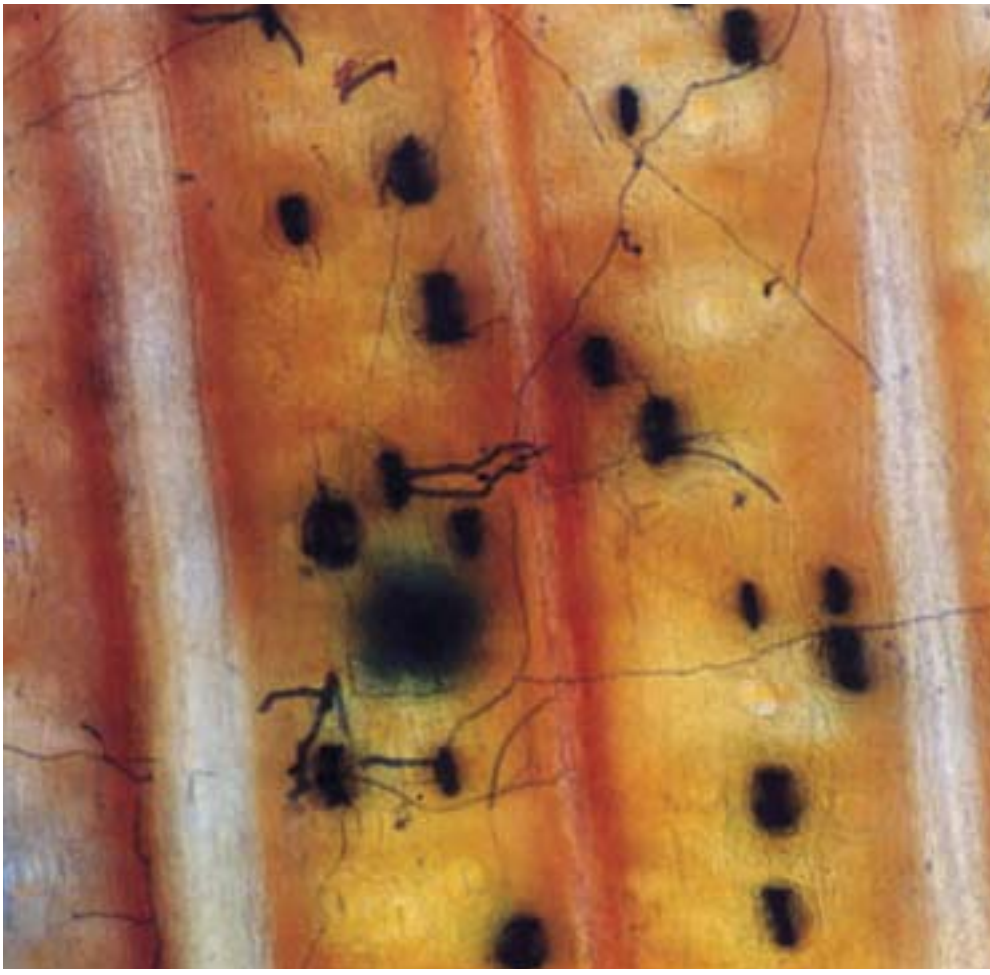
Mycosphaerella leaf spot diseases of bananas: present status and outlook

Proceedings of the 2nd International workshop on *Mycosphaerella* leaf spot diseases held in San José, Costa Rica, 20-23 May 2002

L. Jacome, P. Lepoivre, D. Marin, R. Ortiz, R. Romero and J.V. Escalant, editors



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- To coordinate, facilitate and support the production, collection and exchange of information and documentation related to banana and plantain.

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Cover illustration: Microscope photo of the causal agent of black leaf streak disease, *Paracercospora fijiensis* (photo: J. Carlier, CIRAD).

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Session 5 – Integrated disease management

Foreword

The rapid expansion in the 1980s of black leaf streak disease, which is caused by *Mycosphaerella fijiensis,* resulted in such damage to small producers that it encouraged INIBAP to organize the 1st International workshop on Sigatoka leaf spot diseases of Banana held in San José, Costa Rica in March 1989. Coming 13 years after this meeting, the 2nd International workshop on *Mycosphaerella* leaf spot diseases of bananas provided a timely opportunity to analyse the current situation regarding *Mycosphaerella* leaf spot diseases at the global level.

Black leaf streak disease has been spreading for more than 20 years and is now reported in most parts of the world. During that time, considerable research efforts have been expended to develop alternatives to allow small producers to continue producing banana and plantain. Efforts to create new resistant varieties were part of a broad spectrum of activities including classical and modern tools for genetic improvement. Studies are ongoing to develop a better understanding of host-pathogen interactions. The epidemiology, distribution and population structure of the *Mycosphaerella* pathogens are being investigated at national, regional and international levels. Research is also conducted to develop new methods to control the disease based on a rational use of fungicides.

All those who are involved and interested in the sustainability of the small banana and plantain producers know that the state of the research and the impact of *Mycospharella* leaf spot diseases have radically changed over the last decade. Sigatoka disease *(*caused by *Mycosphaerella musicola)* is still important in some parts of the world and a previously undescribed leaf spot disease, eumusae leaf spot disease *(*caused by *Mycosphaerella eumusae)*, has recently been discovered in southern and southeastern Asia.

By organizing this workshop, in collaboration with EARTH, CORBANA and CATIE and in the framework of PRO*MUSA*, INIBAP hopes to strengthen collaborations to ensure that the benefits of the research efforts reach the smallholders and to accelerate the creation of new varieties resistant to *Mycospharella* leaf spot diseases.

Editorial note

Throughout the proceedings, **black leaf streak disease,** also known as black Sigatoka, is used to refer to the disease caused by *Mycosphaerella fijiensis*, **Sigatoka disease**, also known as yellow Sigatoka, refers to the disease caused by *Mycosphaerella musicola*, and **eumusae leaf spot disease,** which was called *Septoria* leaf spot disease when first identified, refers to the disease caused by *Mycosphaerella eumusae*.

Introduction to workshop

Overview of progress and results since the first international workshop on *Mycosphaerella* leaf spot diseases of bananas in 1989

X. Mourichon

At the San José workshop, 1989, the main topics about banana pathogens were:

1) improvements in knowledge,

2) geographical distribution,

3) epidemiology,

4) mechanisms of host-parasite interactions,

5) sources of resistance and genetic improvement in *Musa* and

6) efficacy of new fungicides and their use.

This paper reviews and evaluates progress made in recent years, especially the major developments, and highlights topics in which efforts have perhaps not been sufficiently sustained.

In general, the main results obtained on *Mycosphaerella* leaf spot diseases over the past 10 years have been widely published in refereed journals together with several reviews. The last book edited by CABI (Jones 2000) is a very good synthesis of present knowledge of *Mycosphaerella* leaf spot diseases of bananas.

CIRAD-AMIS, Montpellier, France

Identification, taxonomy and diagnosis

In 1989, the discussions at the San José meeting concentrated on the availability of diagnostic methods for the pathogens causing Sigatoka disease, black leaf streak disease and black Sigatoka. The main observation was the risk of confusing the species when diagnosis was based on the observation of symptoms alone.

In situ or *in vitro* observation of the anamorph was considered to be the most reliable method to identify the diseases:

1) the anamorph *Cercospora musae*, now *Pseudocercospora musae*, for Sigatoka disease caused by *Mycosphaerella musicola*,

2) the anamorph *Cercospora fijiensis*, now *Paracercospora fijiensis*, for black leaf streak disease caused by *Mycosphaerella fijiensis*,

3) the two anamorphs for black Sigatoka which at the time were attributed to *Mycosphaerella fijiensis* var. *difformis* were in reality the anamorphs of *M. fijiensis* and *M. musicola*. The species was described only in Latin America.

There has been much work on these aspects in recent years, using the methods developed for molecular taxonomy on different populations of the pathogens (Carlier *et al.*, 1994, 2000; Johanson and Jeger, 1993; Johanson *et al.*, 1994).

Molecular markers are highly sensitive at discriminating between fungal species and have clarified the taxonomy of the banana pathogens.

Molecular markers have made it possible to distinguish clearly between *M. fijiensis* and *M. musicola* and to confirm that *M. fijiensis* and *M. fijiensis* var. *difformis* are synonymous (Carlier *et al.*, 1994).

More recently, markers have been used to identify a new species pathogenic to banana, *Mycosphaerella eumusae*. Initially, *M. eumusae* was thought to have a *Septoria* anamorph (Carlier *et al.*, 2000) therefore the new disease was called *Septoria* leaf spot disease. Additional detailed work on the morphotaxonomy attributed *M. eumusae* with a *Pseudocercospora* anamorph and the name was changed to eumusae leaf spot disease, ELSD (Crous and Mourichon, 2002).

Other methods based on serology are still being developed. The methods are intended above all to be sufficiently simple to diagnose the early stages of disease, for example, within the framework of preventive control measures with rational use of fungicides (Etienne *et al.*, 1995). More specific methods to identify pathogens as well as opportunist or non-pathogenic species of *Mycosphaerella* are also being developed.

The use of molecular markers has provided a great deal of information in recent years. In particular, molecular markers have made it possible to perform many analyses of genetic diversity, mainly in *M. fijiensis*.

A high level of genetic diversity has been demonstrated in *M. fijiensis* particularly in the genetic structure of populations at a macrogeographical scale (Carlier *et al.*, 1996). The diversity and geographical distribution of popu-

lations are mainly explained by genetic recombination in the teleomorph of *Mycosphaerella*. Genetic differences have also been observed at smaller scales e.g. at a field scale (Müller *et al*., 1997).

M. musicola also shows considerable intraspecific diversity, again involving sexual reproduction (Hayden *et al*., 2000, 2002).

There is universal agreement about the extent of genetic diversity in *M. fijiensis* and *M. musicola,* and the implications for the capacity of the two fungi to evolve. Thus, genetic diversity must be taken into account when devising strategies to improve disease resistance in banana. A knowledge of the genetic variation in different geographical populations of the pathogens is important for the management of resistance genes.

Geographical distribution of *Mycosphaerella*

Black leaf streak disease (BLSD) and Sigatoka disease (SD) are widespread in the main banana production zones, particularly Southeast Asia, which is the zone of origin of the pathogens, and the Pacific, and also in Latin America and Africa (Jones, 2000).

Since the San José workshop, the main change has been the rapid spread of BLSD in Latin America from Central America northwards to Mexico and Florida, USA, and southwards to Colombia, Peru, Venezuela, Bolivia and Brazil. BLSD has spread to the Caribbean e.g. Cuba, Jamaica, the Dominican Republic and Haiti, and the rest of the Caribbean arc is threatened.

BLSD has also spread to the western central and eastern parts of the African continent and, recently, to Madagascar. *M. fijiensis* is no longer confined to northern Australia and is a new constraint that has to be managed in other Australian commercial plantations.

Very little information is available on the geographical distribution of the new species, *M. eumusae*, cause of ELSD. The species appears to be centred in India but probably has a wider distribution and requires detailed study.

Finally, a fourth species, *M. musae*, causing speckle disease, is widespread throughout the world but generally causes little damage except in the sub-tropical areas of Australia and South Africa.

Epidemiology of *Mycosphaerella* leaf spot diseases

At the San José meeting, it was recognized that knowledge of the epidemiology of *Mycosphaerella* leaf spot diseases was weak. Just one recommendation regarding "the urgent need to concentrate efforts on a better understanding of the different epidemiological components" was made.

Nevertheless, important research (unpublished) had been done on various epidemiological aspects of BLSD in Latin America and Africa. The work was mainly on the effects of abiotic factors on the different phases of monocyclic infection by *M. fijiensis* i.e. infection processes, incubation period, rates of symptom development, reproduction and dispersal (Gauhl, 1994; Fouré, 1992; Rutter *et al*., 1998; Smith *et al*., 1997).

At the time, this work aimed to make the control strategies more scientific but the question is whether the results were sufficiently exploited and used.

Other biotic factors e.g. genetic host resistance also affect parts of the infection cycle (see: management of genetic resistance).

Host-pathogen interactions

In the past, host resistance was evaluated in the field under natural, and hence variable, pathogen pressure, and in environmental conditions that differed considerably between locations. Environmental factors can have major effects on the expression of resistance, especially partial resistance.

Variability was reduced by using tissue-cultured plants inoculated with different isolates of *Mycosphaerella*. The plants could be maintained in controlled environmental chambers but the system was criticised for such things as using host plants that were too young, which could give rise to resistant phenotypes that were not true-to-type and results that were not reproducible. A miniaturized technique was thus developed. Using leaf fragments kept under artificial survival conditions, made it possible to work with older leaves, to work under closely controlled environmental conditions, and to allow the different banana genotypes to express the type and level of resistance to *M. fijiensis* typical of those expressed in the field. Crucially, all stages of development in a monocyclic infection were represented in the miniaturised system. The method can be used to analyse the nature of host-parasite interactions and to study the variability in virulence and aggressiveness in different populations of the pathogens.

Research over the last 10 years has made use of various models: banana plants in natural conditions, young plants or leaves under artificial survival conditions, providing complementary data about the nature of compatible and incompatible interactions.

Host-pathogen interactions occur in nature as the following phenotypes: very or highly resistant banana cultivars and partially resistant bananas with resistance varying from very marked partial resistance to very susceptible. All banana germplasm can be classified by these types of behaviour (Fouré *et al.*, 2000). Only a few wild, and cultivated diploid and triploid *acuminata* are resistant to BLSD. High resistance in the triploid *acuminata* is present only in cultivars of the Ibota subgroup.

Phenotypes with different levels of partial resistance appear to be widely distributed among all diploid and triploid *acuminata* and *balbisiana* genotypes. Genome B seems to give higher levels of partial resistance.

The idea that the relationships between *Mycosphaerella* and banana were based on compatible and incompatible interactions was proposed at the San José workshop.

Compatible interactions refer to susceptible, or partially resistant bananas that display different degrees of partial resistance. *M. fijiensis* can complete its entire infection cycle under this type of interaction. It was also suggested that partial resistance might result from a constitutive polyphenolic compound.

The various cytological, ultrastructural and biochemical studies, and genetic analyses of different varieties of banana with different levels of partial resistance, clearly show that proanthocyanidins play a role in partial resistance. It is also clear that the compounds are not essential for the expression of partial resistance. Proanthocyanidin is probably involved in the rate of lesion elongation, but other factors may be involved at other stages of the infection monocycle and should be identified (Beveraggi *et al.*, 1995; Mourichon *et al.*, 2000).

Using a model under controlled conditions, it is fairly easy to dissect partial resistance and evaluate the importance of certain sequences of monocyclic infection, e.g. incubation period, rate of lesion development, effectiveness of infection, latent periods and different parameters of sexual and asexual sporulation. Recent studies suggest that the presence of several components of resistance act on different stages of the infection cycle. Depending on the banana cultivar, partial resistance may be the result of different mechanisms. Thus, similar expressions of partial resistance could depend on different genetic interactions, e.g. efficacy of infection or level of sexual reproduction. These possibilities should be taken into account by breeders, for whom partial resistance is a major objective.

Partial resistance in banana is important because it is considered to be more durable. However, the great diversity of pathogen populations and their capacity to evolve should be taken into account. Specific interactions between the pathogen and the host plant must be established for certain sequences of the infectious monocycle (Abadie *et al.*, 2001a, b). It is possible that some specific interactions may select for more aggressive pathotypes of *Mycosphaerella*. The result would be gradual erosion, rather than a sudden decrease, of partial resistance.

Incompatible interactions. Banana cultivars that are very resistant rapidly block progress of the fungus in the early stages of disease. At the San José workshop, it was suggested that such behaviour could be governed by an active defence mechanism.

Studies of host-parasite interactions using cytology, particularly at the ultrastructural scale, provided accurate images of the interactions that occur after inoculation. There is clear evidence of active mechanisms such as cell collapse occurring after penetration of stomata (Beveraggi *et al.,* 1995; Mourichon *et al.*, 2000). Similarly, hypersensitive reactions have been elicited, and necrosis induced experimentally by fungal compounds of high molecular weight.

Other research reported at the San José workshop demonstrated that phytotoxic compounds or toxins were released by *M. musicola* and *M. fijiensis*. This raised the question of the role of these compounds in the infection process. Breeders were interested in the use of toxic compounds in schemes for the early selection of bananas resistant to *M. fijiensis*, in particular.

During the last decade, a large number of phytotoxic compounds produced by *M. fijiensis* have been described in the literature, e.g. juglone which displays a high level of biological activity (Stierle *et al.*, 1991).

Several research projects have shown that such compounds are not primary determinants of the disease but are secondary determinants of pathogenicity (Harelimana *et al.*, 1997). The role of these compounds as agents in the selection of resistance, as originally considered, deserves discussion, in particular for studies on partial resistance.

Breeding for resistance to *Mycosphaerella* leaf spot diseases

At the San José meeting, it was stated that "little is known of the genetics and inheritance of resistance to Sigatoka diseases". Several strategies and programmes for genetic improvement were presented but these had been developed mainly for the control of Fusarium wilt.

A great deal of effort has been made over the past decade by several institutions that started programmes to breed for resistance to BLSD in dessert and cooking bananas. The priority of the breeding programmes has been to search for high levels of partial resistance, which is considered to be more durable in the presence of diverse and evolving populations of a pathogen. In some breeding programmes, molecular marker–assisted selection was developed to introduce resistance. Several partially resistant hybrids were produced and tested in multi-site setups such as INIBAP's International *Musa* testing programme. Some hybrids survived the validation stage and were distributed more widely.

Other breeding approaches were developed using biotechnology, and in particular the production of transgenic plants using genes coding for antifungal proteins (AFPs) (see session 4: R. Swennen). However, this approach has the disadvantage of conferring monogenic resistance and is considered unstable in the presence of diverse populations of *Mycosphaerella* species. Nevertheless, the strategy deserves further study. For example, introducing specific resistance genes in bananas that possess a high level of partial resistance might be an attractive approach.

Control strategies

In San José, there was much discussion about the potential of new fungicides, rational ways of using them, the advantages of forecasting systems, and the management of resistance to fungicides. It was emphasized that effective and rational control required a greater knowledge of different aspects of the epidemiology of the pathogens.

It is generally agreed that this theme is probably the one which received the least attention and hence could still produce important results. The use of fungicides remains the strategy by which other strategies are compared. In the past, the selection pressure by different active ingredients has given rise to the disastrous situation where fungicide-resistant pathotypes are continuously selected. This strategy is no longer acceptable in a society increasingly concerned about the environment (Romero, 2000).

In future, the aim will be to propose alternatives to chemical control but without excluding them completely. Integrated control strategies are needed that combine several different methods of control, methods which individually are only partially effective. Integrated control strategies would have to be adapted to the different farming systems of banana production on a large scale and production of plantains and cooking bananas on smallholdings where rational chemical control is difficult.

1) Chemical control can still be considered, provided that its use is strictly limited. Forecasting methods should be improved or adapted to different environmental conditions.

2) Control measures based on cultural practices are known to affect inoculum pressure in the field e.g. leaf removal, methods of irrigation, management of planting density. There is potential for the information to be used more effectively.

3) The use of genetic resistance is important for the future. With the production of resistant varieties as an objective, it is necessary to consider strategies to manage resistance, in order to maximize the durability of resistance.

The key link between these three aspects is the need to obtain more information about the epidemiology of these pathogens, to make better use of what has been achieved in the past and to propose new lines of research into qualitative and quantitative aspects of epidemiology. A particular effort in modelling is expected to take into account the dynamics of pathogen populations.

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Session 1

Impact of *Mycosphaerella* leaf spot diseases of bananas

Introduction

The spread, detection and impact of black leaf streak disease and other *Mycosphaerella* species in the 1990s

R. A. Romero

Abstract
By the end of the 1980s, black leaf streak disease caused by *Mycosphaerella fijiensis* was present in all continents where bananas or plantains were grown, although distribution in some regions was limited to a few countries. In this presentation, the spread of the disease during the 1990s in several countries and regions, and the important socio-economic consequences are discussed. From 1990 to 1999, new records of black leaf streak disease were reported from six countries in Africa, eight in Asia, eight in Latin America and the Caribbean, and one from Australasia/Oceania. *M. fijiensis* has also spread within countries to ecological niches that were previously occupied by *M. musicola*, the causal agent of Sigatoka disease, thus threatening the survival of this pathogen. This presentation also discusses the methods, developed in the last decade, to identify the species of *Mycosphaerella* that cause leaf spot diseases in banana. The methods were used to confirm the synonymy of *M. fijiensis* var. *difformis* and *M. fijiensis,* and have provided the opportunity to study the genetic diversity of pathogen populations among isolates from different geographical regions.

Resumen – Propagación, detección e impacto de la Sigatoka negra y otras enfermedades foliares causadas por *Mycosphaerella* de bananos en la década de los 90
Al finales de la década de los 80, la Sigatoka negra, causada por *Mycosphaerella fijiensis,* ya se encontraba presente en todos los continentes donde se cultivan bananos y plátanos, pero su distribución en algunas regiones estaba limitada a unos pocos países. En esta presentación, se discutirá brevemente la propagación de la enfermedad durante los 90 abarcando varios países y regiones, y su impacto socioeconómico. De 1990 a 1999, se reportaron nuevos registros de la enfermedad desde seis países en Africa, ocho en Asia, ocho en América Latina y el Caribe, y solo un informe de un país en Australasia/Oceania. *M. fijiensis* también ha estado progresando en los países para llegar a los nichos ecológicos que anteriormente estaban ocupados solo por

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M. musicola, agente causal de la Sigatoka amarilla, amenazando la supervivencia de este patógeno. La presentación también discutirá el desarrollo de los métodos más precisos para identificar las diferentes especies de *Mycosphaerella* que causan las enfermedades de las manchas foliares en banano en la última década, que han mejorado nuestra habilidad de detectar el patógeno. Los mismos métodos permitieron confirmar la sinonimia entre *M. fijiensis* var. *difformis* y *M. fijiensis,* así como el estudio de la diversidad genética de las poblaciones del patógeno entre los aislados de diferentes regiones geográficas.

