 μm (avg. 54.7 μm) by 3.1-5.2 μm (avg. 3.8 μm).

Conidial production was observed on the seventh day of incubation and became profuse on the ninth day. Consequently, the colonies appeared light grey (Figs. 19 and 22).

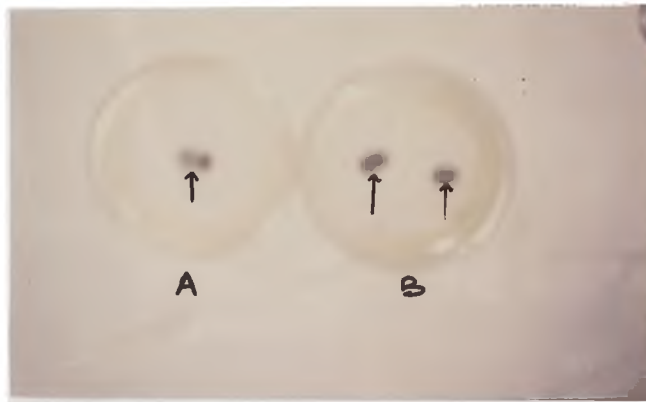


FIG. 22: Sporulating colonies of *M. fijiensis* on CDA.

A - Plantain isolate

B - Banana isolate

Note: Dark colour of colonies due to reflections from the undersurface of the colonies which were originally black

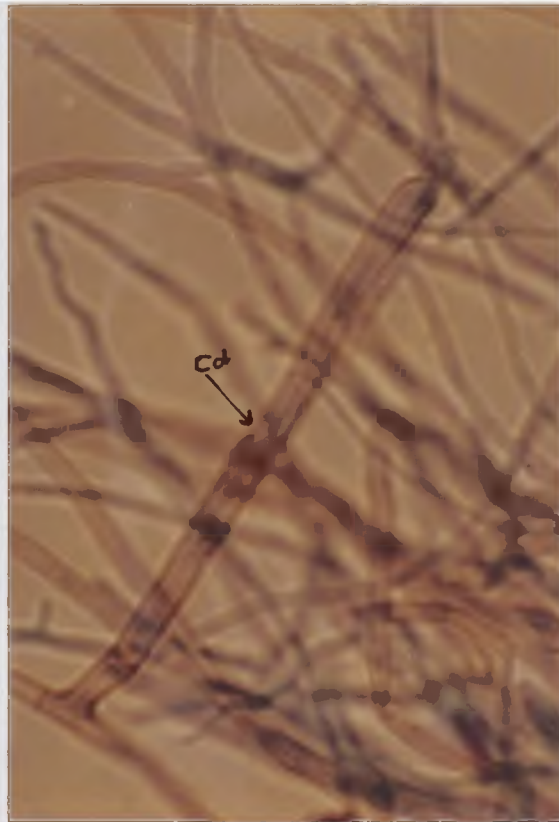


FIG. 23A: Conidiophore (cd) of *M. fijiensis* in culture intermingled in hyphae (x 1000).

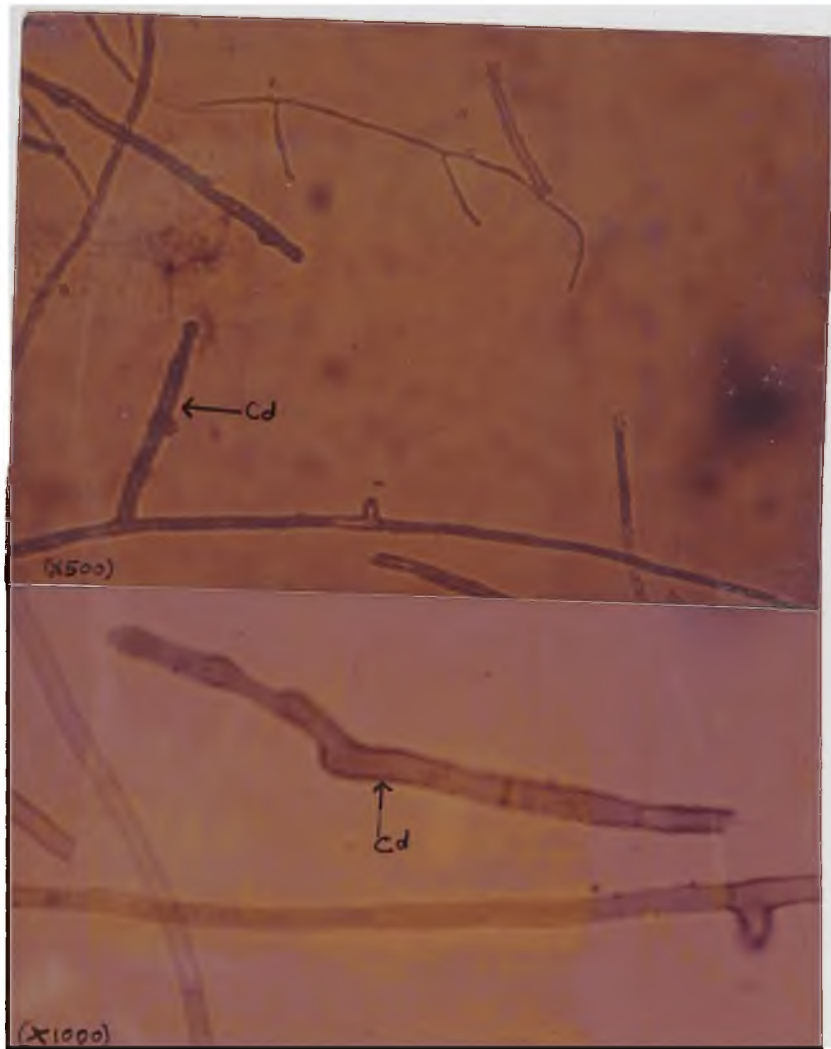


FIG. 23B Conidiophore (cd) of plantain *M. fijiensis* in culture (x 500, x 1000)

Entire colonies sporulated unlike in other reported cases that sporulation was only at the fringes of the colony. Cercospora-type conidia were formed (Fig. 24a, b). Conidia were very pale greenish or olivaceous, obclavate to cylindro-obclavate, 1-12-septate (commonly 3-8-septate), straight or slightly curved (Fig. 24a,b) with a short obconically truncate base having a visible and slightly thickened hilum 1.7-3.5µm diameter (mean 2.5µm), occasionally bulbous tip but commonly obtuse tip 1.7-3.5µm diameter (mean 2.4µm), 31.1-158.7µm long (mean 78.1µm), 3.5-4.5µm diameter (mean 3.6µm) at the broadest point near the base, (Fig. 25). In older cultures, some cells of the conidia became bulbous (Fig. 26). The above conidial description and observation in culture agreed well with that of Meredith and Lawrence (1969) for *Mycosphaerella fijiensis*, the causative organism of black sigatoka disease on banana in Hawaii.

Thus, based on these morphological characteristics, *Mycosphaerella fijiensis* was suspected. With time, sporulating colonies developed very whitish superficial hyphae as a result of conidial germination in culture (Fig. 27a,b). Below these white hyphae was found a very tough rind within which were embedded the dumb-bell-shaped cells which were responsible for the dark appearance of the undersurface of the colonies. Microscopic examination of structures within this portion of the culture revealed round perithecia-like bodies, the walls of which were dark-brown with pseudoparenchymatous cells.

Crushing of these bodies revealed hyaline, rectangular-shaped cells believed to be spermatia which stained deep-blue in lactophenol cotton-blue. In general, growth of the fungus in culture was very slow, colonies measured only 4.5mm on CDA after nine days of incubation, and 1.9cm in diameter after six weeks. Colonies were erumpent after one week and appeared dome-shaped when viewed from the side, with irregular zonation.

4.3. ISOLATION AND CULTURAL CHARACTERISTICS OF THE BANANA ISOLATE

4.3.1. SINGLE CONIDIUM ISOLATION

Microscopic examination of diseased banana leaves revealed pathogens similar to those found on infected plantain (Fig. 15). Isolation was not possible due to the same difficulties encountered in the case of the plantain isolate.

4.3.2. INDUCED MYCELIAL GROWTH

Isolation was impossible due to the slow growth of the fungus and other difficulties similar to those encountered in the case of isolation from plantain.

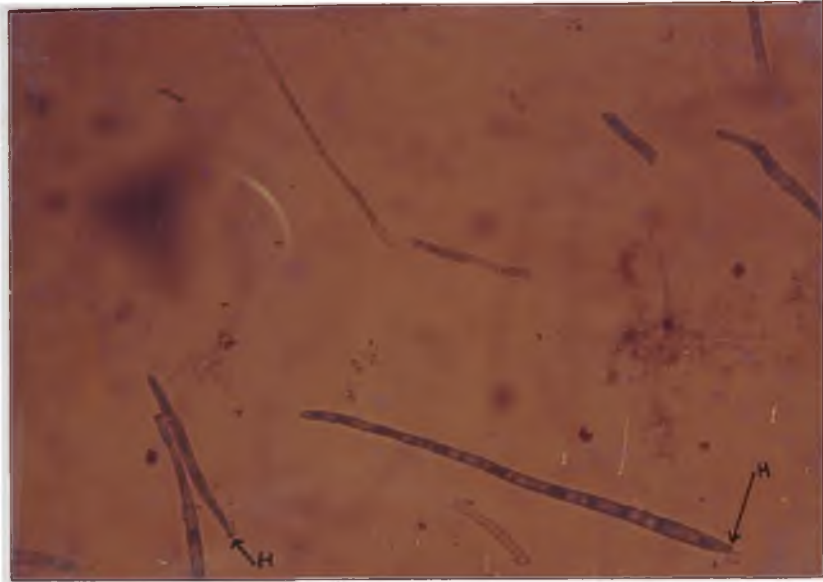


FIG. 24A. Conidia of plantain *M. fijiensis* in culture. Note thickened hilum (H) arrowed. (x 500).