Résumé – La propagation, la détection et l'impact de la maladie des raies noires et d'autres espèces de *Mycosphaerella* dans les années 1990

A la fin des années 1980, la maladie des raies noires, causée par *Mycosphaerella fijiensis,* était présente sur tous les continents où les bananes et les plantains étaient cultivés, bien que dans certaines régions sa distribution était limitée à quelques pays seulement. Dans cet exposé, nous présenterons la propagation de la maladie dans plusieurs pays et régions dans les années 1990 ainsi que les prinicipales répercutions socio-économiques. Entre 1990 et 1999, de nouveaux cas de maladie des raies noires ont été enregistrés dans six pays d'Afrique, huit d'Asie, huit d'Amérique latine et des Caraïbes et un d'Australasie/Océanie. Dans certains pays, *M. fijiensis,* s'est également répandu dans les niches écologiques occupées au préalable par *M. musicola*, l'agent provoquant la maladie de Sigatoka, mettant ainsi en danger la survie de ce pathogène. Nous présenterons également les méthodes développées dans la dernière décennie qui permettent d'identifier les espèces responsables des maladies foliaires causées par les *Mycosphaerella* chez la banane. Ces méthodes ont été utilisées afin de confirmer la synonymie entre *M. fijiensis* var. *difformis* et *M. fijiensis,* et ont permis l'étude de la diversité génétique des populations de pathogènes parmi des isolats de différentes régions géographiques.

Introduction

This paper describes the most important events that have characterized the situation of *Mycosphaerella* leaf spot diseases in the decade 1990-2000. Emphasis is given to black leaf streak disease because of its greater importance in comparison with other *Mycosphaerella* leaf spot diseases. Black leaf streak disease continued to spread to new areas and remains a threat to other countries and regions. From the end of the 1980s to 1999, black leaf streak disease was newly reported in six countries in Africa, eight in Asia, eight in Latin America and in one location in Australasia/Oceania. In America, the French West Indies and the Windward Islands, two regions that produce bananas for export are threatened by black leaf streak disease.

In the near future, it is possible that black leaf streak disease may attain a distribution similar to that of Sigatoka disease caused by *M. musicola*. Details of the spread and the distribution of *Mycosphaerella* leaf spot diseases are described by Pasberg-Gauhl *et al.* (2000) and as updated by D. Jones elsewhere in this volume. The continued spread of black leaf streak disease poses two questions 1) are there preventative measures that could delay or prevent further spread of the disease? and 2) are those countries that are threatened by the disease taking measures to prevent the entry of the pathogen?

During the last decade there have been considerable advances in the development of precise techniques to detect *Mycosphaerella* spp. pathogens (Carlier *et al.*, 1994; Johanson and Jeger, 1993a, 1993b). However, there are few examples where these

molecular methods are being used to prevent or delay the spread of these pathogens to new areas. Little is known about the integration of these methods with strategies to prevent the dissemination of the diseases caused by *Mycosphaerella* spp. A possible limitation to the use of molecular methods is a lack of adequate infrastructure and trained personnel in developing countries.

The arrival of black leaf streak disease to a new area has resulted in the replacement of Sigatoka disease as the predominant disease of bananas and plantains. The ability of *M. fijiensis* to displace *M. musicola* has not been adequately studied. However, it is known that *M. fijiensis* has several biological characteristics that make it more competitive than *M. musicola*, e.g. greater ascospore production, more sexual cycles a year, and a higher rate of colonization of host tissue (Stover, 1980; Mouliom Pefoura *et al*., 1996). Little information is available about differences in pathogenicity between the two species.

The replacement of *M. musicola* by *M. fijiensis*, i.e. the elimination of one species by another in a short time, is interesting from an ecological and evolutionary point of view. Unfortunately, there are no studies that show whether *M. musicola* has in fact disappeared. It is possible that *M. musicola* coexists with *M. fijiensis* at low frequencies that are difficult to detect. If both pathogens survive in mixed populations, with *M. musicola* at a low frequency and *M. fijiensis* as the predominant species, it would be preferable to refer to a complex of *Mycosphaerella* leaf spot diseases rather than to black leaf streak disease. Breeding strategies for resistance would need to take this possibility into consideration because resistance to *M. fijiensis* does not necessarily imply resistance to *M. musicola*. Similarly, resistance genes incorporated by genetic engineering would need to be evaluated against both pathogens. Current molecular techniques may be able to clarify whether *M. musicola* survives at low frequencies with *M. fijiensis*.

Reports describe how *M. musicola* is better adapted than *M. fijiensis* to low temperatures, and how *M. musicola* is prevalent at higher altitudes where temperatures are cooler (Mouliom Pefoura *et al*., 1996; Romero and Gauhl, 1988; Tapia, 1993). However, over the years, *M. fijiensis* has been replacing *M. musicola* at higher altitudes in Costa Rica (Gauhl *et al*., 2000) suggesting that *M. fijiensis* may be adapting to the environment. Plantains and other cooking bananas commonly grown at higher altitudes, sometimes in combination with coffee, can be severely affected due to the greater aggressiveness of black leaf streak disease in comparison with Sigatoka disease.

The socio-economic impact of black leaf streak disease has continued to increase as the pathogen reaches new areas. The impact has also increased as the disease becomes more difficult to control because of increased resistance of the pathogen to new fungicides. Thus, fungicide resistance is increasingly an important constraint to control black leaf streak disease in plantations dedicated to the export market.

Recently *M. eumusae*, a new species pathogenic to bananas, has been described (Anon., 1995; Carlier *et al*., 2000). Little is known about the biology and epidemiology of this species, and hence it is not possible to evaluate whether or not it represents a threat to small-scale and large–scale banana production. Research is urgently needed to characterize the pathogenicity and aggressiveness of *M. eumusa*. In particular, studies are needed on its competitive ability with respect to *M. musicola*

and *M. fijiensis* to determine the potential of *M. eumusae* as a pathogen of bananas and other *Musa* genotypes.

During the course of this workshop, we will have the opportunity to examine how these events have taken place or are currently underway. We may be able to learn from the difficulties and progress on technical assistance and research and hence increase our ability to improve the control of these diseases in the future.

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The distribution and importance of the *Mycosphaerella* leaf spot diseases of banana

D. R. Jones

Abstract

The three main fungal leaf spot pathogens of banana are *Mycosphaerella musicola*, *M. fijiensis* and *M. eumusae*. All result in serious economic damage to cultivars in the Cavendish subgroup grown for local consumption and export. *M. musicola* was the first foliar pathogen to be identified as a major problem. Its spread from the Southeast Asian/Pacific region, where it was established by the 1920s, to North and South America in the 1930s, resulted in widespread disruption to the export trade. Since the 1960s, *M. musicola* has been steadily and largely replaced worldwide by *M. fijiensis*. This pathogen is more damaging to Cavendish cultivars than *M. musicola* and attacks a wider range of banana clones. It was first recognised in the Pacific region and has since become a major problem in most banana-growing areas. The susceptibility of plantain and other subsistence banana types is of special concern in Africa. By the late 1980s, reports in the scientific literature of records of *M. fijiensis* and *M. musicola* in Asia led to some confusion as to the true distribution of the two pathogens in this region. Many specimens collected in the region were identified as *M. eumusae*, a new leaf spot pathogen of banana. This fungus was observed damaging Cavendish cultivars and plantain. Most records for *M. eumusae* have been from Asia but the pathogen has also been found on islands in the Indian Ocean and in West Africa. Because of the similarity of symptoms caused by *M. musicola*, *M. fijiensis* and *M. eumusae*, it is proposed that the diseases caused by these three fungi be known collectively as *Mycosphaerella* leaf spot diseases.

Resumen – Distribución e importancia de las enfermedades de las manchas foliares causadas por *Mycosphaerella* en banano

Los tres principales patógenos fungosos de las manchas foliares del banano son *Mycosphaerella musicola*, *M. fijiensis* y *M. eumusae*. Todos ellos producen serios daños económicos a los cultivares en el subgrupo Cavendish cultivados para el consumo local y para la exportación. *M. musicola* fue el primer patógeno foliar identificado como el principal problema en la región productora de bananos de América Latina y el Caribe. Se propagó de la región de Sudeste asiático y el Pacífico, donde se había establecido allá por la década de los 20, al Nuevo Mundo en la década de los 30, resultando en una quiebra del comercio de exportación. Desde los años 60, *M. musicola* fue reemplazada firme y extensamente en todo el mundo por *M. fijiensis*. Este patógeno es más dañino para los cultivares

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Cavendish que *M. musicola* y ataca un rango más amplio de cultivares. Por primera vez esta enfermedad fue reconocida en el Pacífico. Actualmente, *M. fijiensis* es uno de los principales problemas en la mayoría de las áreas productoras de banano y ha reemplazado a *M. musicola* como patógeno dominante de la mancha foliar. La susceptibilidad del plátano y otros tipos de bananos de subsistencia representa la principal preocupación en Africa. A finales de la década de los 80, los informes encontrados en la literatura científica de los registros de *M. fijiensis* y *M. musicola* en el Sudeste de Asia llevaron a algunas confusiones en cuanto a la verdadera distribución de los dos patógenos en esta región. Muchos especímenes recolectados en la región fueron identificados como *M. eumusae*, un nuevo patógeno de la mancha foliar del banano. Este hongo se observó como dañino para los cultivares Cavendish y plátano. Aunque hasta la fecha la mayoría de los registros sobre la *M. eumusae* provienen de las regiones de Sudeste y Sur de Asia, el patógeno también fue encontrado en las islas del Océano Indico y en Africa Occidental. Debido a la similitud de los síntomas causados por *M. musicola*, *M. fijiensis* y *M. eumusae*, se sugiere que las enfermedades causadas por estos tres hongos se denominen manchas foliares causadas por *Mycosphaerella*.

Résumé – La distribution et l'importance des maladies foliaires causées par les *Mycosphaerella*

Les trois principaux pathogènes responsables des maladies foliaires de la banane sont *Mycosphaerella musicola*, *M. fijiensis* et *M. eumusae*. Tous trois provoquent de sérieux dégâts économiques aux cultivars du sous-groupe Cavendish, cultivés pour la consommation locale et l'exportation. *M. musicola* a été le premier pathogène de *Mycosphaerella* a être identifié provoquant des problèmes majeurs. Sa propagation de l'Asie du Sud-Est/région du Pacifique, où il était établi dans les années 1920, à l'Amérique du Nord et du Sud dans les années 1930, a sérieusement affecté le commerce international. Depuis les années 1960, *M. musicola* a été graduellement et largement remplacé à travers le monde par *M. fijiensis*. Ce pathogène cause plus de dommages aux Cavendish que *M. musicola* et attaque un spectre plus large de cultivars de bananiers. Il a d'abord été identifié dans la région du Pacifique avant de devenir un problème majeur dans la plupart des zones cultivées de bananes. La sensibilité du plantain et d'autres types de bananes servant d'aliment de base est particulièrement préoccupante en ce qui a trait à l'Afrique. A la fin des années 1980, les études rapportant la présence de *M. fijiensis* et de *M. musicola* en Asie, ont prêté à confusion quant à la véritable distribution des deux pathogènes dans cette région. De nombreux échantillons récoltés dans la région ont été identifiés comme étant *M. eumusae*, un nouveau pathogène responsable d'une maladie foliaire des bananiers. Il a été observé que ce champignon attaquait les Cavendish et le plantain. C'est en Asie que la majorité des observations de *M. eumusae* ont été faites mais ce pathogène a également été trouvé dans des îles de l'Océan Indien et en Afrique de l'Ouest. Du fait des symptômes similaires causés par *M. musicola*, *M. fijiensis* et *M. eumusae*, nous proposons que les pathologies causées par ces trois champignons soient connues collectivement en tant que maladies foliaires causées par les *Mycosphaerella*.

Introduction

The most serious leaf spot diseases of banana are caused by three species of *Mycosphaerella*. All three were discovered and became important constraints to commercial production in the 20th century. One has long since reached the limits of its distribution; a second is now approaching the limits of its distribution and a third is possibly only now beginning its global spread. The evidence suggests that all three may have arisen in the Southeast Asian/Australasian region, which is the centre of origin of *Musa* species and also the centre of evolution of cultivated banana (Jones, 2000). Some accessions of *M. acuminata* ssp. *banksii*, which is a wild diploid banana that has contributed genetic components to most edible banana

clones (Carreel, 1995), are known to be susceptible to at least two of the *Mycosphaerella* species causing leaf spot (Carlier *et al*., 2000a) and so co-evolution is a strong possibility.

It is not clear why the *Mycosphaerella* leaf spot pathogens were not disseminated during the first movements of banana planting material out of the Southeast Asian/Australasian region, as they could have been carried as infections of scale leaf tissue associated with sword suckers (Stover, 1978). Possibly, the fungi were not widespread pathogens on subspecies of *M. acuminata* or cultivated banana. Alternatively, the fungi, which initially were saprophytes surviving by colonizing dead leaf tissue, may have evolved to become pathogenic quite recently. Stover (1978) speculated that *M. fijiensis* might have evolved on a susceptible wild diploid and then spread to edible cultivars. The histories of the three *Mycosphaerella* fungi that continue to cause so much damage to cultivated banana are contrasted and compared in chronological order of discovery in the following text.

Mycosphaerella musicola, the cause of Sigatoka disease

First records

Mycosphaerella musicola was the first leaf pathogen to become a serious problem on commercial banana plantations. The fungus, originally named *Cercospora musae* from its imperfect stage, was first described as a pathogen of banana on the island of Java in Indonesia at the beginning of the 20th century (Zimmermann, 1902). However, it wasn't until 10 years later in Fiji that it became prominent as the cause of an important disease. The pathogen was first found in Fiji in the Sigatoka (pronounced 'Singatoka') Valley on the main island Viti Levu and quickly became an important constraint to production (Philpott and Knowles, 1913; Massee, 1914). The disease became known as Sigatoka disease, and more recently as yellow Sigatoka, to distinguish it from the disease caused by *M. fijiensis,* which is widely known as black leaf streak disease or black Sigatoka.

Global spread

The subsequent spread of *M. musicola*, as it was called after the perfect stage was discovered (Leach, 1941), around the world to practically all banana-growing regions is well documented (Meredith, 1970) (Table 1). The chronological sequence of disease occurrences led to speculation that ascopores borne on high altitude wind currents may have been responsible for the intercontinental dissemination of the pathogen from Australia to Africa and Central America (Stover, 1962). However, the latest information on survivability and movement of windblown ascospores, which are the spores implicated in long distance spread, indicate that this hypothesis is unlikely (Parnell *et al*., 1998). Stover (1980) changed his opinion on the distances ascospores of *Mycosphaerella* pathogens could spread by stating that wind dispersal of *M. fijiensis* from small areas of infection to new areas was probably slight when distances exceeded 50 km. Natural ultraviolet radiation, which would be a factor limiting high-altitude dispersal on clear days, kills ascospores of *M. fijiensis* within

Table 1. Year Sigatoka disease was first reported in a given country until 1970 (modified from information published in Table 2 of Meredith,1970).

Region/Country	Year
Australasia/Oceania	
Fiji	1912
Australia (Queensland)	1924
Australia (New South Wales)	1927
Solomon Islands	1946
Papua New Guinea	1951
New Caledonia	1951
Indonesia (Irian Jaya)	1953
Norfolk Island	1954
Wallis Island	1954
USA (Hawaii)	1958
Samoa	1961
American Samoa	1961
French Polynesia	1962
Tonga	1965
Asia	
Indonesia (Java)	1902
Sri Lanka	1919
Philippines	1921
Malaysia (Peninsula)	1933
China	1936
India (Assam)	1946
Taiwan	1953
Malaysia (Sabah and Sarawak)	1959
Cambodia	1960
Thailand	1962
Hong Kong	1966
Vietnam	1966
Laos	1967
Brunei	1968
Latin America/Caribbean	
Guadeloupe	1932 ?
Surinam	1933
Trinidad	1933
Guyana	1935
Honduras	1935
Jamaica	<1936
Belize	1936
Grenada	1936
Martinique	1936
Mexico	1936
Colombia	1937
Costa Rica	1937
Dominican Republic	1937
Guatemala	1937
Haiti	1937
Nicaragua	1937
Panama	1937
St. Vincent	1937
Venezuela	1937-41
Cuba	1938
Dominica	1938
Puerto Rico	1938-39
St. Lucia	1938
Brazil	1944
El Salvador	1944
Peru	1946
Ecuador	1952
Montserrat	1955
USA (Florida)	1955
Bolivia	1968
Africa	
Uganda	1938
Tanzania (mainland and possibly Zanzibar)	1939
Kenya	?
Cameroon	1941
Congo	1948
Mozambique	1948
Guinea	1952
Nigeria	?
South Africa	1954
Zimbabwe	1954
Ghana	1955
Malawi	1955
Sierra Leone	1956
Madagascar	1957
Côte d'Ivoire	1959
Nigeria	?
Mauritius	1960
Angola	1962
Somali Republic	1962
Zambia	1966

?: Pathogen recorded as present, but uncertainty over year of first detection.

Note: Meredith 1970 indicates that there is a possibility that some reports of Sigatoka disease in the Pacific area (such as in Hawaii in 1958) could have been black leaf streak disease. Sigatoka disease has still not been reported in the Canary Islands, Egypt and Israel banana-growing areas. The dry summer climate in these countries may not be conducive for the establishment of the disease.

6 hours (Parnell *et al*., 1998). Ascospores may not spread more than a one or two hundred kilometres and then only in strong winds and heavy cloud, or at night. In summary, many pathologists now believe that airborne ascospores of *Mycosphaerella* leaf spot pathogens spread disease between growing area within countries and between countries, but that airborne spread between continents is unlikely.

Movement between continents and between isolated countries, such as those found in the Pacific, is probably the result of the transfer of diseased material by humans. The appearance of the disease in Australia in the mid-1920s may have been due to the same movements of planting material from the South Pacific that are believed to have introduced bunchy top disease (Magee, 1953). Another possibility is that diseased banana leaves may have been used as packing material for goods shipped into Australia from the South Pacific.

Sigatoka disease may have been introduced to East Africa in the late 1930s by the movement of planting material/diseased leaves from Asia. During the colonial period, there was much migration to East Africa from India and settlers and/or British administrators undoubtedly transported infected suckers of their favourite cultivars from one location to another.

The appearance of Sigatoka disease in European colonies in the eastern Caribbean region in the mid-1930s could also have been due to colonial-inspired movements of *Musa* germplasm from Asia. The almost simultaneous discovery of the disease at several locations may have been due to multiple introductions of the pathogen or to rapid spread from one point after an undetected build-up of inoculum. Prevailing landward winds off the Caribbean Sea would have carried spores of *M. musicola* across the north of South America and into Central America.

Today, Sigatoka disease is regarded as having a worldwide distribution, although it has not been recorded in the Canary Islands, Egypt or Israel (Meredith, 1970). The dry summer climates of these countries may make the local environment unsuitable for disease establishment.

Impact

The most efficient leaves for photosynthesis on a vegetatively growing banana are the second to fifth counting down from the top of the plant. Therefore, if optimal assimilation potential of the plant is to be maintained, it is important that leaves 2-5 are free of excessive shade, severe leaf tearing and disease. In a vigorous plant growing in the tropics, this critical leaf area is renewed monthly (Robinson, 1996) and the pathogen does not cause enough damage to have an appreciable effect on growth (Leach, 1946). Damage comes after bunch emergence when leaf production ceases and leaf tissue cannot be renewed. The greater the damage on remaining leaves and the earlier it occurs after shooting, the greater the effects on yield. Sigatoka disease also affects the physiology of developing fruits causing premature ripening (Meredith, 1970). This occurs in the field if the plant is severely diseased or in transit to markets if moderately affected. For these reasons it was important to control Sigatoka disease in commercial plantations.

The economic impact of Sigatoka disease has been twofold. First, there was the direct effect on production when the disease first became established and control measures were being developed. Between 1937 and 1941, production in Mexico was halved as a direct result of Sigatoka disease. In Honduras, production declined to less than one third of the pre-disease level (Meredith, 1970). These were enormous losses. Secondly, there were the on-going costs associated with leaf spot control once effective measures were developed and adopted. The struggle against Sigatoka disease

in this regard has been well documented (Meredith, 1970; Stover 1972, 1990; Carlier *et al*., 2000a). Bordeaux mixture, the first effective fungicide, had to be applied as a high volume spray and large pipeline systems were installed at great expense to deliver the chemical in plantations. Later, petroleum oil applied as a low-volume spray from aircraft proved effective and costs were reduced, especially when combined with forecasting systems. Protectant fungicides, such as dithiocarbomates, and systemic fungicides, such as benomyl, improved the standard of control. However, control measures, which included labour intensive cultural practices, such as pruning old diseased leaves, was an expense that was borne by growers. In 1990 in Queensland, where Sigatoka disease is still the dominant leaf spot disease, control measures were estimated as 14% of total production costs.

There are no real figures on the impact Sigatoka disease had on small-scale growers in developing countries producing fruit for families or local markets. Plantains and other cooking bananas favoured by many subsistence farmers are resistant to Sigatoka disease in lowland areas, and therefore the impact in these cases would be minimal. Those growing the more susceptible dessert banana cultivars, such as in the Pome subgroup (AAB), would either have had to change to cooking types, accepted a loss in yield, or invested in backpack sprayers. However, the worst was yet to come.

Mycosphaerella fijiensis, the cause of black leaf streak disease

First records

The first report of *M. fijiensis* causing damage was in the same Sigatoka valley on the island of Vitu Levu in Fiji where *M. musicola* was first recognised as a major pathogen of banana fifty years earlier. In February 1963, the disease caused by *M. fijiensis* was reported to be spreading rapidly in the Sigatoka Valley (Rhodes, 1964) and was predicted to affect the whole island by the end of 1964 (Leach, 1964a). The causal organism was also described for the first time from material collected in Fiji (Leach, 1964b). The disease caused by *M. fijiensis* was called black leaf streak disease by Rhodes (1964). Leach (1964b) described the risk of spread of this new disease of banana as "a grave threat" and feared that the abundance of airborne ascospores produced by the pathogen may lead to dissemination around the world faster than for Sigatoka disease. He also warned that an outbreak of a new, perhaps worse, disease might happen again in the future in another location.

The problem caused by *M. fijiensis* in Fiji became apparent when the mist-sprays of light mineral oil being used to control *M. musicola* lost their effectiveness. The recognition of yet another important banana pathogen in Fiji before anywhere else can probably be attributed to the fact that at this location there were sizeable plantations of susceptible dessert banana cultivars and an efficient plant protection service experienced in banana problems.

Surveys undertaken after black leaf streak disease was discovered in Fiji led to the conclusion that the pathogen had most likely been present in the Pacific and parts of the Pacific rim for many years (Meredith, 1970; Stover, 1976; Stover, 1978;

Long, 1979). It was suggested that *M. fijiensis* may have been in the Hawaiian Islands in 1958 (Meredith and Lawrence, 1969). An analysis of herbarium specimens by Stover (1976), showed that *M. fijiensis* was present in Papua New Guinea by at least 1957 and in Taiwan as early as 1927. The similarity of symptoms with those of Sigatoka disease most likely masked the arrival of this new disease in many countries. Because of this, the year that black leaf streak disease was first discovered in many countries in this region (Table 2) does not reflect the order of spread of the pathogen.

Global spread

When the black leaf streak pathogen was first found in Honduras in 1972, it was thought from its morphology to be a variant of *M. fijiensis* and was named *M. fijiensis* var. *difformis* (Mulder and Stover, 1976). However, it was later shown that *M. fijiensis* and *M. fijiensis* var. *difformis* were synonymous (Pons, 1987). Black leaf streak disease has precedence as the common name for the disease caused by *M. fijiensis* and was adopted by Carlier *et al.* (2000a) in the publication 'Diseases of Banana, Abacá and Enset', however it is widely known in as black Sigatoka or *Sigatoka negra* in Spanish. The choice of which name to use in publications is one of personal preference.

A measure of the rate of spread of *M. fijiensis* between countries can be gained from an examination of the records from the Latin American/Caribbean region (Table 2). Within three years of its detection in Honduras in 1972, *M. fijiensis* was reported in Belize to the north and by 1977 had arrived in Guatemala to the west. Local spread was quicker in the direction of prevailing winds from the east and northeast (Stover, 1980). In 1979, it appeared in El Salvador, Nicaragua and Costa Rica and by 1981 had spread north to Mexico and south to Panama and northern Colombia (Carlier *et al.*, 2000a). Spread was believed to have been accelerated in Central America by the movement of diseased banana leaves and leaf trash across international boundaries with road-transported banana and plantain fruit (Stover, 1980). By 1986, commercial plantations in northern Ecuador were affected and plantains in western Venezuela succumbed in 1991. Spread to northern Peru occurred in 1994 and to Bolivia in 1996. The first report from western Brazil came in 1996 and since then *M. fijiensis* has been advancing in a southwesterly direction towards the Brazilian coast. In 2001, movements of banana fruit and associated banana leaves from inland areas where *M. fijiensis* was found to coastal cities were being controlled in an effort to delay spread (R. S. Moreira, Brazil, personal communication).

In the Caribbean, black leaf streak disease was first found in Cuba in 1990, Jamaica in 1995 and the Dominican Republic in 1996. The first authenticated report from Haiti, which has a dry climate, was in 1999. Natural spread to other Caribbean countries is inevitable, but may be slowed considerably by the prevailing winds, which blow from the east. Recent investigations involving the analysis of isolates have revealed that the source of inoculum for the outbreak in Jamaica may have come form Central America and not windblown from Cuba as originally suspected (G. Rivas, Costa Rica, personal communication). The outbreak in the

Table 2. Countries where black leaf streak disease has been detected (modified from Carlier *et al.*, 1999 with information supplied by X. Mourichon and D. R. Jones).

Region/Country	Year[1]	Region/Country	Year[1]
Australasia/Oceania		Colombia	1981
Solomon Islands	1957 (1946)	Ecuador	1986
Papua New Guinea	1957 (1951)	Cuba	1990
Fiji	1963	Venezuela	1991
French Polynesia	1964-67	Peru	1994
Micronesia	1964-67	Jamaica	1995
New Caledonia	1964-67	Bolivia[3]	1996
Vanuatu	1964-67	Dominican Republic	1996
Tonga	1965	Brazil	1998
Samoa	1965	USA (Florida)	1998
USA (Hawaii)	1969 (1958)	Haiti	1999
American Samoa	1975	**Africa**	
Cook Islands	1976	Zambia	1973[4]
Niue	1976	Gabon	1978
Norfolk Island	1980	Cameroon (south-east)	1980
Australia (Torres Strait/Cape York)	1981	Cameroon (south-west)	1983
Wallis and Fortuna Islands	1996	São Tomé	1983
Australia (North Queensland, currently under eradication)	2001	Côte d'Ivoire	1985
Asia		Congo	1985
Taiwan	1927	Nigeria	1986
Philippines (Luzon)	1964	Ghana	1986
Singapore	1964-67	Rwanda	1986
Philippines (Mindanao)	1965	Burundi	1987
Malaysia (Peninsula)	1965	Tanzania (inc. Pemba and Zanzibar)	1987
Thailand	1969	Democratic Republic of Congo (highlands)	1987
Indonesia (Java)	1969	Democratic Republic of Congo (lowlands)	1988
Indonesia (Halmahera)	1970	Togo	1988
China (Hainan)	1980	Kenya	1988
Bhutan	1985	Malawi	1990
China (Guangdong)	1990	Uganda	1990
China (Yunnan)	1993	Benin	1993
Vietnam	1993	Comoros	1993
Indonesia (Sumatra)	1993	Mayotte	1993
Indonesia (Kalimantan)	1996	Central African Republic	1996
East Malaysia (Sarawak)	1996	Madagascar	2000
Latin America/Caribbean			
Honduras	1972 (1969)		
Belize	1975		
Guatemala	1977		
Nicaragua	1979		
Costa Rica	1979		
El Salvador	1979[2]		
Mexico	1980		
Panama	1981		

[1] Most likely year of introduction, but no definite record published at this time.
[2] Year disease was first reported or year present from herbarium specimens (earliest year disease was believed present in hindsight within brackets). [3] In 2001, black leaf streak disease was spreading south-eastwards across Brazil from the Mato Grosso towards the Atlantic coast. [4] Authenticity of this record has been challenged.

Dominican Republic has also been linked to Central America, though the evidence is more circumstantial. In both examples, the disease appeared shortly after banana fruit was shipped to the islands. Could inoculum on the surface of fruit or in associated leaf trash have introduced black leaf streak to these countries?

The first record of black leaf streak disease in Africa was from Zambia in 1973 (Raemaekers, 1975). The publication of this outbreak is convincing but the identity of the pathogen could not be confirmed from specimens sent to the UK, therefore doubt remains as to the authenticity of the report (Dabek and Waller, 1990). The next record was from Gabon in 1978. Frossard (1980) believed it might have been introduced on planting material from Asia. The disease then spread steadily through Central and West Africa reaching Côte d'Ivoire, Nigeria and Ghana in 1985-1986, and Uganda and Malawi in 1990 (Table 2). A second, separate introduction of *M. fijiensis* into Africa is thought to have occurred in 1987 on the island of Pemba. This outbreak is believed to have led to the pathogen spreading to the island of Zanzibar and coastal areas of Tanzania and Kenya (Carlier *et al*., 2000a). In 2000, *M. fijiensis* was recorded in Madagascar for the first time.

The Australian experience

Stover (1978) believed that *M. fijiensis* may have originated in the Papua New Guinea-Solomon Islands area and disseminated around the South Pacific with banana leaves or planting material. This possibility is suggested by the discovery that isolates of *M. fijiensis* are more diverse in the Papua New Guinea /Philippines region than elsewhere, an indication that the area may be the centre of origin of the pathogen (Carlier *et al*., 2000a). Therefore, it is likely that *M. fijiensis* may have been present on banana on islands in the Torres Strait and on the tip of Cape York Peninsula, Australia long before its discovery on the first plant pathological survey of the area in 1981 (Jones and Alcorn, 1982). The pathogen may not have spread further south in Australia because of the barrier presented by the Cape York Peninsula, which is a large, remote area of native bush with comparatively few communities and banana plants. After 1981, better land and air communications, which encouraged more tourists and people seeking an alternative lifestyle, led to a higher risk of spread. During the 1990s, *M. fijiensis* was regularly eradicated from isolated outbreaks on small plantings within the Peninsula. In all cases, the origin of the inoculum could not be positively determined. In 2000, an outbreak occurred on a commercial banana planting at Daintree on the northern fringe of the more heavily populated coastal strip centred on Cairns. The grower was compensated for the destruction of his crop by the Australian banana industry. Although eradicated, the close proximity of this outbreak to the main banana growing area was worrying.

Towards the end of the wet season in April 2001, *M. fijiensis* was detected on unmanaged (feral) banana plants and also on cultivated plants in an adjacent farm in the Tully Valley, which is in the heart of the commercial banana-growing area in North Queensland centred south of Cairns. Subsequently, the pathogen was reported from other locations in the same area. An eradication campaign was immediately mounted. This campaign gathered momentum when the governments of banana-growing states and the Commonwealth Government pledged funds. Measures included: (1) establishment of a special banana quarantine area, (2) a ban on the movement of fruit from this area to other banana-growing areas in Australia, (3) close monitoring of crops and the diagnosis of any leaf spots detected, (4) destruction of fields where affected plants were found, (5) drastic pruning of all

banana plants in the growing area, (6) regular application of systemic fungicides and (7) zero tolerance for leaf spot disease. The campaign was conducted during the 2001 dry season, which also markedly reduced the chances of spore release and infection, with the co-operation of most growers.

A total of 25 plants have been found infected with *M. fijiensis* in the Tully area. The last seven plants were either growing in private gardens or were unmanaged. At the time of the International Sigatoka Workshop on 20-23 May 2002, *M. fijiensis* had not been detected for over five and a half months. There are hopes for the successful eradication of *M. fijiensis*, which will be the first time that this will have been achieved anywhere in the world.

Impact

Black leaf streak disease is the major constraint to cultivation in commercial plantations producing dessert banana fruit for export. It is also a limiting factor for small-scale and subsistence farmers growing plantain. The disease has had a much greater impact than Sigatoka disease because the life cycle and epidemiology of the causal pathogen makes it more difficult to control and it attacks a wider range of banana clones. *M. fijiensis* attacks younger leaves on more susceptible banana clones than those affected by *M. musicola*. On dessert clones in the Cavendish subgroup, which are extremely susceptible, the pathogen can kill much more leaf tissue in the critical 2-5-leaf range than *M. musicola*. After flowering, remaining leaves are rapidly killed resulting in premature ripening and large reductions in yield.

The arrival of black leaf streak disease in Latin America coincided with the introduction of systemic fungicides to control Sigatoka disease on plantations. The fungicides were also effective against black leaf streak disease, but many more applications were needed to maintain control with corresponding increases in production costs. It also became much more important to prevent the disease building-up too much as loss of control could have serious consequences. Good disease control management became essential and those that couldn't were in serious difficulties.

Initial effects on commercial banana production soon after the arrival of the disease were devastating. In the South Pacific, only 49% of unsprayed fruit reached export quality (Firman, 1972). Fiji ceased exporting banana fruit in 1974 and Samoa in 1984. Exports also dropped in Tonga and the Cook Islands because export quality could not be achieved (Fullerton, 1987). In Central America, export banana cultivation has survived, but at a price. Costs of control in Costa Rica are now US$900-1500 hectare/year. The overuse of fungicides with the same mode of action, and the use of fungicides below recommended doses has led to increased resistance to fungicides in *M. fijiensis* populations. Different strategies such as alternation of fungicides with different modes of action and the use of fungicide mixtures had to be adopted. Monitoring the resistances of local populations of *M. fijiensis* to a range of fungicides is now routine. In Costa Rica, the rising costs of labour and leaf spot control are making commercial banana cultivation increasingly uneconomical. Other dessert bananas grown for local consumption, such

as clones in the Pome subgroup (AAB) and 'Silk' (AAB), which are popular in Brazil and India, are also susceptible to black leaf streak disease.

Plantain cultivation has also been seriously affected by *M. fijiensis*. The pathogen has caused a considerable decrease in the availability of fruit for local consumption with a corresponding substantial increase in market price. In many areas of Central America, growers have either gone out of business or have formed cooperatives to share resources to cover the costs of spray equipment and chemicals. In Panama, plantain production was estimated to have decreased by 69% and prices to have increased by 50% between 1979 and 1984 (Bureau, 1990). In Costa Rica, black leaf streak disease was calculated to have reduced production by 40% by 1982 (Romero, 1986). Similar effects occurred in the plantain industry in Colombia, South America. After the introduction of black leaf streak disease, plantains became scarce and expensive and consumers changed to cheaper foods (Belalcazar, 1991). Black leaf streak disease also affects plantain production in Africa and endangers food security for many poor people. On poor sandy soils in West Africa, it has been estimated that yield losses are 33% and 76% during the plant and ratoon cropping cycle respectively (Mobambo *et al*., 1996). This has led to small-scale farmers abandoning plantain cultivation.

Plantain is not the only cooking banana type grown by subsistence farmers and other small-scale growers to be affected by black leaf streak. In the Great Lakes area of Africa, the East African highland cultivars in the Lujugira-Mutika subgroup (AAA) are susceptible. Losses of 37% due to the combined affects of black leaf streak disease and Cladosporium speckle have been reported (Tushemereirwe, 1996). In the Pacific, cultivars in the popular Maia Maoli-Popoulu subgroup (AAB) are susceptible (Carlier *et al*., 2000a).

Interactions between *Mycosphaerella fijiensis* and *Mycosphaerella musicola*

Soon after *M. fijiensis* was discovered on Fiji, it was reported to be displacing *M. musicola* as the dominant leaf spot. Displacement occurred in many coastal areas in the tropics particularly in Latin America. At elevation, *M. musicola* has an advantage being suited to cooler conditions and there are a number of reports (include references here) of the two pathogens co-existing at heights of around 1200m to 1500m with *M. musicola* dominating at higher altitudes and *M. fijiensis* at lower ones. There is also evidence that *M. fijiensis* may be slowly adapting to higher elevations (Carlier *et al*., 2000a).

M. musicola has probably not disappeared completely from banana in coastal areas dominated by *M. fijiensis*. Evidence of small amounts of survival within the leaf spot population comes from Nigeria where 'SH-3362' (AA), a hybrid resistant to *M. fijiensis* and susceptible to *M. musicola*, was found to be affected by *M. musicola* at planting (C. Pasberg-Gauhl and F. Gauhl, Nigeria, personal communication). In the Philippines, *M. musicola* has also still been reported to be present despite the dominance of *M. fijiensis* in commercial plantations. This may be because the great genetic diversity of cultivated banana in this country has meant some clones, like 'Amas' (AA, syn. 'Sucrier'), which is more susceptible to *M. musicola*

than *M. fijiensis,* have maintained the former pathogen. In other areas of Southeast Asia, the situation has been more difficult to understand. This is discussed more fully under "*Mycosphaerella eumusae*".

Mycosphaerella eumusae, the cause of eumusae leaf spot

Confusion between leaf spots in Asia

The latest of the three *Mycosphaerella* leaf spot pathogens to affect banana was only recognised in the mid-1990s (Carlier *et al*., 2000a). This was because of uncertainties concerning the distribution of Sigatoka disease and black leaf streak disease in Southeast and South Asia. The uncertainty arose because the usual rapid displacement of *M. musicola* by *M. fijiensis* in tropical lowland areas, as had occurred in the Americas and West Africa, did not seem to have happened in Java (Indonesia), West Malaysia and Thailand (Jones, 1990). Although *M. fijiensis* had first been recorded at these locations in the mid to late 1960s, *M. musicol*a was still present near Bogor in Java and Kuala Lumpur in West Malaysia in 1976 (Stover, 1976). From observations ten years later in 1988, the author believed Sigatoka disease was still the dominant leaf spot occurring in Java, West Malaysia and Thailand. If *M. fijiensis* was present, what was stopping it from becoming the dominant leaf spot? There was also a problem concerning the leaf spot situation in South Asia. If *M. fijiensis* had been recorded in Bhutan in 1985 (Peregrine, 1989), then why hadn't it since been found in neighbouring India?

First records

It became possible between 1992 and 1995 for the author, who was employed at the time by the International Network for the Improvement of Banana and Plantain (INIBAP), to collect specimens of leaf spot in the Southeast Asian/South Asian region during visits for diagnosis. He hoped that this would help determine the distribution of the two main *Mycosphaerella* leaf spot pathogens, which may help explain the apparent lack of expansion of *M. fijiensis* in the region. Specimens, which were thought to be mainly of *M. musicola*, were collected at different locations and on different banana clones in southern India, Sri Lanka, West Malaysia and Thailand. The specimens were sent by courier to Drs Xavier Mourichon and Jean Carlier at the *Centre de coopération internationale en recherche agronomique pour le développement* (CIRAD) in Montpellier for identification. Unexpectantly, *M. musicola* was not identified from any of the specimens collected. Some specimens from Johore in West Malaysia were found to be *M. fijiensis*, but the majority of leaf spots from all countries were caused by a fungus that was unknown. This fungus had *Mycosphaerella* as the perfect (teleomorph) stage and what first appeared to be *Septoria* as its imperfect (anamorph) stage (Anon., 1995; Carlier *et al*., 2000b). Initially, it was believed that the pathogen might have been *Phaeoseptoria musae* (Anon.,1995), which has been reported to have *Mycosphaerella* as a perfect stage and is fairly widespread

(Carlier *et al.*, 2000a). However, this turned out not to be the case (Carlier *et al.*, 2000b). The new fungus was named *M. eumusae* (Carlier *et al.*, 2000b).

Because of the similarity of symptoms with those of Sigatoka disease (Carlier *et al.*, 2000a), it is likely that the leaf spot caused by *M. eumusae* was seen by the author in Malaysia and Thailand in 1988. Evidence suggested that it might have been the common leaf spot of banana in the South Asia and parts of Southeast Asia. If so, *M. eumusae* competed effectively with *M. fijiensis* and prevented it from becoming dominant.

A specimen of a leaf spot collected in the Mekong Delta in Vietnam by Ivan Buddenhagen in 1995 was later identified as *M. eumusae* at CIRAD, as were specimens from Mauritius in 1997 (Carlier *et al.*, 2000b) and Réunion in 2000 (X. Mourichon, France, personal communication). Re-examination of specimens collected at Onne, Nigeria in 1989 and 1990, when *M. fijiensis* was presumed to be present, also revealed the presence of *M. eumusae* (Carlier *et al.*, 2000b). Details of findings of *M. eumusae* that are documented by CIRAD are summarised in Table 3.

Table 3. Countries where *Mycosphaerella eumusae*, the causal agent of eumusae leaf spot disease, has been detected in chronological order of records (modified from Table 1 of Carlier *et al.*, 2000b with additional information supplied by X. Mourichon). All identifications made by J. Carlier and M.F. Zapater, CIRAD.

Country	Banana host	Year specimen was collected and collector
Nigeria (Onne)	AAB clone, most likely in plantain subgroup	1989 (IITA)
Nigeria (Onne)	AAB clone, most likely in plantain subgroup	1990 (IITA)
India (Bangalore)	'Grande naine' (AAA, Cavendish subgroup)	1992 (D.R. Jones, INIBAP)
Malaysia (Johor State)	'Pisang kapas' (AA or AAB)	1993 (D.R. Jones, INIBAP)
Thailand (Sukothai)	'Grande naine' (AAA, Cavendish subgroup)	1994 (D.R. Jones, INIBAP)
Thailand (Surat Thani)	'Williams' (AAA, Cavendish subgroup)	1994 (D.R. Jones, INIBAP)
Thailand (Tha Yang)	'Kluai hom thong' (AAA)	1994 (D.R. Jones, INIBAP)
India (Kannara)	'Grande naine' (AAA, Cavendish subgroup)	1995 (D.R. Jones, INIBAP)
Sri Lanka (Gannoruwa)	AAA clone in Cavendish subgroup	1995 (D.R. Jones, INIBAP)
Sri Lanka (Nugahena)	'Anamala' (AAA, syn. 'Gros Michel')	1995 (D.R. Jones, INIBAP)
Vietnam (Mekong Delta)	'Sucrier' (AA)	1995 (I. Buddenhagen)
Mauritius	'Grande naine' (AAA, Cavendish subgroup)	1997 (S.P. Beni Madhu, AREU)
Réunion	'Grande naine' (AAA, Cavendish subgroup)	2000 (C. Lavigne, CIRAD)

Recently, a re-examination of *M. eumusae* specimens and cultures has revealed that the imperfect stage of the fungus is likely to be *Pseudocercospora* and not *Septoria* as originally thought. However, the fungus can be distinguished from *M. musicola* and *M. fijiensis* on morphological grounds (Crous and Mourichon, 2002) and by ITS sequence analysis (Carlier *et al.*, 2000b). A change in the common name of the disease from Septoria leaf spot disease to eumusae leaf spot disease has been proposed (Crous and Mourichon, 2002).