FIG. 24B. Conidia of plantain *M. fijiensis* in culture. Note thickened hilum (H), arrowed. (x 1000).

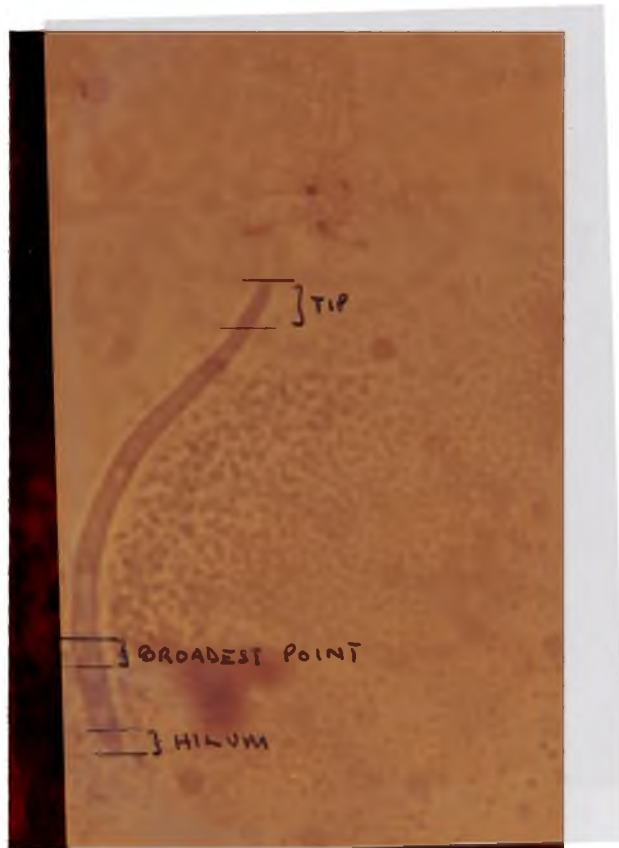


FIG. 25. Sections on conidia of *M. fijiensis* from which measurement were taken.



FIG. 26. Conidia of *M. fijiensis* from plantain showing bulbous cell (arrowed). Similar observations in banana (x 500, x 1000).

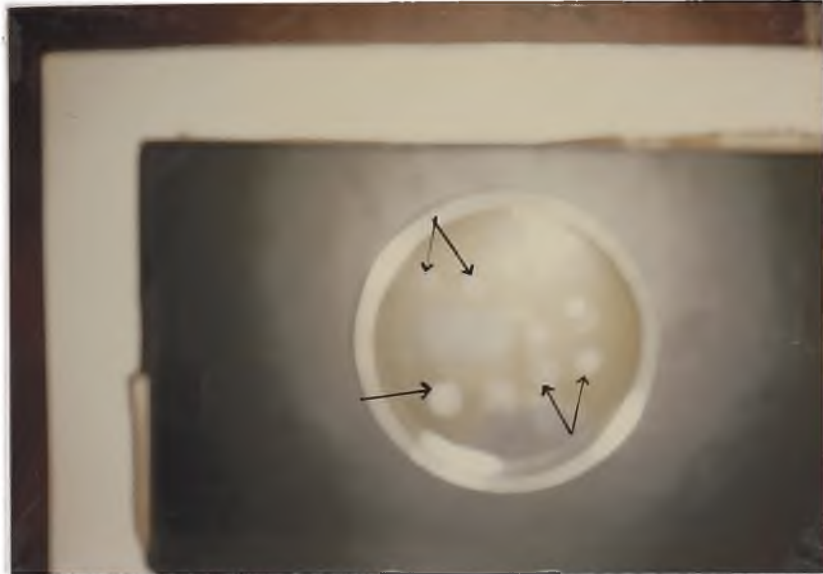


FIG. 27A. 14 days old colonies of plantain isolate of *M. fijiensis* on CDA. Note the very whitish nature of colonies due to conidial germination in culture (arrowed).

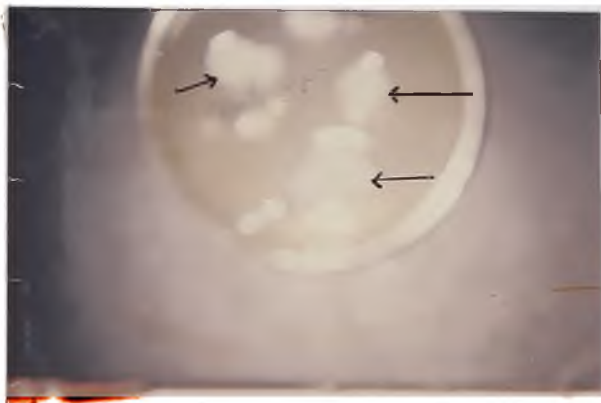


FIG. 27B. 24 days old colonies (arrowed) of plantain isolate of *M. fijiensis* on CDA whitish nature of colonies as a result of germinated conidia in culture.

4.3.3. ISOLATION BY ASCOSPORE DISCHARGE

Ascospores were ejected into SDW, onto PDA and CDA 15-20 minutes after plating of infected leaf. Ascospores appeared hyaline to olivaceous green, two-celled, one of which was larger than the other, with a septum which was not well-marked. Septum was however clearly visible in stain. Ascospores stained deep-blue in lactophenol cotton blue and measured 10.8-16.7 μ m (avg. 13.9 μ m) by 2.5-3.9 μ m (avg. 3.2) (Fig. 28).

4.3.4. GERMINATION OF BANANA ISOLATE IN SDW

Ascospore germination, growth, hyphal septation and other characteristics were similar to those reported for the plantain isolate (Fig. 16b).

4.3.5. GERMINATION OF THE BANANA ISOLATE ON PDA

Ascospore germination and other cultural characteristics such as growth of colony, colour and other structures were similar to those observed for the plantain isolate (Fig. 29 and 30). However, no sporulation occurred on media, neither were conidiophores formed.

4.3.6. CULTURAL CHARACTERISTICS OF THE BANANA ISOLATE ON CDA, PH 7.3, TEMPERATURE OF INCUBATION 26°C.

Ascospore germination began after 12 hours on CDA and became pronounced after 18 hours, with germ tubes measuring 8.6-38.2 μ m (avg. 25.6 μ m) by 3.45 μ m. Germ tubes in culture were robust and the tips began swelling after 24 hours as a sign of dumb-bell-shaped cell development.



FIG. 28. Ascospores of *M. fijiensis* from local tall banana (x 500)



FIG. 29. Non-sporulating 7 days old colonies (arrowed) of banana isolate of *M. fijiensis* on

A - CDA

B - PDA



FIG. 30. 14 days old colonies of banana isolate of *M. fijiensis* on PDA. Note whitish nature of colonies (arrowed)

Additional germ tubes emerged from either or both cells of the germinating ascospores after 18 hours and frequently from the tip of the cells. Maximum of four germ tubes emerged from an ascospore on CDA.

Colonies resulting from ascospores on CDA media were first rarely visible to the unaided eye as fluffy white colonies only after four days of incubation under continuous light. Hyphae from the fluffy colonies were hyaline under the microscope, stained deep blue in lactophenol cotton blue, septate and were highly branched (Fig. 32).

After 5 days of incubation, the under surface of the colonies appeared very dark black and hyphae in this portion were dark-brown, with the cells dumb-bell-shaped (Fig. 32) measuring 3.45µm in diameter. Both hyaline and dumb-bell-shaped cells were septate and frequently branched, (Fig. 31 and 32). The initial hyaline hyphae of 5-day old colonies gradually changed olivaceous brown with time and it was from such light brown hyphae that conidiophores arose. Conidiophores arose from the end of a hyphal cell very near to the septum of the adjoining cell (Fig. 33).

Conidiophore were septate, 1-12, but frequently three (3) and measured 24.2-163.9 µm (avg. 54.9µm) by 3.2-5.4µm (avg. 3.8µm) diameter. Conidiophores were light brown but became paler towards the tip and showing conidial scars, they were straight to slightly bent especially at the tip (Figs. 33,34). Colonies of the isolate from banana measured 4.5mm on CDA after nine days and 1.9cm in diameter after six weeks on media. Colonies were erumpent and appeared dome-shaped when viewed from the side.

Conidial production was observed on the sixth day of incubation and became profuse on the eighth day, with the colonies appearing light grey (Fig. 22b). Whole colonies sporulated as in the case of the plantain isolate. *Cercospora*-type conidia were formed in culture similar to those found in the isolate from plantain.

Conidia were pale greenish or olivaceous, but not quite colourless, obclavate to cylindro-obclavate, 1-13-septate (commonly 3-8-septate), straight or mildly curved, with a short obconically truncate base having a visible and slightly thickened hilum

1.7-3.5 μ m diam. (mean 2.6 μ m), occasionally bulbous tip or cells of the conidia (Fig. 35). Commonly obtuse tip 1.6-3.5 μ m diam. (mean 2.6 μ m), 31.0-158.9 μ m long (mean 78.4 μ m), 3.5-4.5 μ m diam. (mean 3.6 μ m) at the broadest point near the base, (Fig. 25.). Sporulating colonies developed very whitish superficial hyphae with time as a consequence of conidia germination in culture (Figs. 29,30). Below these white mass of hyphae was a very hard rind within which were embedded the dumb-bell-shaped hyphal cells and spherical perithecia-like bodies, the walls of which were dark-brown with pseudoparenchymatous cells.

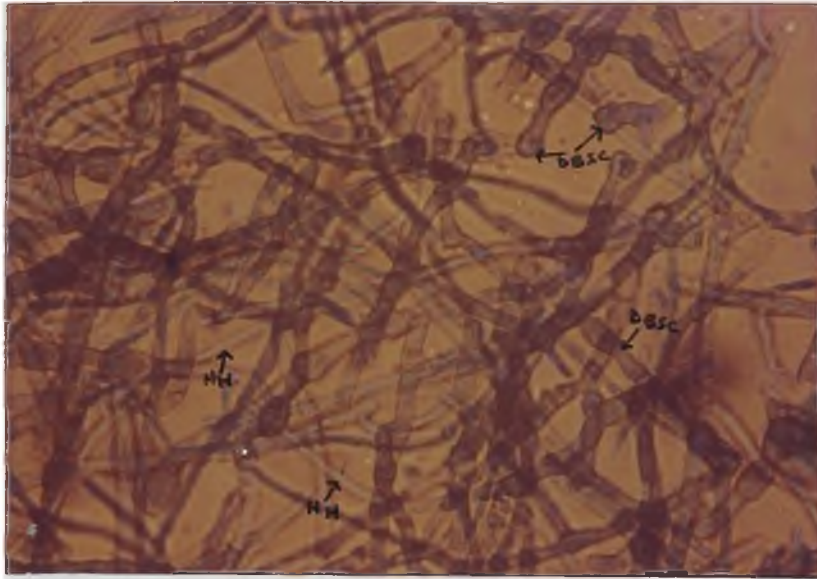


FIG. 31. Hyphae of *M. fijiensis* from LTB showing dumb-bell-shaped cells (DBSC) arrowed, and hyaline hyphae (HH) (x 500)

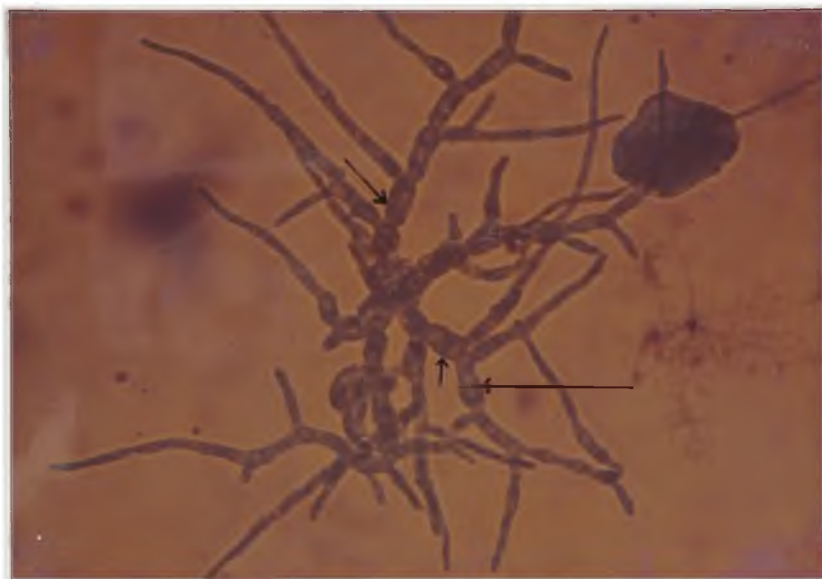


FIG. 32. Hyphae of *M. fijiensis* from LTB, on CDA after 48 hours. Culture from ascospores. (x 500).
Note the beginning of dumb-bell-shaped cells development (arrowed).



FIG. 33. Conidiophore (cd) of banana *M. fijiensis* in culture. Note conidial scar (CS) (x 500, x 1000).

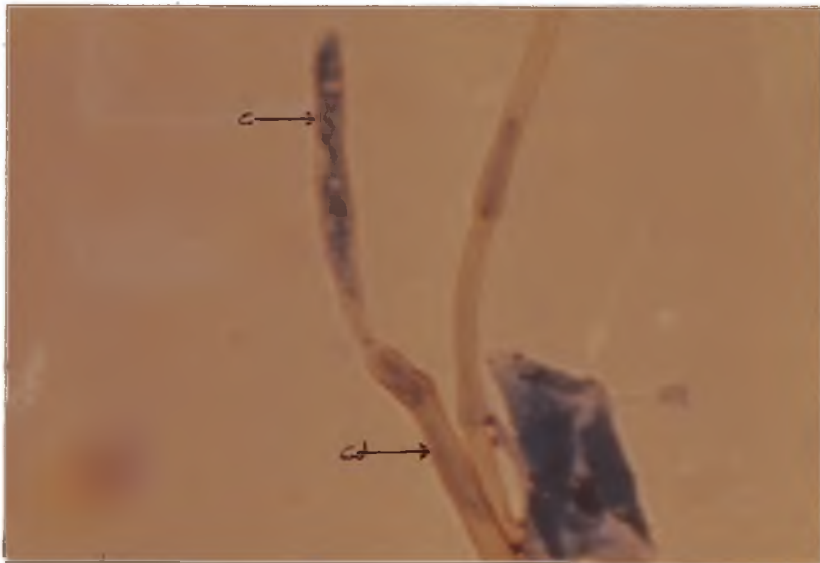


FIG. 34. Conidiophore (cd) of banana *M. fijiensis* bearing a young conidium (c) in culture. (x 500).

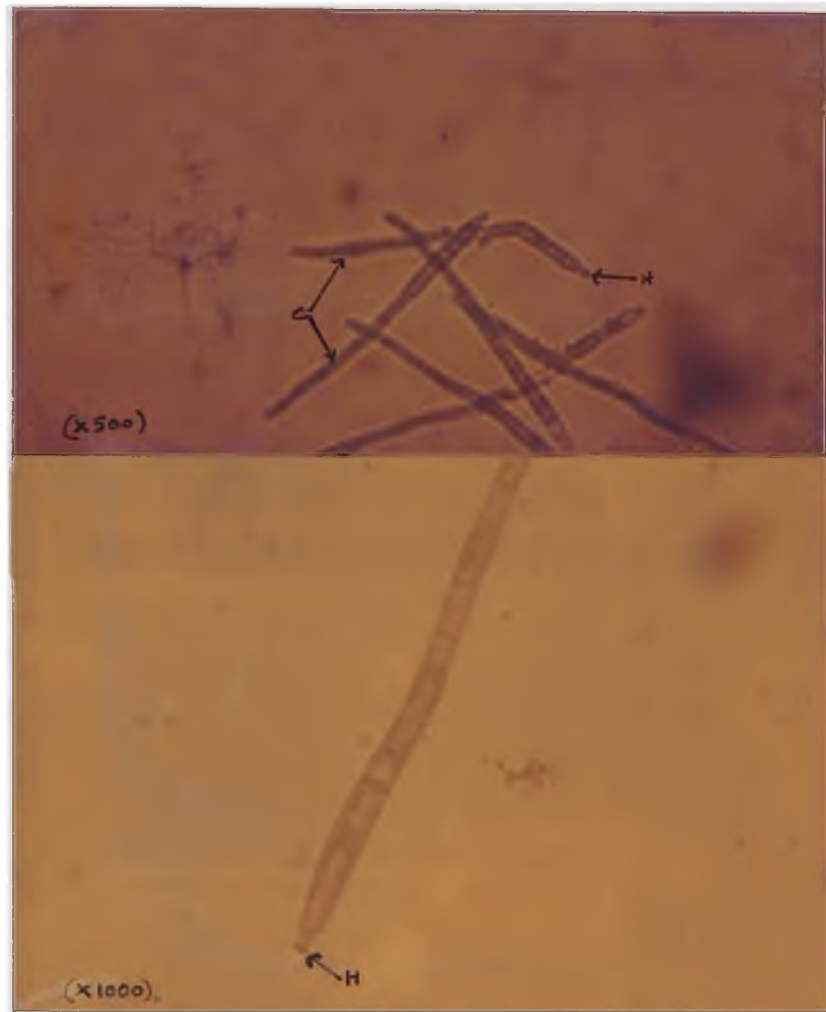


FIG. 35. Conidia of banana *M. fijiensis* in culture. Note thickened hilum (H) arrowed. (x 500, x 1000).

Crushing these colonies revealed hyaline, rectangular shaped cells believed to be spermatia. The dark-brown dumb-bell hyphae in association with the round perithecia-like bodies are responsible for the very dark appearance of the undersurface of the fungal colony.