Impact

The economic impact of *M. eumusae* as a leaf pathogen of banana has still to be evaluated. However, it is known to seriously affect cultivars in the important AAA Cavendish and AAB plantain subgroups in southern India. It has also been observed

causing large areas of necrosis on leaves of 'Anamala' (AAA, syn. 'Gros Michel') in Sri Lanka. Other cultivars/clones seen with symptoms are 'Kluai lep mu nang' (AA), 'Pisang mas' (AA), 'Pisang kapas' (AA) and 'Mysore' (AAB) (Carlier *et al.*, 2000a). More research needs to be undertaken on the effect of *M. eumusae* on important clones.

Summary and discussion

The three *Mycosphaerella* species causing leaf spot diseases of banana reported above are serious pathogens. *M. musicola* and *M. fijiensis* were well documented in the scientific literature because of their steady global or near global spread and impact on banana cultivation. However, *M. fijiensis*, the more recent has had a far greater impact. It is more difficult to control on plantations of dessert bananas for export and local consumption, and also affects the production of cooking bananas grown by resource-poor farmers. The arrival of *M. fijiensis* in West Africa and the perceived threat that it posed to the livelihood of the peoples there led to the formation of INIBAP and the plantain breeding programmes of the International Institute of Tropical Agriculture (IITA) and the *Centre africain de recherches sur bananiers et plantains* (CARBAP).

The third *Mycosphaerella* leaf spot pathogen is not so well known because it has been overlooked until fairly recently. Evidence on distribution suggests that it arose, like the others, in the Southeast Asian/Australasian region. It is not known how long *M. eumusae* has affected banana in South and Southeast Asia, nor the extent of its distribution. Extensive surveys of leaf spots in the region would help clarify the situation. An estimate of the severity of the disease and the name of the clone affected would help determine host reactions. Basic information on the biology and epidemiology of the pathogen is also needed.

M. eumusae has been present in Onne, Nigeria for at least 13 years but was not found in a recent thorough survey of leaf spot organisms in neighbouring Cameroon (C. Abadie, France, personal communication). Therefore, in West Africa, the pathogen may still be confined to southeast Nigeria. If so, one must speculate on how it got here in the first place. Was *M. eumusae* introduced with planting material from Asia?

M. eumusae seems to be an important pathogen and may be able to compete with *M. fijiensis*. The apparent dominance of *M. eumusae* in parts of Asia suggests that it was established before the introduction of *M. fijiensis* and thus perhaps able to resist intrusion by the latter. The situation at Onne, Nigeria, may be different with *M. eumusae* because arrival was after *M. fijiensis* became dominant over *M. musicola* and subsequent competitive interactions favoured the established pathogen. Work is needed to determine if this speculation has any basis in fact. The identification of the leaf spot pathogens found on plantain at Onne may help determine relative competitiveness on the main susceptible host in the area. The identification of leaf spot pathogens on cultivars in the IITA germplasm collection may help to determine the nature of leaf spot interactions on other clones.

When *M. fijiensis* first appeared, some believed that it might have arisen in Fiji by mutation from *M. musicola*. Stover (1969) initially considered *M. fijiensis*

to be a physiological strain of *M. musicola*. The latter hypothesis is now considered to be unlikely. How do we view the appearance of *M. eumusae*? How are the leaf spot pathogens evolving? Recent phylogenetic studies indicate that *M. musicola*, *M. fijiensis* and *M. eumusae* may once have had a common origin (P. Crous, South Africa, personal communication). All may have arisen from similar saprophytic or weakly pathogenic fungi growing on damaged or weakened leaf tissues of banana. Stover (1969) reported *M. minima* as a saprophytic co-inhabitant with *M. musicola* in leaf spots. He has also recorded *M. musae* was an endophyte in Sigatoka leaf spots (Stover, 1994). Other fungi could be evolving as parasites in senescing leaf tissue. Further investigations of speciation in *Mycosphaerella* and other related genera found on banana leaves in the centre of origin of banana and elsewhere may prove interesting.

The similarity of the necrotic symptoms caused by *M. musicola*, *M. fijiensis* and *M. eumusae* and the fact that all three have *Mycosphaerella* as the perfect stage suggests that the diseases should be grouped together as Sigatoka leaf spot diseases. These diseases warrant fundamental research to clarify their evolution and adaptability, and to help to find ways of breeding resistant bananas.

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Integrating morphological and molecular data sets on *Mycosphaerella*, with specific reference to species occurring on *Musa*

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Abstract

The genus *Mycosphaerella* (= *Sphaerella*) is one of the largest genera of ascomycetes, containing more than 3000 named taxa. Approximately 23 anamorph genera have been linked to *Mycosphaerella* via cultural studies. Several of these anamorph genera have recently been reduced to synonymy based on phylogenetic studies derived from ITS1, 5.8S and ITS2 DNA sequence data. In addition, several genera not previously associated with *Mycosphaerella*, have also been shown to cluster within *Mycosphaerella*, which has proved to be largely monophyletic. From these results, as well as a re-evaluation of the criteria upon which anamorph genera are distinguished in this complex, a reduced set of informative criteria and genera are proposed. The degree of scar thickening, darkening and refraction, as well as the presence or absence of pigmentation in conidiophores and conidia appear to be useful features delimiting anamorph genera of *Mycosphaerella*. Species, however, are still separated on a combination of characters such as conidiomatal structure, the nature and arrangement of conidiophores, conidiogenesis, dehiscence scars and pigmentation. For the species that occur on *Musa*, anamorph morphology appears to be more informative than the more conserved teleomorph morphology, and can be used to separate the major pathogens, namely *M. fijiensis* (the causal agent of black leaf streak disease), *M. musicola* (the causal agent of Sigatoka disease), *M. eumusae* (the causal agent of eumusae leaf spot disease), as well as other reputedly less important pathogens.

Resumen – Integración de los conjuntos de datos morfológicos y moleculares en *Mycosphaerella*, con referencia específica a las especies que ocurren en *Musa*

El género *Mycosphaerella* (= *Sphaerella*) es uno de los géneros más grandes de ascomicetos, que contiene más de 3000 taxa nombrados. Aproximadamente 23 géneros anamorfos diferentes han estado vinculados con *Mycosphaerella* mediante estudios culturales. Varios de estos géneros anamorfos fueron reducidos recientemente debido a la sinonimia basada en estudios filogenéticos

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derivados de los datos de las secuencias de ADN ITS1, 5.8S y ITS2. En adición, se ha demostrado que varios géneros que no estaban asociados previamente con *Mycosphaerella*, actualmente están agrupados dentro del género *Mycosphaerella*, el cual está comprobado, que es extensamente monofilético. De estos resultados, así como de una nueva evaluación de los criterios de acuerdo a los cuales se distinguen géneros anamorfos en este complejo, se propone un conjunto reducido de criterios y géneros informativos. El grado del espesor, oscurecimiento y refracción de las cicatrices, así como la presencia o ausencia de pigmentación en conidióforos y conidios parecen representar características útiles que delimitan los géneros anamorfos de *Mycosphaerella*. Sin embargo, las especies aún están separadas en una combinación de caracteres como la estructura conidiomatal, la naturaleza y el arreglo de los conidióforos, conidiogénesis, cicatrices de dehiscencia y pigmentación. Para las especies que ocurren en *Musa*, la morfología anamorfa parece ser más informativa que la morfología teleomorfa más conservada, y puede ser utilizada para separar los principales patógenos, a saber *M. eumusae* (agente causal del ELSD), *M. musicola* (agente causal de la enfermedad de Sigatoka), *M. fijiensis* (agente causal de la raya negra de la hoja).

Résumé – L'intégration des données morphologiques et moléculaires sur *Mycosphaerella*, particulièrement celles des espèces présentes sur *Musa*

Le genre *Mycosphaerella* (= *Sphaerella*) est un des genres les plus représentés des ascomycètes avec plus de 3000 taxa. Environ 23 genres anamorphes ont été liés à *Mycosphaerella* à l'aide d'études sur les cultures. Des études phylogénétiques à partir de séquences d'ADN ITS1, 5.8S et ITS2, ont permis d'identifier les synonymes parmi ces genres anamorphes. De plus, plusieurs genres qui n'étaient pas associés auparavant à *Mycosphaerella*, se regroupent dans ce genre qui s'est avéré être principalement monophylétique. A partir de ces résultats ainsi que par la réévaluation des critères à partir desquels on peut distinguer les genres anamorphes, nous proposons une réduction du nombre de critères pertinents et de genres. Le degré d'épaississement, de noircissement et de réfraction des cicatrices, ainsi que la présence ou l'absence de pigmentation dans les conidiophores et les conidies, semblent être des critères intéressants pour délimiter les genres anamorphes de *Mycosphaerella*. Les espèces se distinguent toutefois par une combinaison de caractères tels que la structure des conidioma, la nature et la disposition des conidiophores, la conidiogénèse, les cicatrices de déhiscence et la pigmentation. Pour les espèces qui se trouvent sur *Musa*, la morphologie de l'anamorphe est plus instructive que la morphologie moins variable du téléomorphe et peut être utilisée pour distinguer les principaux pathogènes, soit *M. fijiensis* (l'agent causal de la maladie des raies noires), *M. musicola* (l'agent causal de la maladie de Sigatoka), *M. eumusae* (l'agent causal de l'ELSD, *eumusae leaf spot disease*), ainsi que d'autres pathogènes considérés moins importants.

What is *Mycosphaerella*?

Corlett (1991, 1995) lists more than 2000 species belonging to the genus *Mycosphaerella* Johanson (*Dothideales*: *Mycosphaerellaceae*) (including the fungi described in *Sphaerella* Ces. et De Not.). This makes it one of the largest genera of ascomycetes known. Furthermore, this genus has been confirmed to have anamorphs in at least 23 different genera (Crous *et al.*, 2000), including *Cercospora* Fres. and *Septoria* Sacc., which alone encompass several thousand species (Pollack, 1987; Sutton, 1980). Some species are saprobes, but most are known from necrotic lesions that are associated with leaves, stems or fruit of various hosts (Park and Keane, 1984). Host specificity still plays a major role in the identification of species, where taxa are chiefly compared with those that occur on a specific host genus or family of phanerogamic plants (Chupp, 1954; Braun, 1995). Von Arx (1949)

regarded some species as polyphagous but the concept of host specificity is still strongly adhered to, especially with species shown to be plant pathogens.

Species of *Mycosphaerella* are usually defined as having small, black, immersed or erumpent to almost superficial pseudothecial ascomata, with various degrees of stromatic tissue surrounding the ascomata, and pale to brown superficial mycelium being present or absent, smooth or verruculose. The ascomatal wall consists of 3–6 layers of pseudoparenchyma cells but in some taxa this can also be more prominently developed, especially around the ostiole. Ostioles are singular, central, and usually lined with periphyses; in some taxa, however, this develops further, giving the impression of periphysoids. The hamathecium dissolves at maturity, and no stromatic tissue remains between the asci. Asci are bitunicate, 8-spored, sessile, arranged in a cluster, hyaline, ovoid to obovoid, ellipsoidal or cylindrical, rarely clavate. Ascospores are bi to multi-seriate, thin to thick-walled, guttulate or not, 1-septate, constricted at the septum or not, usually hyaline and smooth, rarely with a mucous sheath, fusoid to obovoid, ellipsoid or elongate. Anamorphs are highly variable, including numerous hyphomycete and coelomycete genera, namely *Cercospora* Fres., *Cercosporella* Sacc., *Cladosporium* Link, *Clypeispora* Ramaley, *Colletogloeopsis* Crous et M.J. Wingf., *Dissoconium* De Hoog, Oorschot et Hijwegen, *Fusicladiella* Höhn., *Miuraea* Hara, *Passalora* Fr., *Phaeophleospora* Rangel, *Phloeospora* Wallr., *Pseudocercospora* Speg., *Pseudocercosporella* Deighton, *Ramularia* Unger, *Septoria* Sacc., *Sonderhenia* H.J. Swart and J. Walker, *Stenella* Syd., *Thedgonia* B. Sutton, *Uwebraunia* Crous et M.J. Wingf., *Xenostigmina* Crous (Crous *et al.*, 2000; 2001).

Given the wide range of variation among anamorphs, it was not clear whether *Mycosphaerella* was monophyletic, paraphyletic or polyphyletic. Crous (1998) hypothesized that *Mycosphaerella* could be split into groups as indicated by the various anamorph genera. Based on the revision of the monograph on *Mycosphaerella* by A. Aptroot (CBS, The Netherlands), several sections are recognized (modified from Barr 1972):

• **Section** *Mycosphaerella* is characterized by cylindrical asci and mostly uniseriate, thin-walled, often small ascospores that are constricted at the septum and inequilateral, with rounded upper ends. Anamorphs: typically *Ramularia* with *Asteromella* spermatial states. Representative species: the common polyphagous *M. punctiformis* (Pers. : Fr.) Starb.

• **Section** *Tassiana* M.E. Barr is characterized by pyriform asci and irregularly arranged, thick-walled ascospores that are often large and constricted at the septum and nearly equilateral, relatively broad with rounded ends. Anamorph: typically *Cladosporium*. Representative species: the common polyphagous species *M. tassiana* (de Not.) Joh. (with large ascospores) and *M. longissima* (Fuck.) Lindau (with small ascospores). Further research is still required to determine whether the teleomorphs of *Cladosporium* subgen. *Heterosporium* (David, 1997) can also be accommodated in this section. Preliminary data suggest, however, that *Cladosporium* may fall outside *Mycosphaerella* (Crous *et al.*, 2001, unpublished data).

• **Section** *Caterva* M.E. Barr is characterized by cylindrical asci and irregularly arranged, thin-walled, often medium-sized ascospores that are rarely constricted at the septum and inequilateral, with more or less pointed ends. *Asteroma* and *Asteromella* spermatial forms are typical. Representative species: the common polyphagous *M. subradians* (Fr. : Fr.) Schroeter.

• **Section** *Longispora* M.E. Barr is characterized by cylindrical asci with aggregated, thin-walled, long and slender ascospores that are rarely constricted at the septum and mostly equilateral, long but slender ascospores, characteristically with rounded upper and pointed lower ends. Anamorphs: *Phloeospora* or *Septoria sensu lato*. Representative species: *M. millegrana* (Cooke) Schroeter (with short spores), *M. latebrosa* (Cooke) Schroeter (with longer spores) and *M. populi* (Auersw.) J. Schröt. (with the longest spores in the genus). The genus *Sphaerulina* Sacc., which differs only by additional septa, may be synonymous.

• **Section** *Fusispora* M.E. Barr is characterized by pyriform asci and irregularly arranged, thin-walled ascospores that are rarely constricted at the septum and mostly equilateral, fusiform, pointed ascospores. Anamorphs have not been proved.

• **Section** *Plaga* M.E. Barr (including Section *Macula* M.E. Barr) includes endophytic species sporulating on leaf spots, many of which are described as plant pathogens. This section is characterized by obovoid to ellipsoidal or cylindrical asci, small to medium sized ascopores, fusiform to obovoid with rounded ends. Many species have been described in these groups, and the majority originate from warm-temperate and tropical areas. Anamorphs include *Colletogloeopsis*, *Mycovellosiella*, *Phaeophleospora*, *Pseudocercospora*, *Pseudocercosporella*, *Sonderhenia*, *Stenella* Syd., *Dissoconium*, *Uwebraunia* and possibly others. Representative species: listed by Crous (1998) on *Eucalyptus*.

How do we separate species in this complex based on morphology?

Mycosphaerella encompasses species with a saprobic, plant pathogenic as well as a hyperparasitic habit. In general, however, most species are found to be associated with leaf spots. Some species have been isolated as endophytes (Crous, 1998) but this is not the norm. Importantly, up to four taxa have been reported from the same lesion on leaves of *Eucalyptus* (Crous, 1998) suggesting that some act as primary and others as secondary or weak pathogens.

In the past, the taxonomy of *Mycosphaerella* relied mostly on aspects such as host, symptom type, and teleomorph morphology (pseudothecium, ascus and ascospore morphology). Very few studies focused on cultures, therefore ascospore germination patterns and cultural characteristics are unknown for most species. Furthermore, no ex-type cultures are available for molecular studies.

Most links to anamorphs have also been based on association. Given the concept of sympatric colonisation of host tissue discussed above, this has led to numerous erroneous reports of anamorph/teleomorph links. Anamorph

morphology has focused strongly on aspects such as conidiomatal structure, and mode of conidiogenesis (von Arx, 1983; Sutton and Hennebert, 1994). An important overlap has been observed between different conidiomatal types (Nag Raj, 1993; Braun 1995), hence species have been transferred from one anamorph genus to another (Sutton *et al.*, 1996; Braun, 1998). The separation of some coelomycete and hyphomycete anamorphs of *Mycosphaerella* is, therefore, debatable.

Subsequent to the wide taxonomic concept employed for the cercosporoid fungi by Chupp (1954), Deighton (1973, 1976, 1987, 1990) recognised the value of pigmentation, conidiogenesis and conidium release. Different types of dehiscence scars (David, 1993) have subsequently been recognised to separate genera such as *Cladosporium*, *Cercospora* and *Stenella*. Conidiogenesis is variable in this complex (Verkley, 1997), as are dehiscence scars (Crous, 1998), but it still remains a useful feature to separate species (Braun, 1995).

Integrating morphological and molecular data sets

The issue of integrating morphological and molecular data sets in *Mycosphaerella* is beset with numerous problems. Several hypotheses have been proposed to divide the genus into separate parts, groups, sections or genera. The molecular work to date, however, mainly supported one major clade of *Mycosphaerella* (Table 1), as well as two minor clades, typified by *Dissoconium* and *Cladosporium* (Crous *et al.*, 2000, 2001). That anamorph concepts have not always correlated with different groups in *Mycosphaerella*, but have been shown to have evolved more than once within the genus, has caused confusion. Sometimes, however, integration has simplified or reduced the genera. For example, species of *Mycovellosiella* (superficial mycelium; conidia in chains), *Phaeoramularia* (no superficial mycelium, conidia in chains), and *Passalora* (no superficial mycelium, conidia solitary) consistently cluster together, leading to synonymy of the genera under the older name *Passalora* (Crous *et al.*, 2001). *Pseudocercospora* is another confusing example. Published work (Crous *et al.*, 2001) indicates that several other genera are also clustered here, including *Pseudophaeoramularia* U. Braun, and *Stigmina*, while the position of *Stenella* was still not clearly resolved. As more taxa have been added to our analyses (unpublished data), it has become clear that *Stenella* and *Stigmina* are in fact good genera, whereas *Pseudophaeoramularia* clusters in *Pseudocercospora*. These findings are still in agreement with those of Crous *et al.* (2001), namely that conidial catenulation is not a good feature at the generic level, and that the degree of thickening of spore scars is important, rather than just presence or absence.

Molecular data will also influence the way we define the teleomorph. Preliminary data suggest that ascospore septation, and the presence of pseudoparenchymatal stromatic tissue around the ostiole is of lesser taxonomic value at the generic level (unpublished data). This will result in many older names having to be re-evaluated, and may even eventually require the conservation of *Mycosphaerella*, to safely preserve the name for future use over older, but lesser known names.

Integrating morphology and molecular data sets on a species level is also beset with problems. Firstly, as discussed above, most species are known from dried herbarium material only and there are no reliable reference strains. Most strains that can be ordered from culture collections are sterile (a common phenomenon associated with species of *Mycosphaerella*) therefore their identities cannot be confirmed. Many of the species already sequenced in GenBank originate from such strains, and thus these data that are routinely used by plant pathologists obscure the issue even further, even for the few species presently known on *Musa*.