The cultural characteristics of the fungal colony, in addition to the appearance of the conidia produced in culture, were similar to those of *Mycosphaerella fijiensis* described for the isolate from plantain. Based on these observations, *M. fijiensis* was suspected and consequently identified.

4.4. PATHOGENECITY STUDIES

4.4.1. PLANTAIN ISOLATE INOCULATED ON PLANTAIN

After 12-22 days (mean 18 days) of inoculating leaves, faint, minute reddish-brown specks, rarely visible to the naked eye were observed on the abaxial (under) leaf surface (Fig. 36A). Within days, the specks elongated and became clearly visible on the lower leaf surface than on the adaxial (upper surface) with their long axes parallel to the leaf veins. These specks referred to as first streaks were more densely aggregated, with some adjacent streaks coalesced (Fig. 36A). The streaks then turned dark-brown from reddish-brown (Fig. 36B) ;and consequently became clearly visible on the adaxial leaf surface, with majority of the streaks coalesced. These symptoms marked the second-streak stage. The dark-brown symptoms broaden with the infected portions heavily water-soaked especially during the early hours of the day. The water-soaking effect disappeared with time on the same day, which is characteristic of a first-spot stage (Fig. 36C.). Subsequently, 6-16 days (mean 11 days) after the appearance of the initial specks, the spot became depressed, marking the beginning of a second-spot stage. This was characterised by a dark-brown or black colour of the central portions of the spot with the immediate margins appearing very black (Fig. 37B). Immediately beyond this black margin was a yellow transition zone (Fig. 38).

The centre of the spot became dry within a further 7 days and appeared grey or buff-coloured. The spot was now surrounded by a narrow well-defined black margin

(Fig. 37A, 38) and between this black border and the healthy green tissue of the leaf was a bright yellow transitional zone. This marked the third or mature-spot stage.

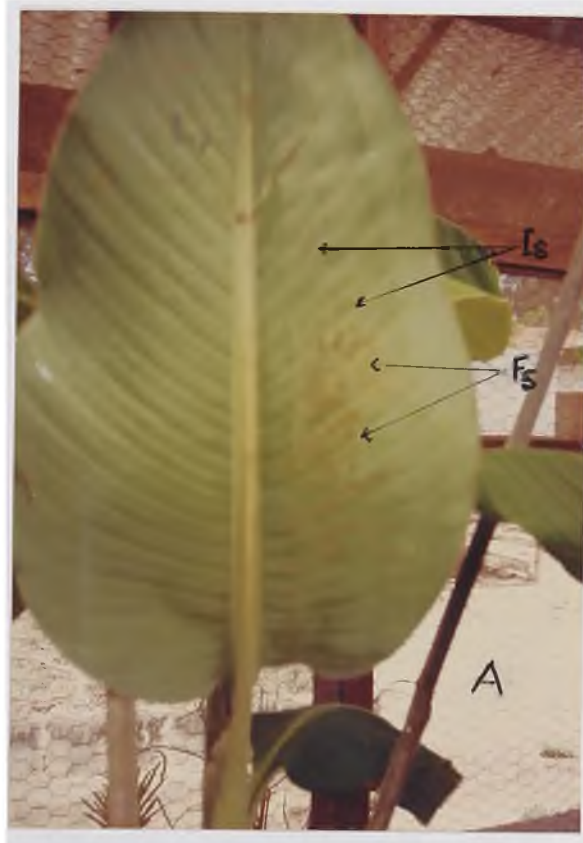
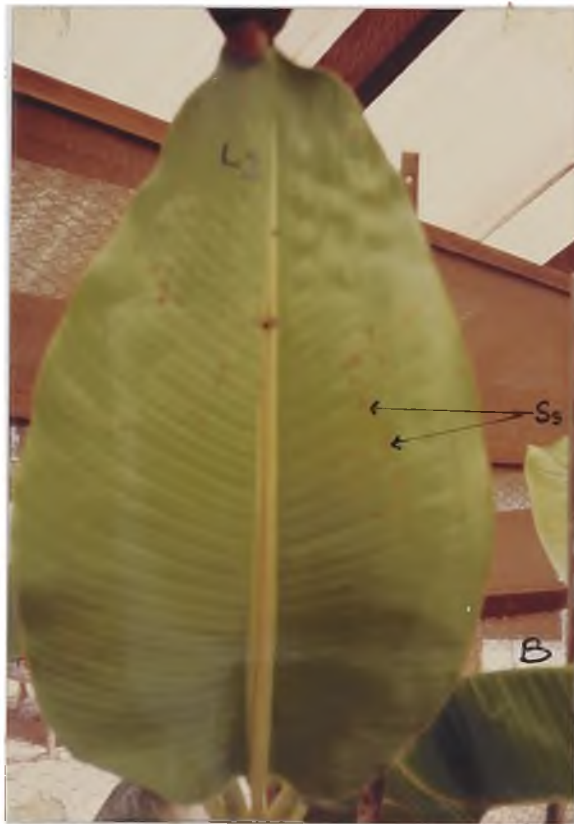


FIG. 36: (A,B,C) Different stages of black sigatoka symptoms on greenhouse-grown plantain leaves inoculated with conidia of *M. fijiensis* isolated from plantain.

A: - Initial specks (Is) and first - streak (Fs) symptoms.

Note: Other halves of leaves that were not inoculated serve as control treatments throughout this experiments.



B: - Second-streak (Ss) symptoms on abaxial leaf surface.

Note: Other halves of leaves that were not inoculated serve as control treatments throughout this experiments.



C: - First-spot stage symptoms (arrowed) on abaxial leaf surface.
Note: Other halves of leaves that were not inoculated serve as control treatments throughout this experiments.



FIG. 37: (A,B) Some stages of black sigatoka on greenhouse-grown plantain inoculated with *M. fijiensis* conidia isolated from plantain.

A:L₁, showing specks and streaks.

L₂ showing mature spot with grey centres.



Fig. 37. B - leaf showing second-spot symptoms. Note halo-margin.



FIG. 38. Array of inoculated plantains showing different stages of black sigatoka symptoms. L₁-second spot symptoms. Note black spot with halo-margin. L₂, L₃, L₄ showing mature spot symptoms with grey centres.

4.4.2. BANANA ISOLATE INOCULATED ON LOCAL TALL BANANA

Symptoms were similar to those found on plantain inoculated with conidia derived from plantain but with variations in the time intervals. Initial specks were evident between 20-31 days (mean 23 days) after inoculation of leaves (Fig. 39A). These specks became clearly visible and elongated on the abaxial leaf surface of the inoculated leaves, and densely aggregated, with adjacent streaks coalesced (Fig. 39B). These symptoms marked the first-streak stage. Streaks turned dark-brown from reddish-brown (Fig. 39C) within four days and became clearly visible on the adaxial leaf surface, with majority of the streaks coalesced. This stage is referred to as the second-streak stage.

The dark-brown portions of the leaf broaden and became heavily water-soaked especially during the early hours of the day. The water-soaking disappeared with time on the same day. This observation was similar to a first-spot stage. The spot became depressed 13 days after the appearance of the initial specks with the infected portion becoming dark-brown or black at the centre, with the immediate margins appearing black (Fig. 40A). Beyond this black margin was a yellow transition zone. The spot within 5 days on the average, became dry and appeared grey or buff-coloured. The spot was now surrounded by a narrow well-defined black margin (Fig. 40B), and between this black border and the healthy green tissue of the leaf was a bright yellow transitional zone. This was the final or mature spot stage of the disease (Fig. 40C).



FIG.39 (a,b,c) Symptoms on greenhouse-grown LTB, inoculated with isolate from Local Tall Banana.
A - initial speck symptoms (arrowed)



Fig.39 B - First-streak symptoms on abaxial leaf surface (arrowed).



C - Second-streak symptoms. Note the coalescing of streaks, with water soaking (W), which is not clearly visible in pictures.
Note the left half of leaves that serve as controls are without symptoms.

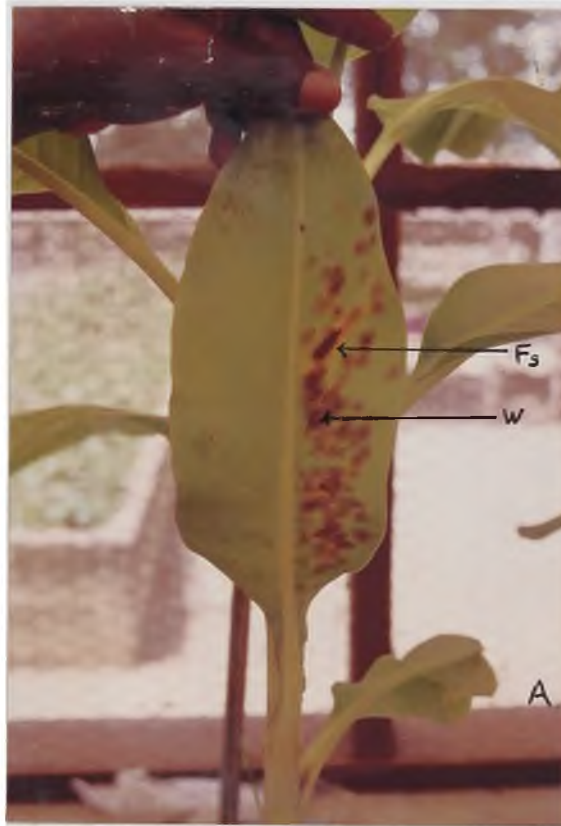


FIG. 40: (A,B,C,) Symptoms on greenhouse-grown LTB, inoculated with *M. fijiensis* isolate from Local Tall Banana.
A - Second streak symptoms with water-soaking (W) and first-spot symptom (Fs). Note the black spot with the forming halo-margin.



Fig. 40B - Second spot stage symptom. Note black spot with the immediate halo-margin.

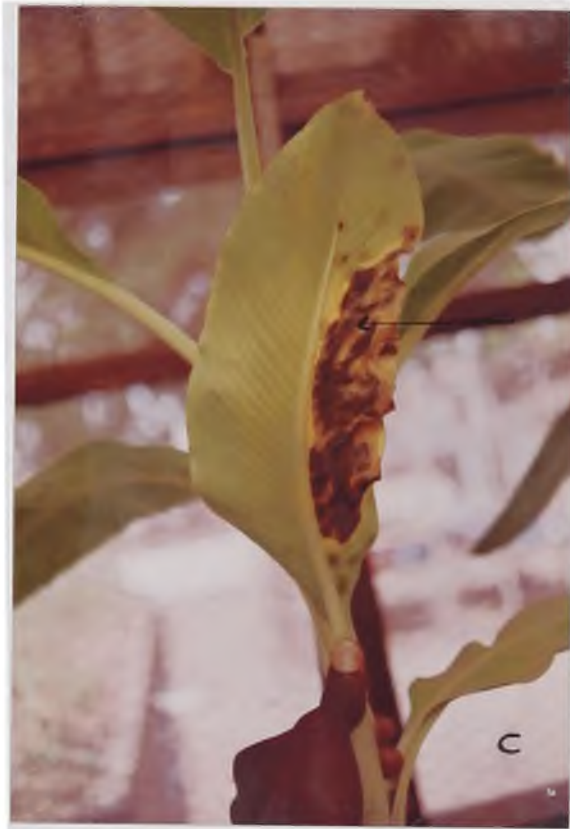


Fig. 40C - Mature or third-stage spot with grey centres (arrowed).
Note the left half of all leaves that serve as control are without symptoms.

4.5. CROSS-INFECTION STUDIES

4.5.1. PLANTAIN ISOLATE ON LTB

Symptom expression was similar to those observed in pathogenecity studies. There were however variations in the incubation period as well as other stages of the disease. Initial specks were observed 29 days on the average after inoculation and were evident only on the abaxial leaf surface (Fig. 41A). Symptoms passed through the first and second streak stages. Within 44 days (on the average) after inoculation, the symptoms entered the first-spot stage (Fig. 41B). Symptoms progressed through the second spot stage, (Fig. 42A) and 51 days after inoculation entered the third or mature spot stage (Fig. 42B and C). Mature spot stage was attained 22 days after the appearance of initial specks (Table 1).

4.5.2. ASCOSPORES FROM PLANTAIN INOCULATED ON LTB

Initial specks were evident 21 days after inoculation and symptoms passed through all the stages described above until mature spots were formed (Figs. 43 A,B,C).

Table 1: Time Course of Disease Progression on Inoculated Plants in the Greenhouse**A. PATHOGENICITY STUDIES**

TYPE OF INOCULATION	INCUBATION PERIOD (DAYS)		AVERAGE NUMBER OF DAYS TAKEN FOR MATURE SPOTS TO APPEAR FROM DAY OF INOCULATION	DAYS TAKEN FOR MATURE SPOTS TO APPEAR AFTER INCUBATION PERIOD
	RANGE	AVERAGE		
Plantain isolate on plantain	12-22	18	36	18
Banana isolate on LTB	20-31	23	31	8

B. CROSS-INFECTION STUDIES

Plantain isolate on LTB	25-36	29	51	22
Banana isolate on plantain	12-38	26	51	25
Plantain ascospores on LTB	-	21	-	-



FIGS. 41 (A,B): Black sigatoka symptoms on greenhouse grown Local Tall Banana inoculated with plantain isolate of *M. fijiensis*
A - initial specks (I_s) and first-streak (F_s) symptoms

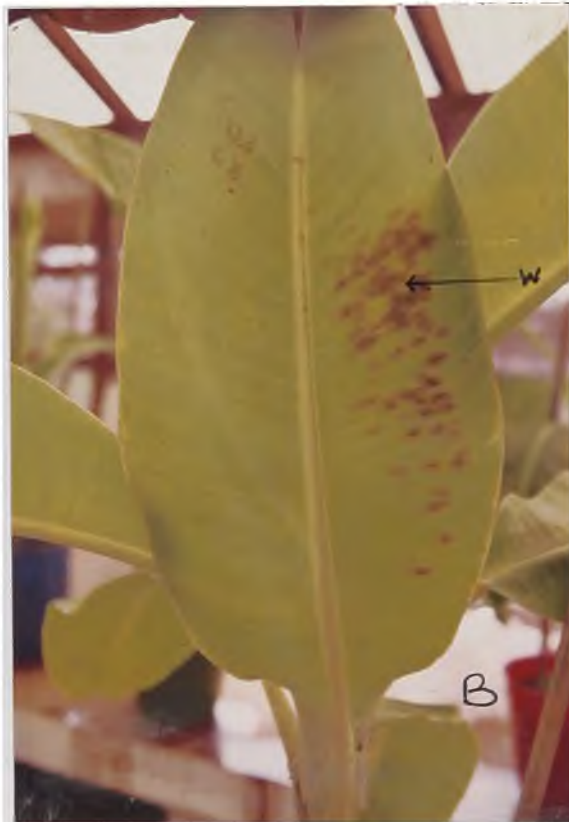


Fig. 41B - second-streak stage developing into first-spot stage symptoms.
Note the coalescing of streaks and water soaking (W) effect.



FIGS. 42.(A,B,C): Black sigatoka symptoms on greenhouse grown Local Tall Banana inoculated with plantain isolate of *M. fijiensis*.
A - Right (R), first-spot stage (Fs) and second spot (Ss) stage symptoms. Left (L) mature (third-spot) stage symptom. Note grey nature of symptom.



FIG. 42B: - Mature spots - Note grey nature of spot and the adjoining halo-margin.



FIG. 42C: - Mature spots of black sigatoka disease - Note grey nature of spot and the adjoining halo-margin.



FIGS. 43 (A,B,C) Black sigatoka symptoms on greenhouse grown Local Tall Banana inoculated with ascospores of *M. fijiensis* ejected from plantain.

FIG. 43A. - Disease specks (rusty spots) on abaxial leaf surface.



FIG. 43B. - Left (L), uninoculated leaf on LTB. Right (R) inoculated leaf showing second spot stage symptom. Note black spots with surrounding yellow leaf tissue.



FIG. 43C - Mature-spot stage symptoms.

Note grey centres (arrowed), with adjoining halo margin.

4.5.3. BANANA ISOLATE INOCULATED ON PLANTAIN

On the average, it took 26 days for initial specks to appear on inoculated leaves (Table 1). Symptoms passed through the various stages described for the pathogenicity studies until mature spots were formed 25 days after the appearance of initial specks (Fig. 45, 46).

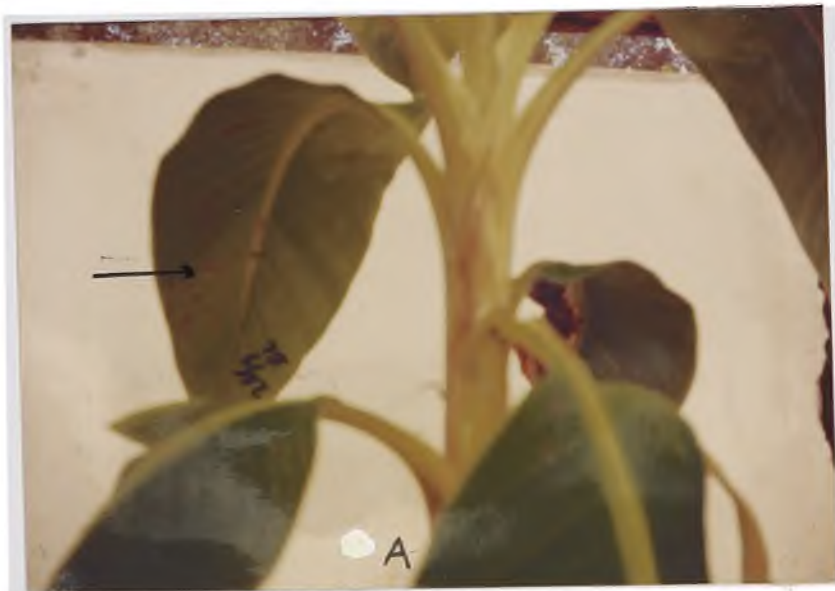


FIG.44 (A, B, C) : Black sigatoka symptoms on greenhouse grown plantain inoculated with banana isolate of *M fijiensis*

FIG. 44A. Second streak symptoms (arrowed) on abaxial leaf surface.