Table 1. Fungal isolates included for ITS sequence analysis

Accession no.	Teleomorph	Anamorph	Origin
U04234	*Leptosphaeria microscopica*	Unknown	ATCC 42652 (*Saccharum officinarum*, Kenya)
PCR18	*Mycosphaerella cruenta*	*Pseudocercospora cruenta*	ATCC 262271 (*Vigna*, Puerto Rico)
PP15	*M. berkeleyi*	*Passalora personata*	MPPD L2121 (*Arachis, Oklahoma*, U.S.A.)
458	*M. eumusae*	*Pseudocercospora eumusae*	*Musa*, Malaysia
487	*M. eumusae*	*Pseudocercospora eumusae*	*Musa*, Thailand
PF7	*M. fijiensis*	*Paracercospora fijiensis*	ATCC 22116 (*Musa*, Philippines)
PF8	*M. fijiensis*	*Paracercospora fijiensis*	ATCC 22117 (*Musa*, Hawaii)
01A	*M. fijiensis*	*Paracercospora fijiensis*	*Musa*, Philippines
009	*M. fijiensis*	*Paracercospora fijiensis*	*Musa*, Ntoum, Gabon
PFD9	*M. fijiensis var. difformis*	*Paracercospora fijiensis* var. difformis	ATCC 36054 (Musa, Honduras)
PM10	*M. musicola*	*Pseudocercospora musae*	ATCC 22115 (*Musa*, Philippines)
PM11	*M. musicola*	*Pseudocercospora musae*	ATCC 36143 (*Musa*, Honduras)
121	*M musicola*	*Pseudocercospora musae*	*Musa*, Indonesia, West Java
090	*M musicola*	*Pseudocercospora musae*	*Musa*, Armenia, Colombia
CA1	*Mycosphaerella* state unknown	*Cercospora apii*	ATCC 12246
CH5, CH6	*Mycosphaerella* state unknown	*Cercospora hayi*	ATCC 12234 (*Musa*, Cuba)
MA12	Unknown	*Mycocentrospora acerina*	ATCC 34539 (*Daucus carota*, Norway)
IMI 271341	Unknown	*Phaeoseptoria musae*	*Musa*, Honduras

Re-evaluating species occurring on *Musa*

Mycosphaerella eumusae, M. fijiensis and *M. musicola*

The *Mycosphaerella* state of *M. eumusae* is morphologically very similar to that of *M. musicola*. As expected, reports from literature also suggest that these two pathogens have commonly been confused in the past, thereby also questioning the value of much of the published literature (and distribution records) on this disease complex. Leaf spot symptoms attributed to *M. eumusae* were first seen after a survey conducted in Southeast Asia during 1992–1995 (Carlier *et al.*, 2000b). The anamorph of *M. eumusae* was initially regarded as a species of

Septoria (Carlier *et al.*, 2000a, b). *Pseudocercospora eumusae*, the anamorph of *M. eumusae*, is characterized by having predominantly epiphyllous sporodochia that form on dark brown substomatal stromata. The sporodochia are mingled with developing spermatogonia. Young sporodochia are subepidermal and substomatal, and initially produce conidia that appear to be exuding from a subepidermal, substomatal pycnidium. In cross section, however, the subepidermal and substomatal structure is seen to be a sporodochium, not a pycnidium. As more stromatal tissue is formed, conidiophores become erumpent, and sporodochia burst through the epidermis, almost appearing acervular, but in fact being subepidermal sporodochia. Conidiophores are subhyaline to pale olivaceous, becoming pale brown at the base, subcylindrical, 0 to 3-septate, 10 to 25 x 3–5 µm, with conidiogenous cells terminating in truncate ends. Sporodochia of *M. eumusae* develop in a similar fashion to those of *M. musicola* but the conidiophores are much longer and more septate in the former. Conidia of *P. eumusae* are subhyaline to pale olivaceous, subcylindrical, (18–)30–50(–65) x (2–)2.5–3 µm, 3 to 8-septate, and have subtruncate ends without visible scars. Conidia can be distinguished from those of *M. musicola* by their more cylindrical shape, subtruncate ends, and shorter dimensions (Crous and Mourichon, 2002). Based on ITS sequence data (Figure 1), the two species are also very closely related. Isolates of *M. fijiensis* (anamorph: *P. fijiensis*) are easily distinguished from *P. musae* and *P. eumusae* by their minutely thickened spore scars (Deighton, 1979). These scars have been shown to be of lesser phylogenetic importance in the cercosporoids (Stewart *et al.*, 1999), and *Paracercospora* should be merged back into *Pseudocercospora* (Crous *et al.*, 2000, 2001), but they are still valuable at the species level and should be used to separate taxa. Nevertheless, the anamorph of *M. fijiensis* should now correctly be referred to as *Pseudocercospora fijiensis*.

Other species of *Mycosphaerella*

As can be seen below, numerous additional species of *Mycosphaerella* (or their anamorphs) have been described from *Musa*. Little is known about many of these taxa, but they are expected to occur on lesions typically associated with the major pathogens discussed above. Some species, e.g. *Cercospora hayi*, appear to have a wider host range than just *Musa*. From general morphology, *C. hayi* resembles what is currently referred to as the *Cercospora apii sensu lato* morphotype. It is suspected that *Cercospora apii* is a weak or secondary pathogen with a wide host range, and has been described from numerous hosts worldwide. Based on morphology, *C. hayi* is indistinguishable from *C. apii*. From the ITS sequence data (Figure 1), the two species are clearly similar, and should be regarded as conspecific, with preference being given to the older name, *C. apii* (Braun *et al.*, unpublished data). *Cladosporium musae*, and several other species of *Cladosporium* have also been recorded from *Musa*. The generic affinities, and circumscription of genera within the *Cladosporium*-complex, however, remain to be resolved pending a full morphological and molecular study (Braun *et al.* unpublished data). *Mycosphaerella musae* is another interesting species and is commonly associated with *Mycosphaerella* speckle. From an analysis of published literature, and

purported anamorphs associated with this species (Stover, 1994; Carlier *et al.*, 2000a), it is clear that several different biological species have in the past been treated as representative of *M. musae*.

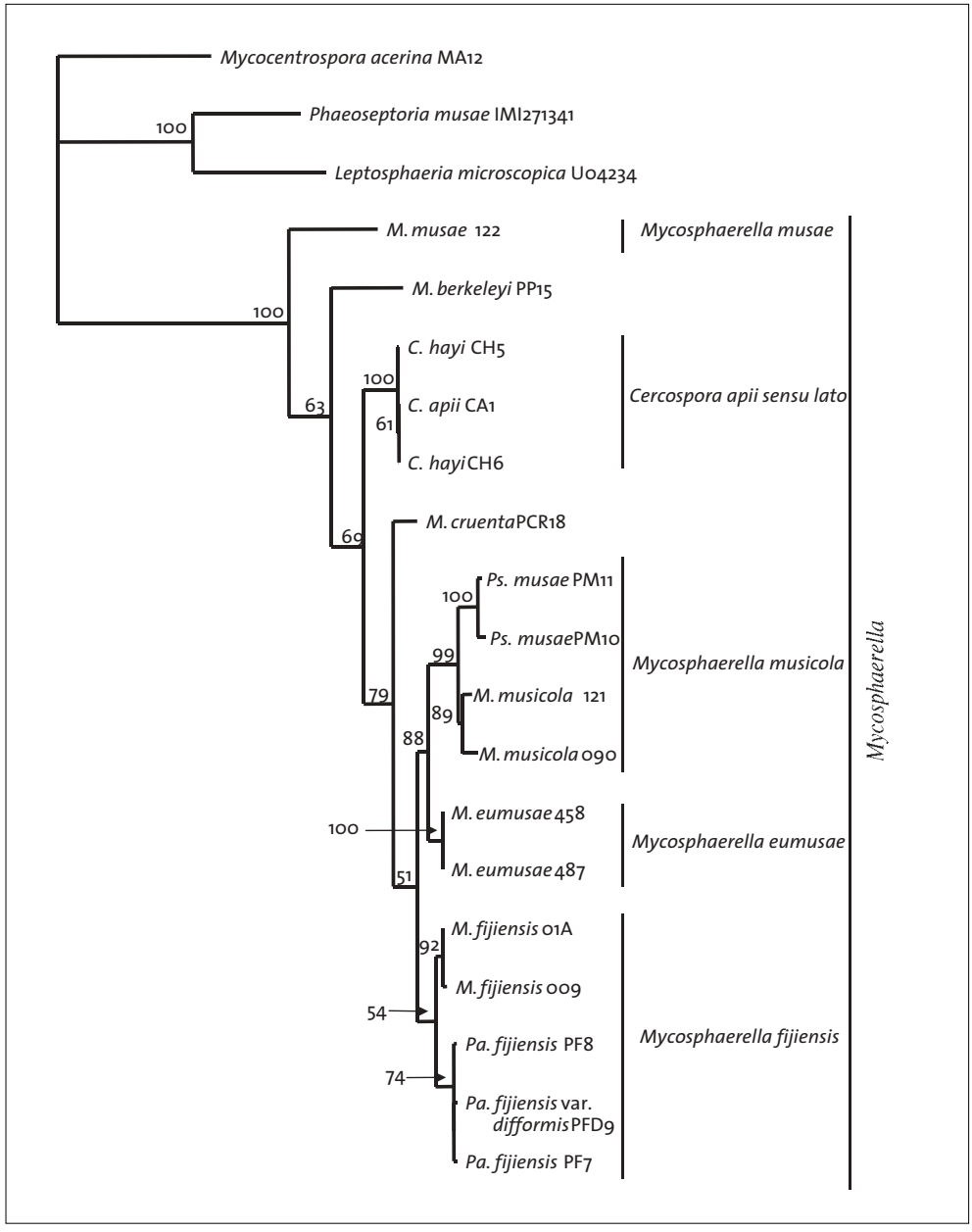

Figure 1. One of eight most parsimonious trees (length = 697 steps, CI = 0.803, RI = 0.795, RC = 0.639). Bootstrap support from 1000 replicates is shown above the lines. *Mycocentrospora acerina*, *Phaeoseptoria lysae* and *Leptosphaeria microscopica* were used as outgroups.
M. = *Mycosphaerella*, Ps. = *Pseudocerospora* and Pa. = *Paracercospora*.

Major *Mycosphaerella* diseases

Black leaf streak disease

Mycosphaerella fijiensis M. Morelet, *Ann. Soc. Sci. Nat. Archéol. Toulon Var.* 21:105. 1969.

= *Mycosphaerella fijiensis* var. *difformis* J.L. Mulder & R.H. Stover, *Trans. Brit. Mycol. Soc.* 67:82. 1976.

Anamorph: *Pseudocercospora fijiensis* (M. Morelet) Deighton, *Mycol. Pap.* 140:144. 1976.

≡ *Cercospora fijiensis* M. Morelet, *Ann. Soc. Sci. Nat. Archéol. Toulon Var.* 21:105. 1969.

≡ *Paracercospora fijiensis* (M. Morelet) Deighton, *Mycol. Pap.* 144:51. 1979.

= *Cercospora fijiensis* var. *difformis* J.L. Mulder & R.H. Stover, *Trans. Brit. Mycol. Soc.* 67:82. 1976.

≡ *Paracercospora fijiensis* var. *difformis* (J.L. Mulder & R.H. Stover) Deighton, *Mycol. Pap.* 144:52. 1979.

Host(s) and Distribution: *Musa acuminata*, *Musa* spp.; American Samoa, Australia, Belize, Benin, Bhutan, Bolivia, Brazil, Burundi, Cameroon, Central African Republic, China, Colombia, Comoros, Congo, Cook Islands, Costa Rica, Côte d'Ivoire, Cuba, Dominican Republic, Ecuador, El Salvador, Fiji, French Polynesia, Gabon, Ghana, Guatemala, Guinea-Bissau, Guyana, Haiti, Honduras, Indonesia, Jamaica, Kenya, Malawi, Malaysia, Mayotte, Mexico, Micronesia, Netherlands Antilles, New Caledonia, Nicaragua, Niger, Nigeria, Niue, Norfolk Island, Northern Mariana Islands, Panama, Papua New Guinea, Peru, Philippines, Rwanda, Samoa, São Tomé and Principe, Singapore, Solomon Islands, Somalia, Tahiti, Taiwan, Tanzania (Pemba, Zanzibar), Thailand, Togo, Tonga, Uganda, USA (FL, HI), Vanuatu, Venezuela, Vietnam, Wallis and Futuna Islands, Zambia.

Eumusae leaf spot disease

Mycosphaerella eumusae Crous et X. Mourichon, *Sydowia* 54:36. 2002.

Anamorph: *Pseudocercospora eumusae* Crous et X. Mourichon, *Sydowia*, 54:36. 2002.

Leaf spots amphigenous, initially visible as faint brown streaks, developing into oval or elliptical light brown lesions with grey centres and dark brown borders, coalescing to form large, brown, necrotic areas under favourable conditions. Grey spots and patches are visible in necrotic areas, and lesions are surrounded by a chlorotic yellow zone. *Pseudothecia* amphigenous, predominantly hypophyllous, black, subepidermal, becoming slightly erumpent, globose, up to 80 µm diam., apical ostiole 10–15 µm wide; wall consisting of 2–3 layers of medium brown textura angularis. *Asci* aparaphysate, fasciculate, bitunicate, subsessile, obovoid, straight or slightly incurved, 8-spored, 30–50 x 9–15 µm. *Ascospores* tri- to multiseriate, overlapping, hyaline, guttulate, thick-walled,

straight, obovoid with obtuse ends, widest in the middle of apical cell, medianly 1-septate or basal cell slightly longer than apical cell, tapering towards both ends, but with a more prominent taper towards lower end, (11–)12–13(–16.5) x (3–)3.5–4(–4.5) µm. *Spermogonia* predominantly hypophyllous, subepidermal, substomatal, globose, dark brown, up to 75 µm diam. *Spermatia* hyaline, rod-shaped, 3–6 x 1–2 µm. Mycelium internal, pale brown, consisting of septate, branched, smooth hyphae, 1.5–2.5 µm wide. *Caespituli* sporodochial, subepidermal, substomatal, predominantly epiphyllous, grey, up to 100 µm wide. *Conidiophores* aggregated in dense fascicles arising from the upper cells of a brown stroma up to 70 µm wide; conidiophores subcylindrical, smooth, hyaline or pale brown below, 0–3-septate, straight to geniculate-sinuous, unbranched or branched below, 10–25 x 3–5 µm. *Conidiogenous cells* terminal, unbranched, hyaline, smooth, tapering to flat-tipped apical loci, proliferating sympodially, or 1–4 times percurrently near the apex, 10–20 x 3–4 µm; scars inconspicuous. *Conidia* solitary, subhyaline to pale olivaceous, thick-walled, smooth, subcylindrical, apex obtuse, base subtruncate, straight to variously curved, 3–8-septate, (18–)30–50(–65) x (2–)2.5–3 µm; hila inconspicuous. *Cultural characteristics*: colonies pale olivaceous grey (23”””d according to Rayner, 1970) to rosy vinaceous (7”d) (surface), and brown vinaceous (5”’m) (bottom), with even margins and moderate aerial mycelium, obtaining 10 mm diam. after 2 months at 25°C in the dark (Crous and Mourichon, 2002).

Host(s) and Distribution: *Musa* spp.; Southern India, Sri Lanka, Thailand, Malaysia, Vietnam, Mauritius, Nigeria.

Sigatoka disease

Mycosphaerella musicola R. Leach ex J.L. Mulder, *Trans. Brit. Mycol. Soc.* 67:77. 1976.

≡ *Mycosphaerella musicola* R. Leach, *Trop. Agric.* 18:92. 1941. (nom. nud.).

Anamorph: *Pseudocercospora musae* (Zimm.) Deighton, *Mycol. Pap.* 140:148. 1976.

≡ *Cercospora musae* Zimm., *Centralbl. Bakteriol. Parasitenk. 2. Abth.* 8:219. 1902.

= *Cercospora musae* Massee, *Bull. Misc. Inform.* 28:159. 1914.

Host(s) and Distribution: *Musa acuminata, M. banksii, M. basjoo, M. liukiuensis, M. paradisiaca, M. textiles*; widely distributed, including American Samoa, Angola, Antigua and Barbuda, Argentina, Australia, Bahamas, Barbados, Belau, Belize, Bolivia, Brazil, Brunei Darussalam, Bhutan, Cambodia, Cameroon, Cape Verde, Cayman Islands, China, Colombia, Congo, Cook Islands, Costa Rica, Côte d’Ivoire, Cuba, Dominica, Dominican Republic, Ecuador, El Salvador, Ethiopia, Fiji, French Guiana, French Polynesia, Gabon, Ghana, Grenada, Guadeloupe, Guam, Guatemala, Guinea, Guinea-Bissau, Guyana, Haiti, Honduras, Hong Kong, India, Indonesia, Jamaica, Kenya, Kiribati, Laos, Madagascar, Malawi, Malaysia, Martinique, Mauritius, Mexico, Micronesia, Montserrat, Mozambique, Nepal, New Caledonia, Nicaragua, Nigeria, Niue, Norfolk Island, Panama, Papua New Guinea, Peru, Philippines, Puerto Rico, Saint Kitts and Nevis, Saint Lucia, Saint Vincent and the Grenadines, Samoa,

Sao Tome and Principe, Sierra Leone, Solomon Islands, Somalia, South Africa, Sri Lanka, Surinam, Taiwan, Tanzania, Thailand, Togo, Tonga, Trinidad and Tobago, Tuvalu, Uganda, USA (FL, HI), Vanuatu, Venezuela, Vietnam, Wallis and Futuna Islands, Yemen, Zambia, Zimbabwe.

Other diseases caused by species of *Mycosphaerella* and its anamorphs

Cercospora hayi Calp., *Studies on the Sigatoka Disease of Bananas and its Fungus Pathogen*, *Atkins Garden and Research Laboratory, Cuba,* p. 63. 1955.

Host(s) and Distribution: *Musa paradisiaca, Musa* sp.; Brazil, Cuba, Philippines.

Notes: Part of the *C. apii sensu lato* species complex.

Cercospora musae **var**. *paradisiaca* Bat. et R. Garnier, *Revista Agric. (Recife)*, 3:54. 1963.

Host(s) and Distribution: *Musa paradisiaca*; Brazil.

Notes: Status unknown, has not been treated.

Cercospora pingtungensis T.Y. Lin et J.M. Yen, *Bull. Soc. Mycol. France* 87:427. (1971) 1972.

Host(s) and Distribution: *Musa acuminata, M. cavendishii*; China, Taiwan.

Notes: Conidia pigmented with thickened hila, not a *Cercospora*.

Cladosporium bisporum, Matsush., *Icones microfungorum a Matsushima lectorum* (Kobe):33. 1975.

Host(s) and Distribution: *Musa paradisiaca*; Japan.

Cladosporium leaf speckle

Cladosporium musae E.W. Mason, *Mycol. Pap.* 13:2. 1945.

Host(s) and Distribution: *Musa paradisiaca, M. textiles, Musa* spp.; Bangladesh, Brunei, Burundi, Cameroon, Congo, Côte d'Ivoire, Cuba, Ecuador, Egypt, Ethiopia, France, Ghana, Ghana, Guinea, Guinea, Honduras, Honduras, Hong Kong, Indonesia, Jamaica, Malaysia, Nepal, Papua New Guinea, Rwanda, Sierra Leone, Solomon Islands, South Africa, Sri Lanka, Sudan, Uganda, Thailand, Togo, Uganda, Vietnam, Western Samoa, Zimbabwe.

Cladosporium oxysporum Berk. et M.A. Curtis, *J. Linn. Soc. Bot.* 10:362. 1868.

Host(s) and Distribution: *Musa paradisiaca*; Brazil, Venezuela.

Mycosphaerella formosana T.Y. Lin et J.M. Yen, *Rev. Mycol.* 35:323. 1971.

Host(s) and Distribution: *Musa* sp.; Taiwan.

Mycosphaerella henriquesiana G. Winter, *Bol. Soc. Brot.* 4:13. 1886.

Host(s) and Distribution: *Musa* sp.; Africa.

Mycosphaerella liukiuensis Sawada, *Special Publ. Coll. Agric. Natl. Taiwan Univ.* 63. 1959.

Host(s) and Distribution: *Musa formosana* (?), *M. liukiuensis*; Taiwan.

Mycosphaerella speckle
Mycosphaerella musae (Speg.) Syd. et P. Syd., *Phillipp. J. Sci.* 8:482. 1913.

≡ *Sphaerella musae* Speg., *Anal. Mus. Nac. Hist. Nat. Buenos Aires* 19:354. 1909.

= *Sphaerella musae* Sacc., *Atti Accad. Sci. Veneto-Trentino-Instriana, Ser.* 3, 10:67. 1917, homonym.

Host(s) and Distribution: *Musa acuminata, M. banksii, M. cavendishii, M. coccinea, M. paradisiaca, M. paradisiaca, M. textilis, M. uranoscopos, Musa* sp.; Argentina, Australia, Malaysia, Philippines, Puerto Rico, USA (HI), Virgin Islands.

Pseudocercospora fengshanensis (T.Y. Lin & J.M. Yen) J.M. Yen & S.K. Sun, *Cryptogam. Mycol.* 4:197. 1983.

≡ *Cercospora fengshanensis* T.Y. Lin & J.M. Yen, *Rev. Mycol.* 35:320. (1970) 1971.

Host(s) and Distribution: *Musa acuminata*; China, Taiwan.

Pseudocercospora musae-sapienti (A.K. Kar & M. Mandal) U. Braun & Mouch., *New Zealand J. Bot.* 37:317. 1999.

≡ *Cercospora musae-sapienti* A.K. Kar & M. Mandal, *Norweg. J. Bot.* 22:105. 1975.

Host(s) and Distribution: *Musa paradisiaca*; India, Wallis.

Pseudocercospora musicola U. Braun, *New Zealand J. Bot.* 37:317. 1999.

≡ *Cercospora musicola* Sawada (*musaecola*), *Rep. Gov. Agric. Res. Inst. Taiwan* 85:116. 1943 (nom. inval.).

≡ *Cercospora musicola* Goh & W.H. Hsieh, *Cercospora and similar fungi from Taiwan* (1990, p. 242). (nom. inval.).

= *Cercospora musae-liukiuensis* Sawada, *Special Publ. Coll. Agric. Natl. Taiwan Univ.* 8:221. 1959. (nom. nud.).

Host(s) and Distribution: *Musa acuminata, M. basjoo, M. liukiuensis, M. paradisiaca*; China, Japan, Taiwan.

Excluded from *Mycosphaerella*

Deightoniella leaf spot
Teleomorph state unknown (presumed not *Mycosphaerella*)
Anamorph: *Deightoniella torulosa* (Syd.) M.B. Ellis, *Mycol. Pap.* 66:7 (1957).

≡ *Brachysporium torulosum* Syd., *Hedwigia* 49: 83 (1909).

= *Cercospora musarum* S.F. Ashby, *Bull. Dept. Agric. Jamaica N.S.* 2:109. 1913.

Host(s) and Distribution: *Musa paradisiaca, Musa* spp.; Australia, Egypt, Jamaica, Malaysia, Sierra Leone, Somalia, Taiwan, Thailand, Vietnam.

Current and future research prospects

1. Given the obvious problems surrounding *Mycosphaerella* research discussed above, it is imperative that all researchers agree that they are working with the same disease. This needs to be firmly established by means of molecular techniques. Primers have been developed to identify *M. musicola* and *M. fijiensis* (Johanson *et al.*, 1994) but little is known about similar, closely related species, and whether they could be separated using these primers which are based on ITS sequence data. Although ITS has thus far proved to be very valuable in *Mycosphaerella* systematics, additional genes also need to be sequenced, as ITS alone indicates similarity, not necessarily conspecificity.

2. The data presently available in GenBank for species occurring on *Musa* indicate some variation within well-known taxa. This could either be due to sequencing errors, intraspecific variation, or to different researchers working with different species. To standardize the taxonomy and pathology research being conducted on these organisms, we need to agree on what they are. This can only be achieved by designating epitypes of the various species following a thorough taxonomic study. These cultures should then be lodged in culture collections and be readily available to those wishing to study the organisms occurring on *Musa*. In all these instances, it is best for mycologists to derive the cultures from fresh specimens, so that the identity can be confirmed on the host material, and once again in culture.

3. To fully understand species within *Mycosphaerella*, we need to collect both species and populations. We need to address questions relating to host specificity, speciation and, in respect to plant pathology, epidemiology, fungicide sensitivity, the importance of the different morphs and mechanisms of variation and dispersal. We also need to learn how the species are migrating around the world. To address these questions, we need to develop the correct molecular markers that can be used to for investigations at a species and a population level. Once again, these populations need to be deposited in reputable culture collections (i.e. CBS, IMI or ATCC) so that they can be studied and re-studied in years to come.

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Improved PCR-based detection of Sigatoka disease and black leaf streak disease in Australian banana crops

J. Henderson[1], K. Grice[2], J. Pattemore[1], R. Peterson[2] and E. Aitken[1]

Abstract

Accurate diagnosis of black leaf streak disease is often complicated by the presence of other fungal pathogens and in particular by the morphological similarity of the related species *Mycosphaerella musicola*, the causal agent of Sigatoka disease. In addition, high rainfall often washes away fungal structures making microscopic identification difficult. Starting in 1998, the Queensland Department of Primary Industries has been using molecular methods to help diagnose black leaf streak disease. A polymerase chain reaction (PCR) assay was used, but the protocol was found to lack specificity when applied to Australian isolates of the fungi. In July 2000, a project aimed at improving the sensitivity and specificity of the PCR as well as streamlining the assay was initiated. Various components of the PCR test were targeted for improvement. Homogenization of banana leaf tissue has eliminated possible cross-contamination while tripling batch throughput. An improved DNA extraction method produces cleaner DNA in less than half the time of the prior extraction method. Flexibility and sensitivity of the PCR has been improved by introducing a new enzyme while the new format PCR thermal cyclers have increased sample throughput. Importantly, specificity has been enhanced with the design of new diagnostic primers. These changes produce a definitive result during the first PCR in more than 98% of samples while increasing daily throughput more than eight-fold.

Resumen – Mejoramiento de la detección de la Sigatoka negra y Sigatoka amarilla basada en PCR en los cultivos bananeros de Australia

A menudo el diagnóstico preciso de la Sigatoka negra es complicado debido a la presencia de otros patógenos fungosos y en particular por la similitud morfológica de la especie relacionada *Mycosphaerella musicola*, agente causal de la Sigatoka amarilla. En adición, fuertes precipitaciones a menudo se llevan las estructuras fungosas dificultando la identificación microscópica. El QDPI ha estado utilizando métodos moleculares para confirmar el diagnóstico de la Sigatoka negra desde 1998. Se utilizó un ensayo de la reacción en cadena de polimerasa (PCR), sin embargo se descubrió que al protocolo le faltaba la especificidad al aplicarlo a los aislados australianos de los hongos. En julio de 2000, se inició un proyecto dirigido a mejorar la sensibilidad y especificidad del PCR así como la modernización del ensayo. Se seleccionaron varios componentes del examen

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PCR para ser mejorados. La homogenización del tejido foliar del banano con la ayuda de una micromano de mortero plástica dentro del tubo ha eliminado una posible contaminación cruzada al triplicar el rendimiento de los lotes. Un método mejorado de extracción de ADN produce un ADN más limpio en menos de la mitad del tiempo, que el método de extracción anterior. La flexibilidad y sensibilidad de PCR fueron mejoradas introduciendo una nueva enzima, mientras que nuevos variadores térmicos para el formateo de PCR han aumentado el rendimiento de las muestras. La especificidad de PCR ha sido mejorada a través del diseño de nuevos iniciadores de diagnóstico. Combinadas, estas mejoras producen un resultado definitivo durante el primer ensayo de PCR en más del 98% de las muestras, mientras que el rendimiento diario de la muestra es 8 veces mayor.

Résumé – Détection améliorée basée sur la PCR de la maladie de Sigatoka et de la maladie des raies noires dans les plantations de bananes en Australie
Le diagnostic exact de la maladie des raies noires est souvent rendu plus difficile par la présence d'autres pathogènes fongiques, et en particulier par la similarité morphologique d'une espèce voisine *Mycosphaerella musicola*, l'agent causal de la maladie de Sigatoka. De plus, de fortes précipitations éliminent souvent des structures fongiques ce qui rend l'identification au microscope difficile. En 1998, le *Queensland Department of Primary Industries* (QDPI) a commencé à utiliser des méthodes moléculaires afin de mieux identifier la maladie des raies noires. Un essai basé sur la réaction en chaîne par polymérase (PCR, *polymerase chain reaction*) a été utilisé mais ce protocole était peu spécifique quant aux isolats australiens du champignon. En juillet 2000, une étude a été initiée afin d'augmenter la sensibilité et la spécificité de la PCR et pour rationaliser le protocole. Divers composants du test PCR ont été ciblés afin d'être améliorés. L'homogénéisation des tissus de la feuille de banane a permis d'éliminer les contaminations extérieures tout en triplant le débit. Une méthode améliorée d'extraction de l'ADN permet d'obtenir un ADN plus pur en deux fois moins de temps. La flexibilité et la sensibilité de la PCR ont été améliorées grâce à l'utilisation d'une nouvelle enzyme. De plus, le nouveau format des thermocycleurs PCR a permis d'accroître le débit. Il est important de noter que la spécificité a été mise en valeur par la conception de nouvelles amorces diagnostiques. Ces changements produisent un résultat définitif dans la première PCR dans plus de 98% des cas et multiplient par huit le nombre d'échantillons traités par jour.

The Tully 2001 black leaf streak disease outbreak

The value of the Australian banana industry is estimated to be A$357 million (US$193 million) per year. In 2000, nearly 250 000 tonnes of bananas were produced by 100 growers in Australia. All Australian bananas are produced for consumption locally and 85% are of the 'Cavendish' variety. The majority of bananas are grown in northern Queensland, with 67% of the crop concentrated in Tully, Cairns and Innisfail (Figure 1).

In April 2001, the Australian banana industry suffered a potentially devastating outbreak of black leaf streak disease (caused by *Mycosphaerella fijiensis)* in Tully (Figure 1). This is the pathogen's first incursion in a major commercial region in Australia; failure to control the pathogen would have far reaching effects on the industry.

The Queensland Department of Primary Industries (QDPI) has had considerable success eradicating previous outbreaks of black leaf streak disease by plant destruction and replacement with resistant varieties. Since the initial discovery of black leaf streak disease in 1981 at Bamaga, an Aboriginal community located 40km

from the tip of the Cape York Peninsula, black leaf streak disease has been detected and eradicated eight times in far north Queensland. This ninth outbreak was in Tully where crops are estimated to be worth A$119 million per year (US$64 million).

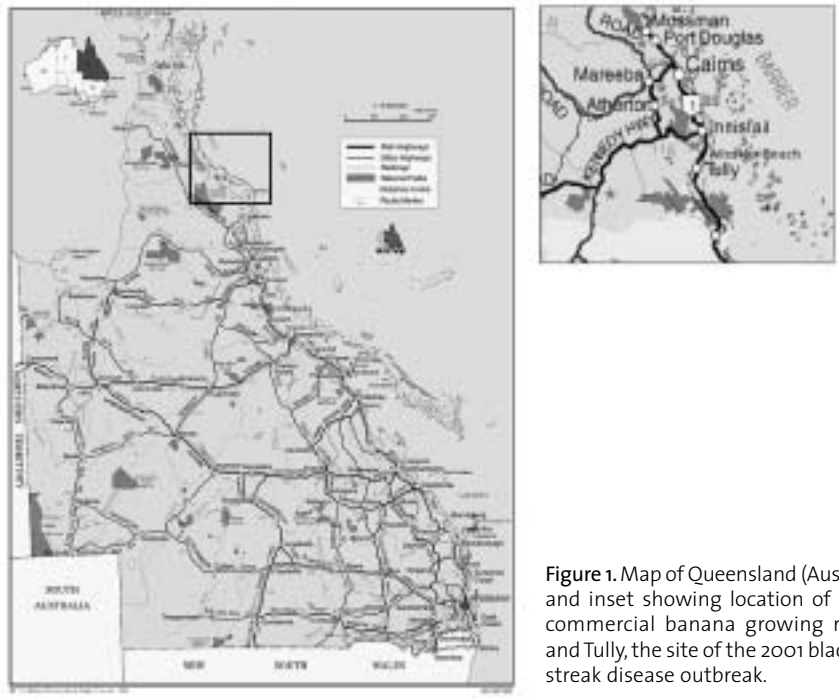

Figure 1. Map of Queensland (Australia) and inset showing location of major commercial banana growing region and Tully, the site of the 2001 black leaf streak disease outbreak.

Diagnosis of black leaf streak disease in Australia

Banana crops are routinely surveyed for black leaf streak disease by QDPI scientists at the Centre for Tropical Agriculture, Mareeba. Accurate diagnosis of black leaf streak disease is complicated by the morphological similarity of *M. fijiensis* to a related species *M. musicola*, which causes Sigatoka disease. Usually, experienced plant pathologists distinguish the two diseases by the development of symptoms and microscopical features of the fungi. In Tully, conidia were absent because of prolonged rainfall, and identification of morphological characters was not possible. Therefore, molecular methods were used for diagnosis.

The QDPI has used the polymerase chain reaction (PCR) to confirm diagnoses of *Mycosphaerella* leaf spot diseases since 1998 (Johanson, 1997). Approximately 10% of laboratory samples required confirmation by PCR. However, the method was slow and lacked specificity to some Australian and Torres Strait Island isolates of the fungi. The lack of specificity was possibly due to the high variability among the Southeast Asian populations of the pathogen. Populations of *M. fijiensis* from the Torres Strait were found to differ from those of the Pacific (Hayden, 2001). However, the Torres Strait populations and Pacific populations were found to be related to those from Papua New Guinea where there is a considerable diversity (Carlier, pers. comm.). In the original

study by Johanson *et al.* (1994) isolates from the Torres Strait were not included and it is possible that these isolates could be the source of the variability not detected by the original PCR primers.

In July 2000, a project between QDPI and the Cooperative Research Centre for Tropical Plant Protection (CRCTPP) was initiated with the aim of improving the specificity of the diagnostic procedure and increasing throughput in readiness for outbreaks of the disease[1]. Aspects of the PCR diagnostic procedure that were targeted for improvement included sample excision, homogenisation of banana leaf tissue, DNA extraction, PCR protocol, PCR primer design and equipment.

Flame sterilised cork-borers 4 mm in diameter have replaced scalpels for the removal of suspect lesions from banana leaves. The method is quick and simple and there is no cross-contamination between samples. Plastic micropestles have replaced ceramic mortar and pestles for homogenising leaf tissue. Micropestles have reduced the potential for cross-contamination between samples, eliminated transfer from mortar to tube, and have tripled the throughput. The rapid cetyltrimethyl ammonium bromide (CTAB) DNA extraction method (Stewart and Via, 1993) was adopted. It produces cleaner DNA in half the time.

New PCR primer sequences specific to *M. musicola* and *M. fijiensis,* and a modification of published ribosomal gene primer sequences (White *et al.*, 1990) to improve specificity to increase duplex stability of the primers with the target DNA (Rychlik, 1993), has improved specificity of the PCR assay. A size difference was also included in the PCR assay with the specific primer for *M. fijiensis* designed to the ITS1 and the specific primer for *M. musicola* designed to the ITS2 (Figure 2). Flexibility and sensitivity was improved by introducing the hot-start enzyme, *Taq*Gold DNA polymerase (PE Biosystems). *Taq*Gold DNA polymerase requires heat-activation before amplification can proceed. Therefore, non-specific amplification products are reduced and reactions can be left at 4°C until the addition of template. New equipment at the Centre for Tropical Agriculture has also improved throughput of assays. New format PCR thermal cyclers have increased tube capacity from 30 to 192 per run, and new electrophoresis equipment can analyse 52 samples for Sigatoka disease and black leaf streak disease at the same time.

The new methods produce a high quality DNA preparation and provide a definitive result during the first PCR in more than 98% samples. In addition, extraction time is more than halved and daily throughput increased by more than eight-fold.

Application of new molecular test

Use of the new methods in April 2001 coincided with Australia's most severe outbreak of black leaf streak disease. This was the first outbreak in a commercial growing area; previous outbreaks had been further north and in places where containment was easy. Fungal structures were absent on banana samples because of high rainfall at Tully. Therefore, diagnosis of up to 50% of samples was confirmed using the PCR assay. The PCR assay provided the Australian government and

[1] The project is co-managed by Ron Peterson (Principal Plant Pathologist, Mareeba QDPI) and Juliane Henderson (Research Officer, CRCTPP).

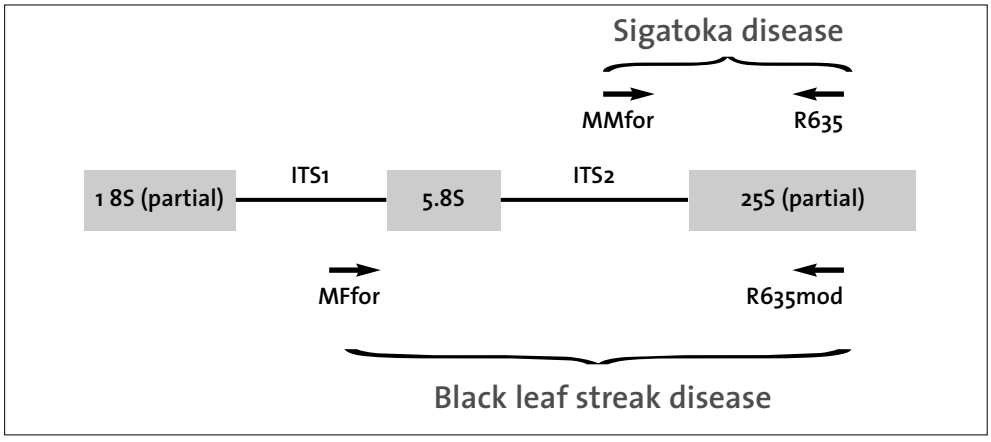

Figure 2. Location of primers for the diagnostic PCR assay. The black leaf streak disease specific forward primer (*MFfor*) is located on the ITS1 region while the Sigatoka disease specific forward primer (*Mmfor*) is located on the ITS2 region.

banana industry the confidence to start a A$20 million (US$10.8 million) surveillance and eradication plan in Tully, and more than 2500 PCR tests have been done.

The CRCTPP and QDPI continue to monitor and improve the PCR diagnostic. Thus, scientists from the CRCTPP improved homogenization of banana tissue by the use of glass beads shaken at high speeds. The commercially available "FastPrep" Instrument (Q-Biogene) processes 12 samples in 45 seconds and eliminates cross-contamination between samples by single-use O-ring tubes. Use of the method at the Centre for Tropical Agriculture is dependant on funds.

Opportunities to automate and improve specificity and sensitivity of the assay are being studied as part of the CRCTPP's plan to use new technology. Development of a real-time PCR assay to detect and differentiate *M. fijiensis, M. musicola* and *M. eumusae* is in progress. A fluorescent PCR format increases sensitivity and specificity, reduces cross-contamination, and increases throughput because post-PCR processing is not required.

To ensure the robustness of the PCR diagnostic and to facilitate development of new diagnostic assays for *Mycosphaerella* leaf spot diseases in Australia, the sequence variability in Australasian isolates of *M. musicola* and *M. fijiensis* will be studied[2]. First, the region incorporating the ITS1, 5.8S ribosomal gene and ITS2 will be cloned and sequenced from *Mycosphaerella* isolates from Australia, the Torres Strait Islands and Fiji. In collaboration with other groups studying sequences pertaining to the disease, we will compare our database with overseas isolates. If further sequence information is required, other conserved fungal genes, e.g. ß-tubulin, histone-4, glyceraldehyde-3-phosphate, will be investigated. The information from this study will help us to understand how the 2001 Australian outbreak arose, e.g. whether from one or several sources.

[2] Drs Elizabeth Aitken (Department of Botany, University of Queensland) and Juliane Henderson will use joint funding from the Australian Banana Industry Protection Board (BIPB) and Horticulture Australia Limited (HAL) to investigate sequence variability of *Mycosphaerella* causing leaf spot diseases on banana.

The status of black leaf streak disease in Australia is yet to be confirmed and the future application of diagnostic tests is uncertain. The method could be used to maintain Australia's disease-free status, as far as black leaf streak disease is concerned, or to monitor pathogen populations for control measures should the disease become endemic. Either would ensure that the best diagnostic assay is available to the Australian banana industry.

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Impact of minor *Mycosphaerella* pathogens on bananas (*Musa*) in South Africa

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Abstract

Of the species of *Mycosphaerella* known to occur on bananas, only *M. musicola* and *M. musae* occur in South Africa. Since both species are less damaging than *M. fijiensis* and *M. eumusae*, they are considered minor *Mycosphaerella* pathogens of this host. However, both *M. musicola* and *M. musae* can cause significant damage to bananas in the subtropics. For several years, *M. musicola* seemed to be the dominant pathogen of banana leaves in South Africa. It was very severe in banana plantations in Southern KwaZulu-Natal in the early 1990s, and caused losses of up to 50% in Cavendish bananas due to early ripening and lower yields in the Komatipoort area in 1999 and 2000. A highly coordinated disease management programme involving severe deleafing and fungicidal sprays has reduced the impact of Sigatoka disease in the country since 2001. However, Mycosphaerella speckle now appears to have replaced Sigatoka disease as the dominant leaf pathogen in all banana growing areas of South Africa. Management strategies for Sigatoka disease seem to be less effective against Mycosphaerella speckle. Although this fungus primarily affects older leaves, the disease has become very severe in southern KwaZulu-Natal during 2002. Its economic impact and epidemiology, however, still have to be determined.

Resumen – Impacto de los patógenos de *Mycosphaerella* de menor importancia sobre los bananos (*Musa*) en Africa del Sur

De las varias especies de *Mycosphaerella* que ocurren en los bananos, solo *M. musicola* y *M. musae,* ocurren en Africa del Sur. Ya que ambas especies causan menores daños que *M. fijiensis* y *M. eumusae*, ellas se consideran patógenos de *Mycosphaerella* de menor importancia en este hospedante. Sin embargo, tanto *M. musicola* como *M. musae* pueden causar daños significativos a los bananos en los subtrópicos. Durante varios años, *M. musicola* se consideró el patógeno dominante en las hojas de los bananos en Africa del Sur. A principios de la década de los 90, este patógeno afectó severamente las plantaciones bananeras en el sur de KwaZulu-Natal, y causó pérdidas de hasta 50% en los bananos Cavendish, debido a una maduración precoz y bajos rendimientos en el área de Komatipoort en 1999 y 2000. Un programa coordinado de manejo de la enfermedad, que incluyó deshoje y rociados de funguicidas redujo el impacto de la

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Sigatoka amarilla en el país desde 2001. Sin embargo, la mancha causada por *Mycosphaerella* parece haber reemplazado actualmente la Sigatoka amarilla como patógeno foliar dominante en todas las zonas productoras de banano en Africa del Sur. Parece que las estrategias de manejo de la Sigatoka amarilla son menos eficaces contra la mancha causada por *Mycosphaerella*. Aunque este hongo afecta principalmente las hojas viejas, la enfermedad se hizo muy severa en el sur de KwaZulu-Natal durante el año 2002. No obstante, aún falta determinar su impacto económico y la epidemiología.