FIG.44B First-spot symptoms with water soaking effect (W) and second-spot symptoms on leaf. Note dark spot with adjoining halo-margin.

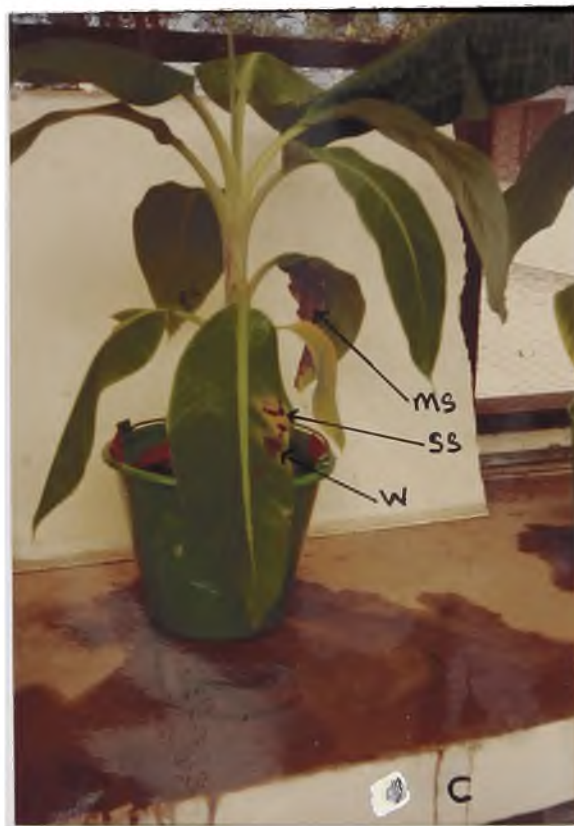


FIG. 44C - Leaves of plantain showing second-spot (SS) and mature-spot (MS) symptoms.



FIG. 45. Mature-spot (Ms) of black sigatoka symptoms on greenhouse grown plantain inoculated with banana isolate of *M. fijiensis*. Note the dried centre with adjoining yellow margin.

4.6. EFFECT OF ENVIRONMENTAL FACTORS ON DISEASE EXPRESSION

Inoculated plants left on the open greenhouse bench without incubation never developed any form of symptoms throughout the course of the experiment. This was a clear indication that high relative humidity was necessary for disease development.

4.7. CONTROL EXPERIMENTS

Uninoculated plants left in the open greenhouse in addition to the other uninoculated halves of all inoculated leaves that were incubated never developed black sigatoka symptoms. This has proven the absence of natural inoculum in the greenhouse.

DISCUSSION

FIELD SYMPTOMS

Leaf symptoms on plantain and banana in the field were similar with those described for black sigatoka by Meredith and Lawrence (1969) and later by Oduro et al. (1991). Symptoms on all greenhouse inoculated plants in this study were also similar to those described for black sigatoka and essentially passed through all the various stages described in literature (Meredith and Lawrence, 1969). The predominance of spotting at the leaf tips and margins in the field is due to pronounced water congestion within the leaf tissues along the margins especially during the night. This water congestion might have increased spore germination, infection and pathogen development within leaf tissues. Thus, based on leaf symptoms in the field and the greenhouse, black sigatoka was identified to be present on both plantain and the local tall banana in Ghana.

SINGLE CONIDIA ISOLATION

The single conidium isolation of the pathogen from infected plantain and banana leaves was made impossible as a result of the abundant spores of other associated fungi such as *Cordana musae* and *Chloridium* spp. This difficulty was complicated by the fact that, the conidia of *Mycosphaerella fijiensis* were seldom found in abundance but rather scattered on the leaf surface. This might be due to spore dispersal either by wind and/or by water. The concurrent presence of reddish-brown *Chloridium* speckles that resemble the first streaks symptoms of black sigatoka also impeded isolation by confusing the symptoms. The repeated washing of infected leaves under tap water could also have removed conidiophores and hence retarded subsequent conidia production on incubated leaves under bell jars. These difficulties accounted for the inability to isolate *M. fijiensis* by single spore isolation. The presence of the three fungal species, *M. fijiensis*, *Cordana musae* and *Chloridium* spp on both plantain and banana, and above all their presence on green leaf tissues might have contributed to the disease (Wardlaw, 1972). This complex might increase

disease severity or otherwise, hence the need for further investigations to identify their roles separately and when in combination with *M. fijiensis* in black sigatoka disease development.

INDUCED MYCELIAL GROWTH

Mycelial growth was not recorded from the cut edges of both infected plantain and banana leaves that were plated on both PDA and CDA. The inability in this case might be due to the very slow growth of the fungus coupled with scorching of the cut edges of the leaf discs, which might have led to the death of the pathogen in these sections. These notwithstanding, inexperience in this isolation technique might also have contributed to this failure.

ISOLATION BY ASCOSPORE DISCHARGE FROM INFECTED BANANA AND PLANTAIN LEAVES

Ascospore discharge from diseased banana and plantain leaves into sterile distilled water (SDW), PDA and CDA was found to be a successful method of isolating *Mycosphaerella fijiensis* Morelet in this study and as was reported by Stahel, (1937); and Meredith and Lawrence (1970); Vasquez et al, (1988); Natural, (1988); and Fouré, (1988).

The quick discharge of ascospores (15 minutes) from wet infected leaves into SDW and their subsequent early germination (1½ hours) in SDW as opposed to 12-18 hours on PDA and CDA might have a lot of epidemiological implications in the field. The early germination of ascospores in water might result in early disease establishment as the spore germ tubes can penetrate stomata and induce disease within a short period of time. It also emphasises the necessity of water for spore germination (Goos and Tschirch, 1963; Stover, 1980; Jacome and Schuh, 1992).

The role of *Leptosphaeria* spores ejected together with ascospores of *M. fijiensis* from both plantain and banana, though not investigated in this study needed further investigation. This is of importance since these spores also germinated very fast (30 minutes) and with multiple germ-tubes. As reported by Stahel (1937) for Yellow Sigatoka and by (Meredith and Lawrence, 1970) for black sigatoka, it seems this fungus association is a constant feature of both Yellow and Black sigatoka diseases.

CULTURAL CHARACTERISTICS OF BANANA AND PLANTAIN ISOLATES IN SDW AND ON PDA

The observed ascospore dimensions (13.8µm avg. by 3.2µm), appearance, septation for the plantain isolate and those for the banana isolate (13.9µm by 3.2µm avg.) agreed perfectly with each other in this study and lie within the ranges reported for *Mycosphaerella fijiensis* Morelet (Meredith and Lawrence, 1970; Natural, 1988). The bipolar nature of ascospore germination in SDW for both plantain and banana isolates was similar and compared with those reported for both *Mycosphaerella*

musicola and *M. fijiensis* on water agar and PDA (Leach, 1941, Meredith and Lawrence, 1970; Natural, 1988). The absence of branching and sporulation of both plantain and banana isolates in SDW as opposed to those on media during this study might be due to the absence of nutrients in SDW.

The time taken for ascospores to germinate (12-15 hours) on PDA for both isolates, and by multiple germtube development was in agreement with that found for *M. fijiensis* germinating on water agar within 24 hours (Vasquez et al, 1988). A further indication of the necessity of nutrient for the growth of the isolates. The longer time taken for ascospores to germinate on media compared to that in SDW gave an indication to the slow growth of the fungus on media in general as reported by Meredith and Lawrence (1970). This observation was substantiated by the finding that fungal colonies of both isolates in this study became visible to the naked eye on media only after 5 days as opposed to 2-3 days reported by (Meredith and Lawrence, 1970) and subsequently grew very slowly with a very compact nature as was reported by Stover, (1937), and Meredith and Lawrence, (1970).

The presence of dumb-bell-shaped cells in culture as being responsible for the dark nature of under surface of colonies characteristic for cultures of *Mycosphaerella fijiensis* and *M. musicola* (Meredith and Lawrence, 1970) were observed. The presence of dumb-bell-shaped cells as an indication of pathogenic isolates of *Cercospora* spp. (Stover, 1976) was also observed in this study for both isolates and later proven in pathogenicity studies in the green house. The absence of sporulation of both plantain and banana isolates on PDA in this study in contrast with other findings (Meredith and Lawrence, 1970; Natural, 1988) might be due to the fact that there might be different strains of the fungus in Ghana, and which cannot sporulate on PDA but rather on CDA. These speculations may be confirmed by the observation in this study that non-sporulating colonies transferred from PDA onto CDA produced typical *Cercospora*-type conidia whereas the vice versa did not. This anomaly in sporulation was also found to be consistent with other *Cercospora* spp that were found to show

difficulty in sporulation (Garman, 1920; Jenkins, 1930; Nagel, 1934; Calpouzos, 1954; Kilpatrick and Johnson, 1956).

The absence of any visual differences between the colonies of both isolates in this study coupled with the fact that they both did not sporulate on PDA gave another indication that they both might belong to the same *Mycosphaerella* sp., however no tentative identification could be done due to the absence of conidia on PDA.

Cultural Characteristics of Plantain and Banana Isolates on CDA

Fungal colonies of both the plantain and banana isolates on CDA were the same in every respect as on PDA; slow growth, hyaline hyphae changing to light brown, presence of dumb-bell-shaped hyphal cells, perithecia-like bodies with associated spermatia-like cells were similarly observed. Based on these, the two isolates appeared to be coming from the same *Mycosphaerella* sp.

However unlike on PDA, profuse sporulation of both plantain and banana isolates was observed on CDA within one week. This observation indicated the suitability of CDA for growth and sporulation of the fungal isolates, which is in contrast to the reported 3 weeks (Natural, 1988) and 5 days (Meredith and Lawrence, 1970) needed for sporulation on PDA. This is the first time in literature that CDA was used to culture either *M. musicola* or *M. fijiensis*.

Conidiophores with evident spore scars and septation in this study conformed to those described for *M. fijiensis* (Meredith and Lawrence, 1970) and those observed on infected leaf tissues from the field in this study.

In this study, the conidia in culture were found to be of the *Cercospora*-type. The thickened hilum which is reported to be characteristic for *M. fijiensis* and the most definitive distinguishing character between *M. musicola* and *M. fijiensis* pointed to this fact (Meredith and Lawrence, 1970; Jones and Mourichon, 1993).

Thus, based on the observation of infected leaf tissues under the microscope, the observation of fungal structures; conidiophores and conidia on infected leaves, *Mycosphaerella fijiensis* (Morelet) was suspected. In addition, based on the colony

appearance, conidiphores and conidia with thickened hilum produced on CDA, and which was found to be similar for both plantain and banana isolates in this study and which compared with those reported by Meredith and Lawrence (1970), *Paracercospora fijiensis* (Morelet) Deighton (anamorph of *Mycosphaerella fijiensis*) was identified as the causative organism for the plantain and Local tall banana leaf spot disease in Ghana.

The findings in this study have scientifically strengthened the report of Oduro et al. (1991) who, based on symptoms alone, reported that the new plantain disease in Ghana is black sigatoka with *M. fijiensis* Morelet as causal agent.

This study further proves that the symptoms found on the Local tall banana in Ghana is also black sigatoka caused by *M. fijiensis* Morelet.

Pathogenicity Studies

In those inoculations where the plantain isolate of *M. fijiensis* was inoculated on plantain and the banana isolate of *M. fijiensis* on Local tall banana in the greenhouse, typical black sigatoka symptoms were recorded on all leaves inoculated and incubated under polythene bags. These symptoms passed through the six stages reported for black sigatoka disease (Meredith and Lawrence, 1970).

The use of high inoculum concentrations as well as inoculating plants at an early stage did not affect symptom expression.

The symptom development in the greenhouse as depicted by photographs followed essentially the patterns described by Meredith and Lawrence (1970) for black Sigatoka.

Though differences existed between the incubation periods (Table 1), this was believed to be either due to the different genetic constitutions between plantain and banana, and/or the different times at which the experiments ran which could affect disease development.

The longer incubation period (23 days) observed when banana isolate was inoculated on the Local tall banana might be due to the period of time (December) in which the inoculations were carried out. This period also recorded the period of least Relative humidity of 35%. However the shorter period (8 days) taken for specks to develop into mature spots might be attributed to the high day temperatures and light intensity during this period. Light is reported to increase disease severity of black sigatoka.

The shorter incubation period (18 days avg.) observed when the plantain isolate of *M. fijiensis* was inoculated on plantain might be attributed to the seeming susceptibility of plantain, and secondly the favourable environmental conditions of high humidity prevailing in the greenhouse during this period of time. The longer period of 18 days taken for spots to develop from streaks on plantain might be due to the favourable environmental conditions at this time, thus making the plants grow more vigorously and enabled the fungus to grow favourably within the leaf. This

vigorous growth might have delayed the rate of cell necroses, thus delaying spot development.

Cross-Infection Studies

On the whole, the cross-inoculation studies in the greenhouse progressed through the different stages of symptom development for black sigatoka disease and was similar to those found during the pathogenicity studies above. Symptoms were first visible as reddish-brown specks on the under surface of all inoculated and incubated leaves and became visible on the adaxial leaf surface only at the second streak stage.

Incubation periods, though varied for the different inoculations (Table 1), might be due to the genetic differences existing among the different varieties.

The wider range for the incubation period, 25-36 days, (29 days on average) when the plantain isolate was inoculated on local tall banana could be due to environmental influence since majority of these inoculations were done in November which also recorded one of the lowest R.H. and high temperatures.

Control Treatments

The absolute absence of symptoms on the uninoculated controls was believed to be due to the unavailability of infective propagules of the pathogen in the open greenhouse environment coupled with the unfavourable environmental conditions such as absence of leaf wetness in the open greenhouse. This observation of the absence of spores in the air was further proven when it was observed that uninoculated leaves that were incubated under polythene bags never developed any form of symptoms.

Absence of symptoms on inoculated, but unincubated, leaves might be due to the death of the fungus on the leaves. Since the leaves were not incubated, the high humidity required for conidia to germinate was not produced, hence stomatal penetration was impossible. The overall observations on the control treatments in this

study agrees with those found by Goos and Tschirch (1963) on greenhouse-grown banana inoculated with *Cercospora musae* Zimm.

GENERAL:

Effect of environment (R.H) on symptom expression/ development

Environmental conditions such as temperature and relative humidity influence incubation period and subsequent disease development. This was evident in inoculations that were carried out when the greenhouse relative humidity was on the high side (87%). This condition which enabled the formation of water films on leaves, had the shortest incubation period of 12 days whereas those inoculations that were carried out when the relative humidity was low (35%) had longer incubation periods of 25-38 days. Further prove was given when there was absence of symptoms on plants left on the open greenhouse bench, without incubation in polythene bags. Other reported incubation periods for *M. fijiensis* on *Musa* spp in the greenhouse include 14 days on banana (Jacome and Schuh, 1992) and 22 days for some *Musa* spp (Vasquez et. al., 1988). Foure (1988) reported 14-24 days for ground mycelium of *M. fijiensis* on plantain whilst for ascospores, 10-32 days were reported. Though the incubation periods reported in this study varied from those reported elsewhere, they were both found to be comparable, and any differences might be due to the genetic differences among the varieties used as well as the different conditions under which the experiments were carried out. Other differences might also be attributed to different strains of *M. fijiensis* used in the inoculation.

The wide variations observed in the incubation periods and rate of disease development in this study might also be due to the activity of the fungus in the leaf after penetration. It is believed that the fungus remains in a latent state of varying duration within the leaf. Goose and Tschirch (1963), and Leach (1946) concluded that such latency could be correlated with anatomical features of the leaf and the number of infections per unit of leaf area. These in this particular case might be different for both plantain and banana.

The difference between disease symptoms on small plants from those on older plants (Simmonds, 1939) were similarly observed in this study as early disease symptoms initially appeared oval on three-month old plants.

It was generally observed that once the first symptoms appeared on susceptible inoculated plants, time for disease development to the mature spot stage varied. This might be due to the internal water relations of the plants which are more crucial than the external environmental conditions. Once the fungus is inside the leaf tissue, the external environmental conditions do not affect the activity of the fungus within the leaf and hence it is capable of inducing disease. (Wardlaw, 1961, Goose and Tschirch, 1963).

Difficulty was encountered in reisolating the fungus from infected plants in the greenhouse and this could be attributed to the method of incubation adopted.

The absence of misting equipment did not allow maintenance of water films on infected leaves for an appreciable length of time which is a necessary requirement for conidia production and perithecia maturation on infected leaf (Jacome and Schuh, 1992). Consequently, no conidia or ascospores were produced on the greenhouse inoculated plants in this study. Inoculated plants were left in the open greenhouse since it became difficult to enclose whole plants under polythene bags due to their large sizes.

It appeared from the greenhouse studies that in Ghana, plantain seemed to be more susceptible than banana to black Sigatoka disease since the shortest period of inoculation, 12 days (Table 1). were observed on plantain when inoculated with both the plantain and banana isolates of *M. fijiensis* isolated in this study. This observation further confirms the fact that the isolates infecting both plantain and banana came from the same species.

In general, the greenhouse inoculations revealed and established the fact that the isolates obtained from both plantain and banana are pathogenic to the respective plants from which they were isolated and also cross-inoculated and produced typical black sigatoka disease symptoms.

The observation in this study that ascospore discharge from wet infected leaves occurred in less than 15 minutes, and those from plantain infect the local tall banana is of much epidemiological importance.

Since ascospores could serve as the primary inocula it is recommended that control strategies should be that of initially removing and burning all leaves from both banana and plantain showing mature (stage six) spots and subsequently controlling the development of streaks to mature spots by fungicidal spray.

In Ghana, since control is mainly carried out on plantain to the neglect of the grooves of local tall bananas, this study has revealed that such control attempts will yield no long-term dividends since the pathogen on the local tall banana will build up and eventually attack plantain.

RECOMMENDATIONS

It is recommended that:

1. Further work be carried out to determine the major spore-type initiating infections in Ghana
2. Investigation of the role of *Cordana musae*, *Chloridium* spp and *Leptosphaeria* spp in disease severity in Ghana be initiated
3. Further research into any possible pathogenic variability of *M. fijiensis* in Ghana and its implication be carried out
4. Control strategies should be expanded to cover all local tall banana grooves in the country.