Résumé – Impact des pathogènes mineurs de *Mycosphaerella* sur les bananiers (*Musa*) en Afrique du Sud

De toutes les espèces connues de *Mycosphaerella* affectant les bananiers, seules *M. musicola* et *M. musae* se trouvent en Afrique du Sud. Vu que ces deux espèces provoquent moins de dégâts que *M. fijiensis* et *M. eumusae*, elles sont considérées comme étant des pathogènes mineurs de cet hôte. Toutefois, *M. musicola* et *M. musae* peuvent toutes deux provoquer des dégâts significatifs aux cultures de bananes dans la zone subtropicale. Pendant plusieurs années, il semblait que *M. musicola* était le pathogène dominant des feuilles de bananiers en Afrique du Sud. L'infection était même très grave dans les plantations du sud du KwaZulu-Natal au début des années 1990 et en 1999 et 2000 a provoqué chez les bananiers Cavendish de la région de Komatipoort des pertes pouvant aller jusqu'à 50% dues à un mûrissement prématuré des fruits et à des rendements réduits. Un programme de gestion de la maladie parfaitement coordonné impliquant un défeuillage massif ainsi que des traitements fongicides a réduit l'impact de la maladie de Sigatoka dans le pays depuis 2001. Toutefois, Mycosphaerella speckle semble maintenant avoir remplacé la maladie de Sigatoka et se trouve être le pathogène dominant dans les régions de culture de la banane en Afrique du Sud. Les stratégies de gestion de la maladie de Sigatoka semblent moins efficaces envers le Mycosphaerella speckle. Bien que ce pathogène affecte en premier les feuilles les plus âgées, la maladie est devenue très grave dans le sud du KwaZulu-Natal en 2002. Son impact économique ainsi que son épidémiologie restent encore à être déterminés.

Introduction

Fungi that cause disease on leaves of banana and plantain include *Mycosphaerella fijiensis* M. Morelet (the causal agent of black leaf streak disease), *M. musicola* Leach ex J.L. Mulder (the causal agent of Sigatoka disease), *M. eumusae* Crous et X. Mourichon (the causal agent of eumusae leaf spot disease) and *M. musae* (Speg.) Syd. et P. Syd. (the causal agent of Mycosphaerella speckle). *M. fijiensis* is the most virulent and economically important of the four *Mycosphaerella* spp. Since its discovery in Fiji in 1963, *M. fijiensis* has replaced *M. musicola* as the main leaf pathogen in all tropical countries that produce banana (Jones, 2000). However, Sigatoka disease is still the main leaf disease in the subtropics and at higher altitudes in the tropics. In 1995, a new disease, eumusae leaf spot, was reported on *Musa* (Carlier *et al*., 2000). Eumusae leaf spot has only been found in Southeast Asia and in Nigeria, West Africa, (Jones, these proceedings) but is very damaging there. Black leaf streak disease, Sigatoka disease and eumusae leaf spot disease comprise the *Mycosphaerella* leaf spot disease complex on banana. Mycosphaerella speckle is not considered to be important on banana. It is limited to the subtropics and is severe only on Cavendish bananas in Australia (Jones, 2000).

The dominant *Mycosphaerella* spp. pathogens that occur in the subtropics are *M. musicola* and *M. musae*. Since neither consistently damage banana leaves, they

are considered minor pathogens. The objective of this manuscript is to report the impact of such minor *Mycosphaerella* pathogens on bananas in South Africa.

Banana production in South Africa

Bananas are produced in six areas in South Africa (Figure 1). The crop was introduced from India at the beginning of the 19th century. Production began along the southern and northern sections of the KwaZulu-Natal (KZN) coast, then introduced in the former Transvaal province and planted in Kiepersol, Tzaneen and Levubu. The largest production area, the Onderberg (4600 ha), became important in the 1990s. The total area of commercial banana production is 12 500 ha, and is with Cavendish cultivars only. Almost 90% of new plantings are from tissue culture, and transport of banana plants between production areas is strictly controlled. All bananas are consumed locally, but there is a possibility of export to the Middle East.

Leaf diseases of banana in South Africa

Since 1999, regular surveys of areas where banana is cultivated have shown that Sigatoka disease, Mycosphaerella speckle and Cordana leaf spot (caused by *Cordana musae* [Zimm.] Höhn.) are present in all production areas. Cladosporium speckle (*Cladosporium musae* E.W. Mason) was found only in Levubu. Sigatoka disease and Mycosphaerella speckle were the most important.

The banana leaf diseases in the southern part of Africa have not been studied very much. Black leaf streak disease is present in most tropical African countries (Jones, 2000), and has been reported as far south as northern Malawi (Ploetz, 1992). However, black leaf streak disease is not known in Zimbabwe (which borders the Levubu area) and Mozambique (which borders the Onderberg area) (Figure 1).

Figure 1.
The six banana production areas of South Africa: Levubu, Letaba (Tzaneen), Hazyview (Kiepersol), Onderberg, and northern and southern KwaZulu-Natal.

A severe outbreak of Sigatoka disease in 1999-2000, prompted an investigation into the identity of the causal agent. Samples were collected in all production areas, and the fungi identified using morphological and molecular techniques (Surridge *et al*., these proceedings). PCR primers developed by Johansen and Jeger (1994) were used to distinguish between Sigatoka disease and black leaf streak disease. Isolates from single conidia were further sequenced (ITS region) and compared with sequence data of *M. musicola*, *M. fijiensis* and *M. eumusae* from GenBank. All local isolates proved to be *M. musicola*, which causes Sigatoka disease. There was no evidence of *M. fijiensis* and *M. eumusae*, suggesting they have not been introduced in South Africa. The severity of the outbreaks was attributed to favourable weather conditions and increases in the amount of inoculum.

The life cycles of *Mycosphaerella* leaf spot diseases have a sexual (teleomorph) stage, which produces ascospores, and an asexual (anamorph) stage, which produces conidia (Jones, 2000). Conidia are the main spore produced by *M. musicola* (Meredith, 1970). Conidia are dispersed within the leaf canopy and to neighbouring plants by rain, which dislodges and washes them onto adjacent leaves. Ascospores are forcefully discharged and spread by wind currents over bigger distances than conidia. Both types of spore require moisture for production, release, infection, growth and sporulation. Most stages in the life cycle take place over a wide range of temperatures; however, minimum night temperatures of 18ºC and 21ºC are needed for the production of conidia and ascospores of *M. musicola,* respectively (Meredith, 1970). Conidia are produced on both leaf surfaces, while ascospore production is almost three times greater on the upper (adaxial) than lower (abaxial) leaf surface (Meredith, 1970).

Climatic conditions in South Africa

The banana production areas of South Africa are located in the east between 25º and 30º latitude and 30º and 32º longitude. The areas have a subtropical climate with cool, dry winters and warm, wet summers. Rainfall and temperature data for the Onderberg over a period of 10 years showed that November and March were the most favourable months for infection and disease development (Figure 2). During this time minimum night temperatures exceed 18ºC, which is necessary for the production of conidia. Minimum night temperatures exceed 21ºC only in January and February, therefore the period for ascospore production is short. Disease development is most rapid between November and March but slows substantially during the cooler months from May through September. Climatic conditions in South Africa provide ample opportunities for the management of Sigatoka disease.

The impact of *Mycosphaerella* diseases

Van den Boom and Kuhne (1969) first reported Sigatoka disease in South Africa, although the disease was also mentioned in 1954 (Meredith, 1970). The first report of Mycosphaerella speckle in South Africa was in 1973 (Brodrick, 1973). Despite these late reports, both diseases have been associated with banana leaves for as long as producers can remember.

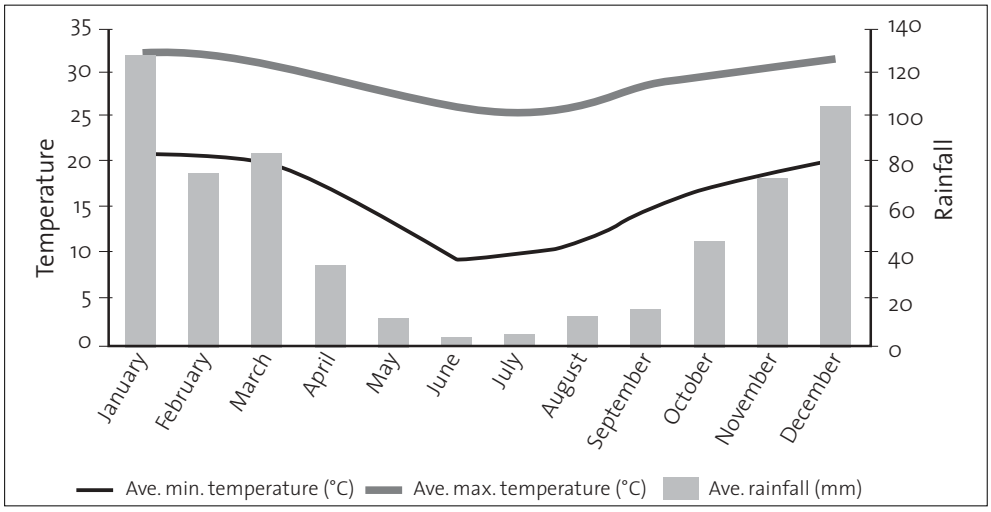

Figure 2. Average annual temperature range and rainfall in the banana growing areas of South Africa.

Sigatoka disease first became severe in South Africa during the 1960's (Van den Boom and Kuhne, 1969). The disease devastated production in southern KZN in the 1990s and in the Onderberg in 2000 (Viljoen, unpublished data). *Mycosphaerella musicola* infects the first three leaves of the banana plant. The symptoms first become visible on the third or fourth leaf (Jones, 2000). Under favourable weather conditions and with large amounts of inoculum, *M. musicola* can destroy all leaves after the stage when bunches are produced. This is what happened during the 1999-2000 outbreaks of Sigatoka disease in the Onderberg. Damage included smaller fruits, smaller bunches, and premature fruit ripening in the field and in boxes. Farmers reported losses of up to 50% of the crop. An extensive disease management programme was implemented in October 2000 to halt the devastation. All leaves with Sigatoka lesions were cut and turned over on the plantation floor to limit the release of air-borne ascospores. Many bunches were sacrificed, in one instance amounting to nearly 18 000 bunches on a farm of about 40 ha. A fungicidal spray programme with protectant and systemic fungicides when the rainy season started and night temperatures exceeded 18°C was recommended to growers. A total of six sprays of systemic fungicide were recommended, interrupted with a protectant fungicide after every second application of systemic fungicide (Peterson *et al.* these proceedings).

None of the farmers applied the recommended number of sprays, and only 2-4 sprays were applied in total. The cost of fungicides, therefore, was small compared with the costs of fungicide sprays used to control black leaf streak disease in the tropics. Sigatoka disease was almost absent from banana fields in 2001, and current control strategies are now limited to cutting leaves and the application of one or two sprays, dependant on pre-seasonal leaf spot incidence, per year.

Mycosphaerella musae infects older leaves of banana plants (Jones, 2000). Mycosphaerella speckle is rarely visible above the fifth fully open leaf, and seldom affects fruit quality and quantity after bunching. Since 2000, Mycosphaerella speckle has become

more severe, and now is the main leaf disease of banana in South Africa. The symptoms are leaf yellowing (chlorosis) and death (necrosis). Necrosis is most visible on the older leaves, but, in 2002, chlorosis affected leaves as young as the third leaf after bunching in southern KZN. The effects on yield have not yet been determined. Control strategies are similar to those for Sigatoka disease, and include removing leaves and applying fungicides.

Conclusion

Mycosphaerella musicola and *M. musae* are the only *Mycosphaerella* leaf pathogens of banana in South Africa. They are considered to be minor pathogens, but become damaging under favourable weather conditions and in the presence of large amounts of inoculum. Subtropical climatic conditions and a clear understanding of the biology and epidemiology of *M. musicola* make the management of Sigatoka disease relatively easy. The increased severity of Mycosphaerella speckle may result from the management of Sigatoka disease. The quantity of *M. musicola* ascospores released into the air is reduced by placing leaves upside down on the ground, but this probably increases the quantity of *M. musae* ascospores, which are mainly released from the lower leaf surface (Jones, 2000). A better understanding of the biology and epidemiology of *M. musae* is needed to develop the necessary management practices for Mycosphaerella speckle in the subtropics.

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Economic impact and management of black leaf streak disease in Cuba

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Abstract
Black leaf streak disease (caused by *Mycosphaerella fijiensis*) is the most damaging disease found in *Musa* plantations in Cuba. Four years after the appearance of the disease in 1990, it had replaced Sigatoka disease (caused by *M. musicola)* in all areas of the country. During 1991 and 1992, plantations of 'Cavendish' bananas received 23 fungicide applications per year, which accounted for 15% of total production costs. A system of integrated control based on cultural practices, a bioclimatic warning system for timing fungicide application and the use of systemic fungicides and mineral oil, reduced the number of applications to 13-15 per year and the cost of control to less than 40%. Black leaf streak disease has seriously affected the production of susceptible varieties. In 1989, more than 40 000 ha of plantain (*Musa* cv. AAB) and 14 000 ha of 'Cavendish' (*Musa* cv. AAA) were treated with fungicide. However, by the end of 1995 the areas had decreased by 69% and 51% respectively. Since 1994, 'FHIA-18', 'FHIA-03', 'FHIA-01-1', 'FHIA-02' and 'FHIA-21' with partial resistance to black leaf streak disease were introduced into Cuba. Currently, there are 10 000 ha planted with these clones resulting in an 80% reduction in the use of fungicide. The severity of black leaf streak disease on 'FHIA-18' has been inversely correlated with the availability of total K and with the ratio K/(K+Ca+Mg) in soil and foliage. Variability in the pathogenicity of *M. fijiensis* populations has been studied in order to identify possible changes that result from large scale cultivation of FHIA hybrids with partial resistance.

Resumen – Impacto económico y manejo de la enfermedad de la raya negra en Cuba
La enfermedad de la raya negra o Sigatoka negra, causada por *Mycosphaerella fijiensis*, es la enfermedad más nociva presente en las plantaciones de musáceas en Cuba. Cuatro años después de su aparición en 1990, reemplazó a la Sigatoka amarilla (*M. musicola*) en todas las áreas del país. Durante 1991 y 1992 se realizaron hasta 23 aplicaciones de fungicida por año en plantaciones de banano 'Cavendish' con un costo de protección alcanzando el 15% del costo total de la producción. Un sistema de manejo integrado basado en prácticas culturales y pronóstico bioclimático de los momentos de tratamiento permitió reducir a 13-15 los tratamientos con funguicidas sistémicos y aceite mineral al año en las principales plantaciones de producción con un costo menor de un 40%. La Sigatoka negra ha tenido un serio impacto en la producción de plátanos susceptibles. En 1989, existían más de 40 000 ha de plátanos

INISAV, Cuba

(*Musa* cv. AAB) y 14 000 ha de banana 'Cavendish' (*Musa* cv. AAA) bajo protección fúngica. A finales de 1995 se habían reducido en un 69 y 51% respectivamente. A partir de 1994, se introdujeron los clones 'FHIA-18', 'FHIA-03', 'FHIA-01-1', 'FHIA-02' y 'FHIA-21' con resistencia parcial a Sigatoka negra. En la actualidad existen 10 000 ha plantadas de estos clones y se ha reducido el consumo de fungicidas en un 80%. Se ha observado una correlación inversa entre la severidad de los ataques de Sigatoka negra en el clon FHIA-18 y la disponibilidad total de K y con la relación K/(K+Ca+Mg) en suelo y hojas. Se desarrollan estudios de la variabilidad genética de las poblaciones de *M. fijiensis* con el objetivo de determinar cambios de patogenicidad a causa del cultivo a gran escala de clones con resistencia parcial.

Résumé – Impact économique et gestion de la maladie des raies noires à Cuba
La maladie des raies noires, causée par *Mycosphaerella fijiensis,* est la maladie la plus destructrice des plantations de bananes à Cuba. Quatre années après l'apparition de la maladie en 1990, elle avait remplacé dans tout le pays la maladie de Sigatoka, causée par *M. musicola*. Pendant les années 1991 et 1992, les plantations de bananes 'Cavendish' ont reçu 23 traitements de fongicides par an , représentant 15% des coûts totaux de production. Un système de lutte intégrée basé sur les pratiques culturales, un système de prévision bioclimatique pour déterminer le moment d'application des fongicides et l'utilisation de fongicides systémiques et d'huile minérale, ont permis de réduire le nombre de traitements à 13-15 par an et les coûts de plus de 60%. La maladie des raies noires a sérieusement affecté la production de variétés sensibles. En 1989, plus de 40 000 ha de plantain (*Musa* cv. AAB) et 14 000 ha de bananes 'Cavendish' (*Musa* cv. AAA) ont été traités avec des fongicides. Toutefois, fin 1995, les surfaces traitées ont été réduites respectivement de 69% et 51%. Depuis 1994, 'FHIA-18', 'FHIA-03', 'FHIA-01-1', 'FHIA-02' et 'FHIA-21', des hybrides possédant une résistance partielle à la maladie des raies noires, ont été introduits à Cuba. Actuellement, 10 000 ha sont plantés avec ces clones ce qui entraîné une baisse de 80% de l'utilisation de fongicides. La gravité de la maladie des raies noires sur 'FHIA-18' a été inversement corrélée avec la disponibilité du K total et avec le rapport K/(K+Ca+Mg) dans le sol et le feuillage. La variabilité du pouvoir pathogène des populations de *M. fijiensis* a été étudiée afin d'identifier des modifications potentielles qui pourraient découler de la culture à grande échelle d'hybrides FHIA partiellement résistants.

Introduction

Many fungal, bacterial and viral diseases affect *Musa* but undoubtedly the one that has had the most socio-economic impact at a world level has been black leaf streak disease caused by the fungus *Mycosphaerella fijiensis* Morelet.

Black leaf streak disease was first reported in Cuba in 1991 (Vidal, 1992). Previously, the main banana and plantain disease in Cuba was Sigatoka disease caused by *M. musicola* Leach ex Mulder, for which a warning system (Ganry and Meyer, 1972b) for timing the application of fungicides in oil emulsions had been established (Perez, 1983, 1989). *Mycosphaerella musicola* causes considerable economic losses in 'Cavendish' cultivars (AAA) and occasionally in plantains (AAB), which generally show an acceptable level of resistance to the pathogen (Vakili, 1968; Perez *et al*., 1981).

This article reviews the economic impact of black leaf streak disease on banana and plantain production in Cuba, and summarises studies on the epidemiology, disease management and resistance of cultivars carried out over several years in Cuba.

Impact of disease

Protection cost

In the 1980s, 15 to 16 applications of mineral oil were carried out each year to control *M. musicola*. A quarter of applications contained mixtures of dithiocarbamate fungicides and two or three contained benomyl or propiconazole. By the end of the 80s, the use of a bioclimatic model to forecast the treatments, based on the method of scoring the rate of development of the disease (Ganry and Meyer, 1972a, b), led to a 30% reduction in the cost of controlling *M. musicola* (Perez, 1989). The cost per hectare in 'Cavendish' plantations varied between US $134 and US $241 (Figure 1). In 1991 and 1992, the first and second years after the first outbreak of black leaf streak disease, the cost per hectare rose to US$640 and US $801. The adoption of a warning system to time the applications of oil-based systemic fungicides (Perez *et al.*, 1993a, 2002) reduced the cost in later years (Perez *et al.,* 1993b, 1997, 2000a, b; Porras and Perez, 1997; Perez, 1998).

Changes in areas occupied by the cultivars

Banana and plantain production in Cuba is for local consumption due to a lack of export markets and to insufficient production levels to satisfy the high demand. The areas planted with different cultivars are shown in Figure 2. In 1990, more than 14 000 ha were planted with 'Cavendish' cultivars and treated with fungicide to control Sigatoka disease. In 2001, ten years after the arrival of black leaf streak disease, the area was reduced to 15% of that figure. In 1990, more than 43 000 ha were planted with plantains but black leaf streak disease reduced this to 18% in later years.

The 'Cavendish' plantations have been replanted with resistant FHIA hybrids, particularly 'FHIA-23' and 'FHIA-18' (AAAB). As a result, the amount of fungicide imported for controlling black leaf streak disease in 'Cavendish' has declined (Figure 3). The plantains have been replaced by 'Burro CEMSA 3/4' (ABB) and 'FHIA-03' (AABB).

Epidemiology of black leaf streak disease: relation betwen weather and disease development

Tables 1 and 2 show the correlation matrix between weather variables – the cumulated quantity and duration of rain over a 10 or 14-day period, the weekly Piche evaporation, the weekly accumulated hours with relative humidity over 95% (RH>95%) - and the state of development of the disease (Fouré, 1988) recorded weekly for eight weeks in 'Grande naine' and 'CEMSA 3/4'. High significative correlations were found between the accumulated quantity and duration of rain and RH>95%, and the state of development of the disease in banana and plantain.