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Appendix 1Ascospore dimensions of Plantain *M. fijiensis* on CDA

<u>Length(μm)</u>		<u>Breadth (Diameter) (μm)</u>	
13.8	13.8	3.45	2.8
12.77	13.11	3.8	3.11
13.8	13.8	3.45	3.45
13.8	13.8	3.11	3.45
13.46	13.8	3.11	3.11
13.8	12.08	2.7	3.45
13.11	13.8	2.7	2.8
12.1	13.8	3.8	2.42
12.1	12.77	3.45	3.8
14.15	13.11	3.8	3.11
12.1	13.8	3.11	2.8
10.35	12.08	3.45	2.42
13.8		3.11	3.45
15.53		3.45	3.11
15.53		3.11	3.45
18.98		3.45	2.42
13.8		2.42	
10.7		3.45	
13.8		3.45	
13.11		3.45	
14.11		3.11	
16.25		3.11	
11.04		3.45	
15.53		3.45	
16.56		3.45	
14.15		3.45	
13.8		2.8	
10.35		3.11	
13.8		2.8	
12.42		2.42	
13.8		2.42	
13.46		3.45	
13.8		3.45	
13.8		3.45	
13.46		2.8	
13.8		2.8	
13.8		2.42	
12.08		3.45	
14.15		3.11	
12.77		3.11	
13.8		3.11	
13.8		2.8	
13.8		3.11	
14.15		2.8	
13.46		2.8	
-----		-----	
Average = 13.8		Average = 3.2	

APPENDIX 2Ascospore dimensions of banana isolate of *M. fijiensis* on CDA.

<u>Length(μm)</u>		<u>Breadth (Diameter) (μm)</u>	
14.08	14.8	2.5	3.86
14.77	16.53	3.9	3.11
15.11	16.53	2.57	2.85
14.77	13.8	3.9	3.9
15.8	14.35	3.42	2.9
15.8	14.2	3.0	3.9
13.8	14.85	2.5	3.75
12.08	10.8	3.8	2.9
15.8	10.8	3.9	3.9
15.8	16.21	3.75	2.9
13.8	16.11	2.85	3.75
15.11	16.7	3.85	2.5
14.8	13.46	3.85	3.45
16.46	14.8	2.8	2.5
15.15	14.8	3.8	2.5
13.8	14.78	2.11	2.45
13.8	13.8	3.8	2.45
14.8		3.7	3.2
14.78		2.5	3.2
14.15		3.9	
13.08		3.45	
13.8		2.5	
13.8		2.9	
15.8		3.9	
13.46		3.78	
13.8		3.5	
14.8		2.58	
16.42		2.58	
13.9		3.8	
13.08		3.16	
13.9		2.8	
12.35		3.45	
15.8		2.85	
14.15		3.85	
16.7		3.45	
15.53		3.48	
14.04		3.11	
16.7		2.86	
16.7		3.86	
15.11			
-----		-----	
Average =13.9		Average = 3.2	

APPENDIX 3Conidia lengths for plantain isolate of *M. fijiensis* on CDA (μm)

31.1	
108.7	
72.5	
84.5	
141.5	44.9
60.5	55.2
48.3	61.1
66.8	86.3
41.4	86.8
123.2	100.1
158.7	100.4
100.1	69.0
98.3	103.5
31.6	46.4
44.9	
100.1	
79.4	
80.0	
361.1	
69.0	
48.3	
88.0	
77.8	
96.6	
86.3	
96.6	
41.4	
103.5	
86.3	
96.6	
41.4	
103.5	
86.3	
86.3	
43.1	
34.5	
87.7	
110.4	
96.6	
56.4	

Average = 78.1

APPENDIX 4

Conidia lengths of banana isolate of *M. fijiensis* on CDA (μm).

59.0	86.3
44.01	69.0
86.3	65.0
158.9	72.5
82.8	75.9
72.5	77.6
82.8	75.9
50.1	63.8
138.0	100.1
100.1	
110.4	
31.0	
69.0	
62.1	
86.3	
38.0	
100.1	
113.9	
107.0	
93.2	
89.7	
103.5	
79.4	
44.9	
46.6	
60.4	
75.9	
104.2	
36.2	
69.0	
93.2	
62.1	
69.0	
72.5	
48.3	
148.4	
103.5	
86.3	
36.2	
65.6	
82.8	

Average 78.4

APPENDIX 5

Conidia breadths (diameters) (μm) for the plantain isolate of *M. fijiensis* on CDA.

<u>Tip</u>	<u>Broadest Point</u>	<u>Hilum</u>
1.76	3.45	2.76
2.1	3.45	2.42
2.1	3.8	1.73
2.1	3.45	2.2
2.42	3.45	3.45
2.42	3.8	3.11
3.11	3.8	3.45
3.11	4.14	2.76
2.42	3.45	3.11
2.42	3.8	1.73
2.1	3.45	2.42
2.76	4.49	3.45
3.45	3.8	3.11
1.73	3.45	3.11
2.1	3.8	3.11
2.42	3.45	3.45
2.76	3.8	3.11
2.76	3.45	1.73
2.1	3.45	2.1
2.42	3.45	2.1
2.1	3.45	3.11
1.73	3.45	3.11
2.73	3.45	2.42
2.76	3.8	2.42
3.11	3.45	1.73
2.42	3.45	2.42
2.76	3.45	2.1
3.45	3.45	2.76
2.10	3.45	2.42
1.73	3.45	3.11
2.1	3.45	2.42
2.1	3.45	2.76
2.1	3.8	1.73
2.1	3.45	2.42
2.42	3.45	2.1
2.1	3.8	1.73
2.1	3.45	2.1
2.1	3.45	2.42
2.42	3.45	1.73
2.42	3.45	2.1
-----	-----	-----
Average 2.4	Average = 3.6	Average = 2.5

APPENDIX 6

Conidia breadths (diameters) (μm). for the banana isolate of *M. fijiensis* on
CDA

<u>Tip</u>	<u>Broadest Point</u>	<u>Hilum</u>
3.1	4.49	3.1
3.1	3.8	2.73
3.42	3.45	3.42
2.42	3.8	3.1
3.11	3.45	1.73
3.11	3.8	2.1
3.42	3.45	2.42
2.42	3.45	1.73
3.1	3.45	2.76
2.76	3.45	2.42
3.45	3.45	3.11
1.63	3.8	2.42
2.2	3.8	2.76
2.42	3.45	2.1
2.76	3.45	2.42
2.76	3.45	1.73
3.1	3.45	3.45
3.42	3.45	3.11
2.1	3.45	1.73
2.73	3.45	2.1
1.73	3.45	2.1
2.76	3.45	3.11
2.76	3.8	2.42
3.45	3.45	2.42
2.76	3.45	3.11
2.42	3.45	3.11
3.11	3.45	3.11
2.1	3.45	3.45
1.73	3.45	2.42
2.1	3.8	1.73
2.1	3.45	3.11
2.42	3.45	2.76
2.1	3.8	3.45
2.1	3.8	3.11
2.1	4.14	3.45
2.42	3.45	2.2
2.1	3.8	1.73
2.42	3.45	2.42
2.1	3.45	2.76
	3.45	
----- Average 2.4	----- Average = 3.6	----- Average = 2.6

APPENDIX 7Conidiophore Dimensions of plantain isolate of *M. fijiensis* on CDA.

<u>Length</u> (μm)	<u>Breadth</u> (μm)	<u>Septation</u>
3.45	163.9	13
3.45	31.1	3
3.8	51.8	4
3.8	48.3	3
4.49	65.6	4
4.49	48.3	1
3.8	76.6	4
3.8	57.8	5
3.8	51.8	4
3.14	24.2	3
3.8	51.8	3
4.14	74.9	4
4.14	38.0	6
3.45	44.9	3
5.18	43.8	1
3.11	51.8	1
3.8	25.5	2
3.11	62.1	0
3.45	56.9	2
5.18	48.3	4
5.18	31.1	3
3.11	25.9	1
3.11	86.3	1
3.8	75.9	5
4.14	65.6	5
3.8	51.8	0
3.45	59.0	1
3.11	57.3	4
3.8	53.5	3
3.45	41.4	3
3.45	48.3	3
3.83		3
----- Average 3.8	----- Average = 54.7	----- Average = 3

APPENDIX 8Conidiophore dimensions of banana isolate of *M. fijiensis* on CDA

<u>Length(um)</u>	<u>Breadth(um)</u>	<u>Septation</u>
52.8	3.8	3
40.6	3.21	3
75.9	3.45	4
44.9	5.18	3
43.8	3.21	12
25.5	5.18	4
51.8	5.4	1
31.1	3.21	5
62.1	3.21	4
56.9	3.8	4
45.3	4.36	3
75.9	3.8	3
86.3	3.45	4
25.9	3.21	6
31.1	3.8	3
65.6	3.8	1
51.8	3.45	1
53.0	3.45	0
59.0	3.8	0
41.4	4.0	2
48.3	4.0	2
53.5	3.9	1
54.3	3.24	3
163.9	3.9	1
31.1	4.14	4
51.4	3.14	5
40.3	3.45	1
41.0	5.18	5
76.6	3.21	4
48.3	3.8	3
51.8	3.45	3
24.2	3.45	3
----- Average 54.9	----- Average = 3.8	----- Average = 3