In 'Cavendish' bananas, Perez *et al.* (1993b, 2000a) found the highest correlations between the cumulated quantity and duration of rain over a 10, 14 or 28-day period, and the state of development of the disease four to six weeks later. Regression

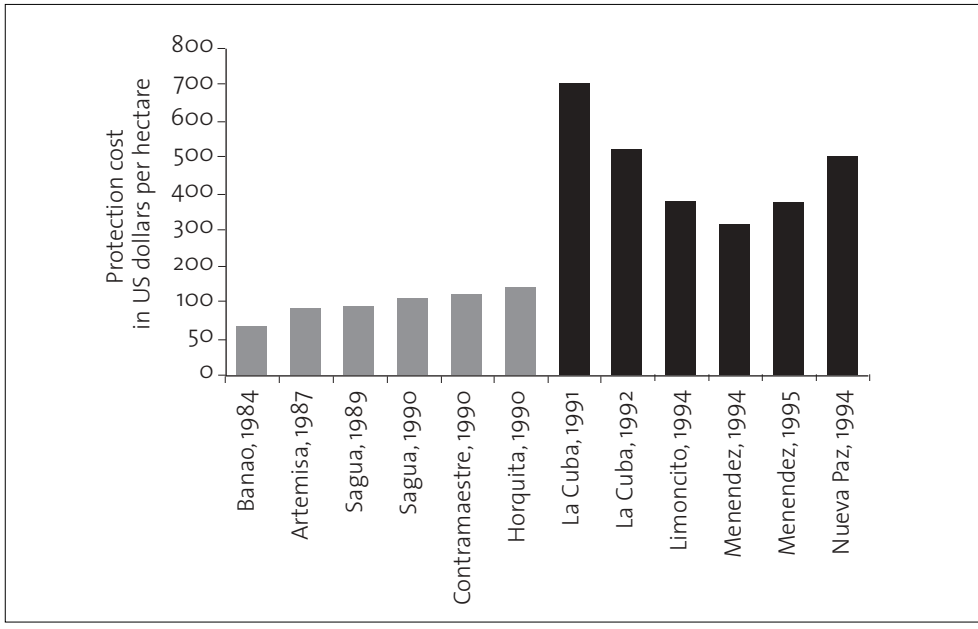

Figure 1. The cost of protection per hectare in various banana producing enterprises before and after the first report of *Mycosphaerella fijiensis* in Cuba. Bars for black leaf streak disease represent the costs in the first and second years after the disease appeared in each plantation.

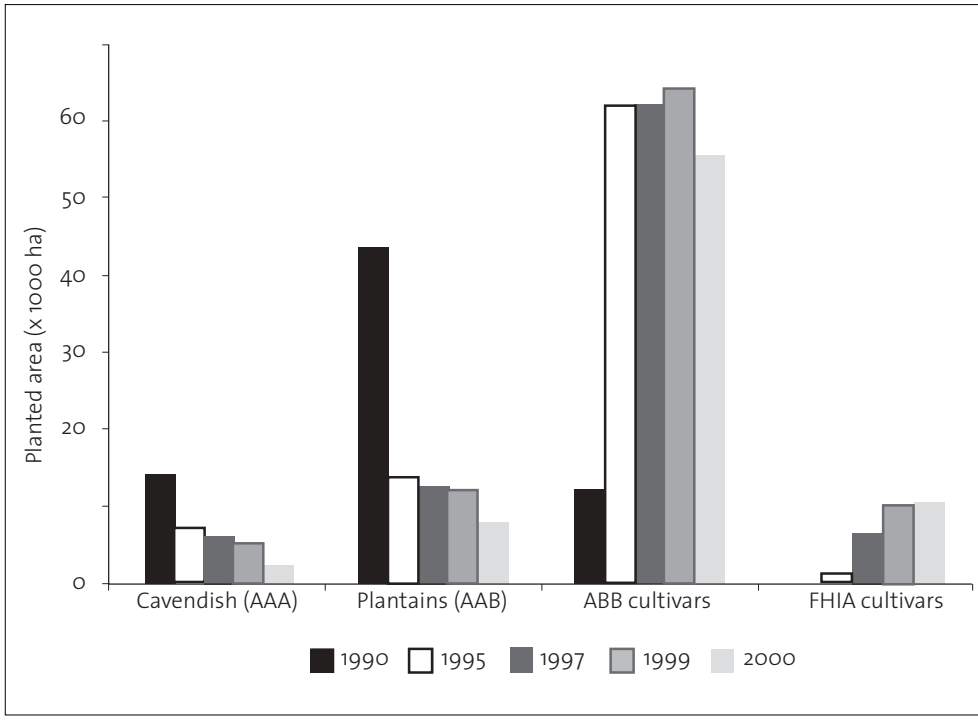

Figure 2. Surface area planted with various types of banana and plantain in Cuba, 1990-2000.

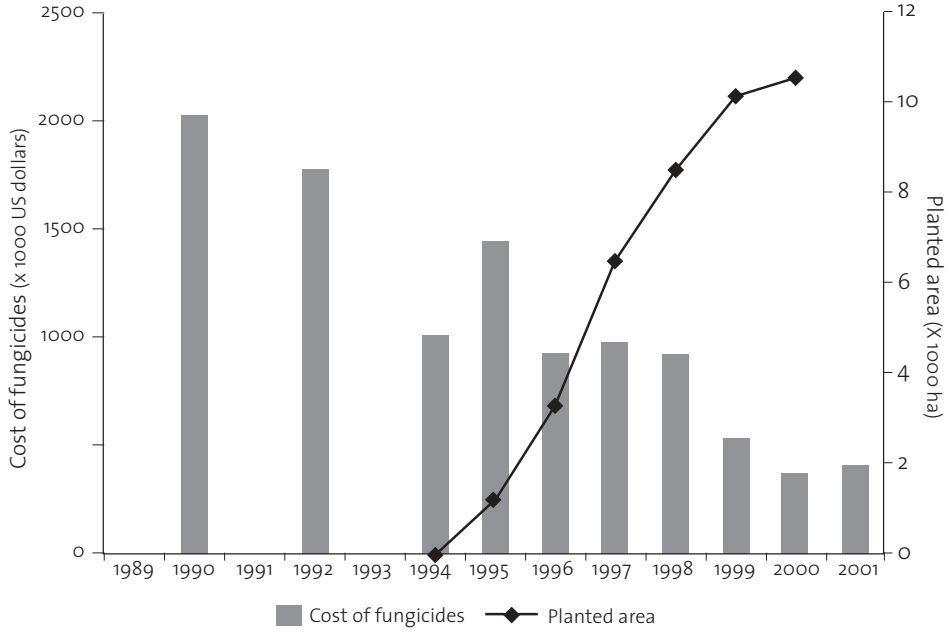

Figure 3. Cost of fungicides to protect 'Cavendish' plantations and area planted with FHIA hybrids that are partially resistant to black leaf streak disease.

equations were used for develop models to predict the development of black leaf streak disease as a function of the duration and intensity of rainfall. The observed and estimated state of development of the disease in 'Grande naine' as a function of rainfall four weeks before is shown in Figure 4.

In 'CEMSA 3/4', Perez e*t al*. (2000b), found high correlations between the state of development of the disease and the cumulated quantity and duration of rain over a 10 or 14-day period four to six weeks before, and the weekly Piche evaporation (PwEv) three to six weeks before (Table 2). The highest correlation coefficient was obtained between the cumulated quantity of rain over a 14-day period and the state of development of the disease five weeks later. In 'CEMSA 3/4', a highly significant negative correlation was found between PwEv and the state of development of black leaf streak disease three to five weeks later.

In general, the state of development of the disease in any week of the year is highly dependent on leaf wetness four and five weeks before. Many of the biological process of the fungus, such as mating between compatible isolates and pseudothecia development (Mourichon and Zapater, 1990), conidia development, ascospore and conidia germ tube growth (Porras y Perez, 1997) and ascospore release from pseudothecia (Stover 1980), are dependent on the presence of a water layer on the leaf surface or on high relative humidity.

In 'CEMSA 3/4', the cumulated duration of rain over a 14-day period and the state of development of the disease four weeks later are shown in Figure 5. The progressions and regressions of the disease were highly correlated with rainfall and leaf wetness.

Table 1. Correlation matrix between climatic factors and the state of development of black leaf streak disease in 'Grande naine', La Cuba 1995–1996. (Adapted from Perez *et al.*, 2000a).

Dependent variable	Independent variable	Number of weeks after the recording of the independent variable							
		0	1	2	3	4	5	6	7
SD4L	Rf7mm	n.s.	0.34*	n.s.	0.56 ***	0.61 ***	0.74 ***	0.41 *	n.s.
	RfD7 min	n.s.	n.s.	n.s.	0.45 **	0.48 **	0.77 ***	0.52 **	n.s.
	Rf10 mm	n.s.	n.s.	0.38 *	0.54 ***	0.80 ***	0.71 ***	n.s.	n.s.
	RfD10 min	n.s.	n.s.	0.37 *	0.39 *	0.74 ***	0.73 ***	n.s.	n.s.
	Rf14 mm	n.s.	n.s.	0.45 **	0.64 ***	0.77 ***	0.69 ***	n.s.	n.s.
	RfD14 min	n.s.	n.s.	0.38 *	0.51 **	0.75 ***	0.73 ***	n.s.	n.s.
	H7	n.s.	n.s.	n.s.	n.s.	0.72 **	0.71 **	0.53 *	n.s
	H10	n.s.	n.s.	0.55*	n.s.	0.79**	0.70 **	n.s.	n.s.
	H14	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	RH>95%	n.s.	n.s.	n.s.	0.316*	0.276*	0.308*	0.36**	0.36**
	PwEv	-0.27 *	-0.29 *	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	T Med	0.32 *	n.s.	0.29 *	0.29 *	n.s.	n.s.	n.s.	n.s.

* Statistically significant at probability 0.05. ** Statistically significant at probability 0.01. *** Statistically significant at probability 0.001. n.s.: Not statistically significant.
SD4L: State of development of black leaf streak disease in the four youngest leaves. Rf(n)mm: Cumulated quantity, in mm, of rain over a period of n days. RfD(n)min: Cumulated duration, in min, of rain over a period of n days. H(n): Cumulated quantity, in mm, of water on the leaves over a period of n days. PwEv: Weekly Piche evaporation.

Table 2. Correlation matrix between climatic factors and the state of development of black leaf streak disease in 'CEMSA 3/4' (*Musa* spp., AAB), La Cuba 1996. (Adapted from Perez *et al.*, 2000b).

Dependent variable	Independent variable	Number of weeks after the recording of the independent variable							
		0	1	2	3	4	5	6	7
SD4L	Rf7 mm	n.s.	n.s.	n.s.	0.46 **	0.48 **	0.60 ***	0.58 ***	0.55 **
	RfD7 min	n.s.	n.s.	n.s.	0.34 *	0.39 *	0.64 ***	0.55 **	0.54 **
	Rf10 mm	n.s.	n.s.	n.s.	0.51 **	0.59 ***	0.74 ***	0.62 ***	0.40 *
	RfD10 min	n.s.	n.s.	n.s.	0.40 *	0.50 **	0.74 ***	0.57 ***	0.38 *
	Rf14 mm	n.s.	n.s.	n.s.	0.52 **	0.65 ***	0.74 ***	0.64 ***	0.39 *
	RfD14 min	n.s.	n.s.	n.s.	0.41 *	0.58 ***	0.72 ***	62 ***	0.38 *
	H_7 (mm)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	H_{10} (mm)	0.49*	0.50*	n.s.	n.s.	0.73**	n.s.	n.s.	n.s.
	H_{14} (mm)	0.57**	0.57**	0.58*	n.s.	0.81**	0.55*	n.s.	n.s.
	PwEv	-0.44 **	-0.58 ***	-0.60***	-0.70***	-0.74***	-0.75***	-0.52**	n.s.
	T Med	n.s.	n.s.	n.s.	0.40 *	0.45 **	0.48 **	0.51 **	0.43 **

* Statistically significant at probability 0.05. ** Statistically significant at probability 0.01. *** Statistically significant at probability 0.001. n.s.: Not statistically significant.
SD4L: State of development of black leaf streak disease in the four youngest leaves. Rf(n)mm: Cumulated quantity, in mm, of rain over a period of n days. RfD(n)min: Cumulated duration, in min, of rain over a period of n days; H(n): Cumulated quantity, in mm, of water on the leaves over a period of n days. PwEv: Weekly Piche evaporation.

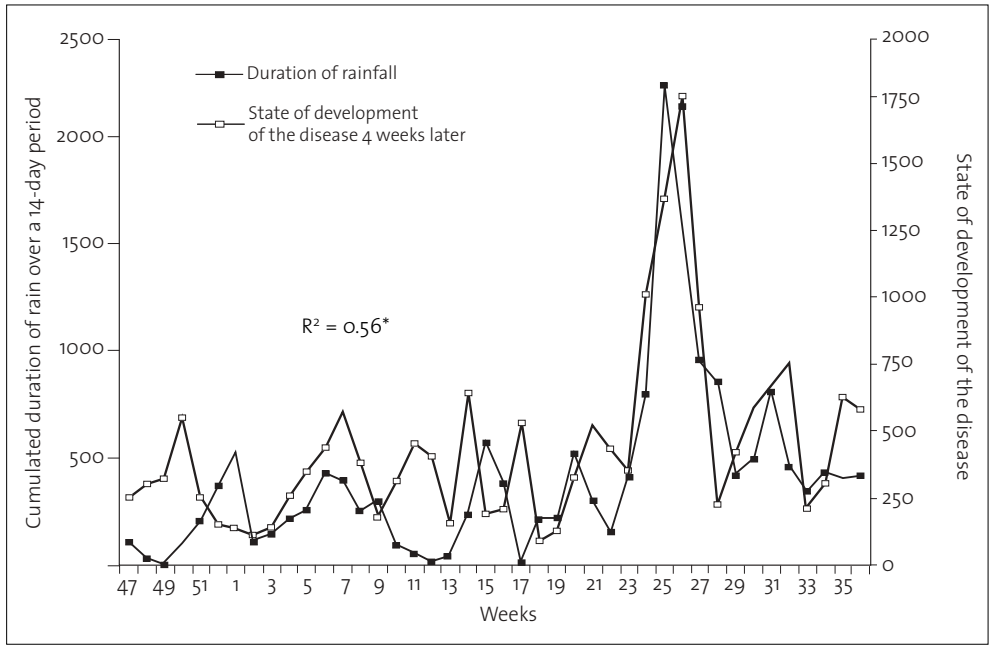

Figure 4. Correlation between the cumulated duration of rain, in minutes, over a 14-day period and the state of development of black leaf streak disease in the four youngest leaves (SD4L) of 'Grande naine' 4 weeks later. (From Perez *et al.*, 2000b).

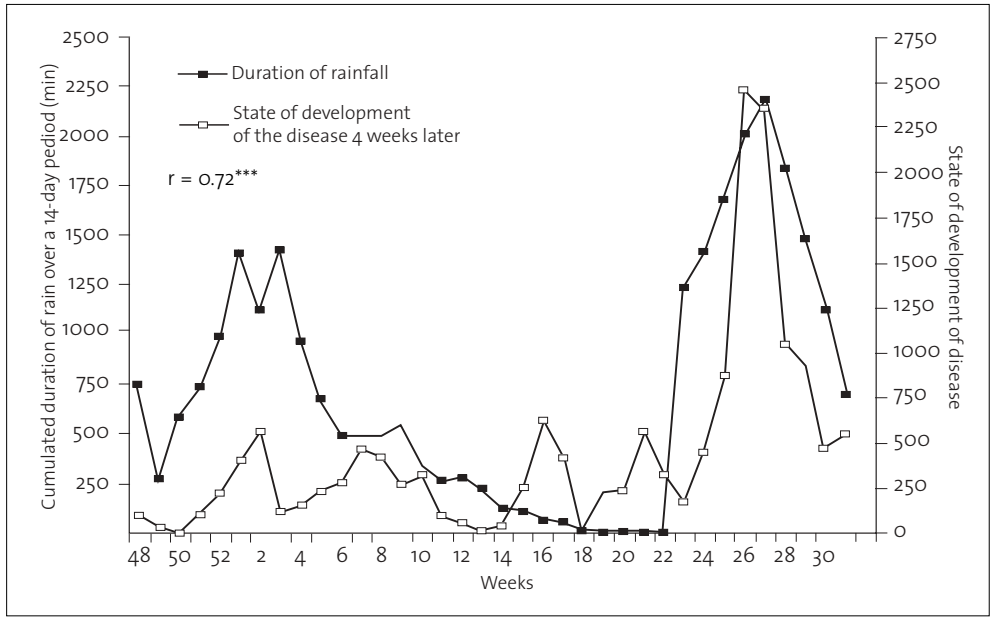

Figure 5. Curves of the cumulated duration of rain, in minutes, over a 14-day period and the state of development of black leaf streak disease in the four youngest leaves (SD4L) of 'CEMSA 3/4' 5 weeks later. (From Perez *et al.*, 2000b).

The curves of the observed and the estimated state of development of the disease using the model SD4L = 6.24 (S_{14d} Rfmm) – 198.2 PwEv + 1812 are shown in Figure 6. The model predicts the progress of the disease three weeks later.

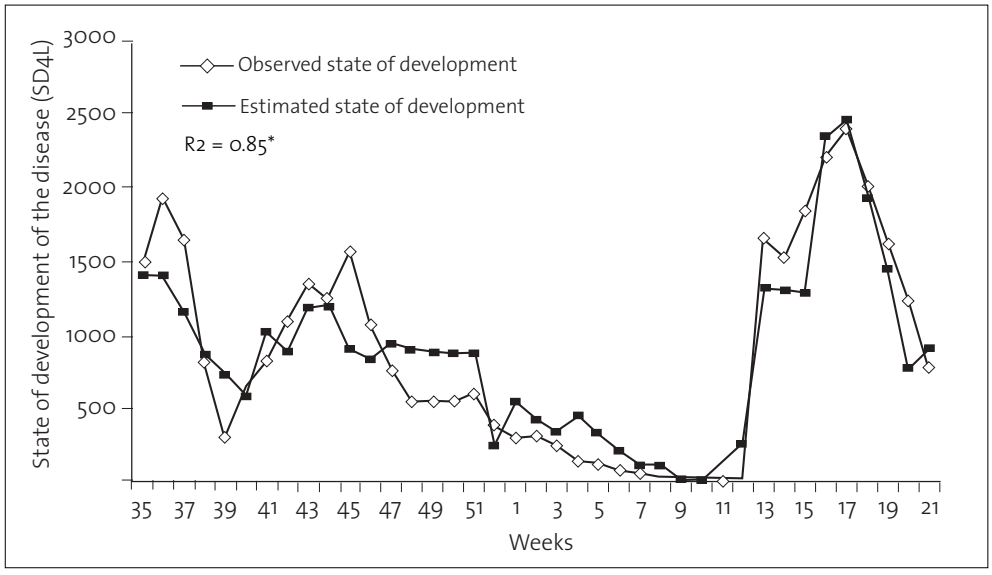

Figure 6. Observed and estimated state of development of the disease in the four youngest leaves (SD4L). The estimated values were derived using the model SD4L = 6.24 (S_{14d} Rfmm) – 198.2 EvPp + 1812. (From Perez *et al., 2000b).*

Between 1993 and 1996, 13 to 15 treatments of systemic fungicides per year were applied in 'Cavendish' plantations using the pattern of rainfall as the variable on which to base the decision of using fungicides. For example, the state of development of black leaf streak disease and the timing of fungicide treatments, using the bioclimatic model, are shown in Figure 7 for La Cuba in 1994.

Between 8 and 10 treatments of fungicides are required each year in plantain plantations in Cuba, to achieve an adequate control of black leaf streak disease. The treatments carried out using a bio-climatic model for the cultivar 'CEMSA 3/4' in La Cuba plantations during 1996 are shown in Figure 8. However, the low yields of plantain cultivars and the low prices paid for the product do not cover the costs of controlling black leaf streak disease.

A comparison of the cost of controlling black leaf streak disease using the bioclimatic model for timing applications with the cost of using a predetermined schedule of fungicide applications are shown in Table 3. The use of the bioclimatic model led to a 40% reduction in total costs, which resulted in an important reduction of the quantity of fungicides imported in Cuba to control black leaf streak disease in 'Cavendish' plantations (Perez *et al.,* 1993a, b, 1997, 2000a, b; Porras and Perez, 1997; Perez, 1998).

The model for timing applications of fungicides has shown to be effective in regions where the total annual rainfall is under 2000 mm. The system depends on the use of systemic fungicides able to inhibit the evolution of infections already established in the host at the time of the application.

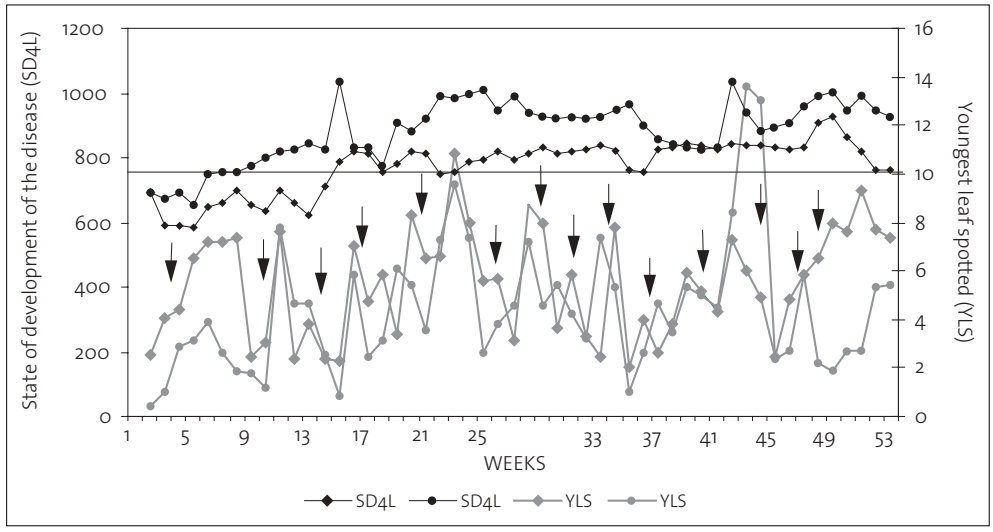

Figure 7. Black leaf streak disease control in a 'Grande naine' (AAA) plantation using the bioclimatic model for timing applications in La Cuba in 1994 (fields 1 and 2). Arrows indicate the moments of the application. (Adapted from Perez, 1998).

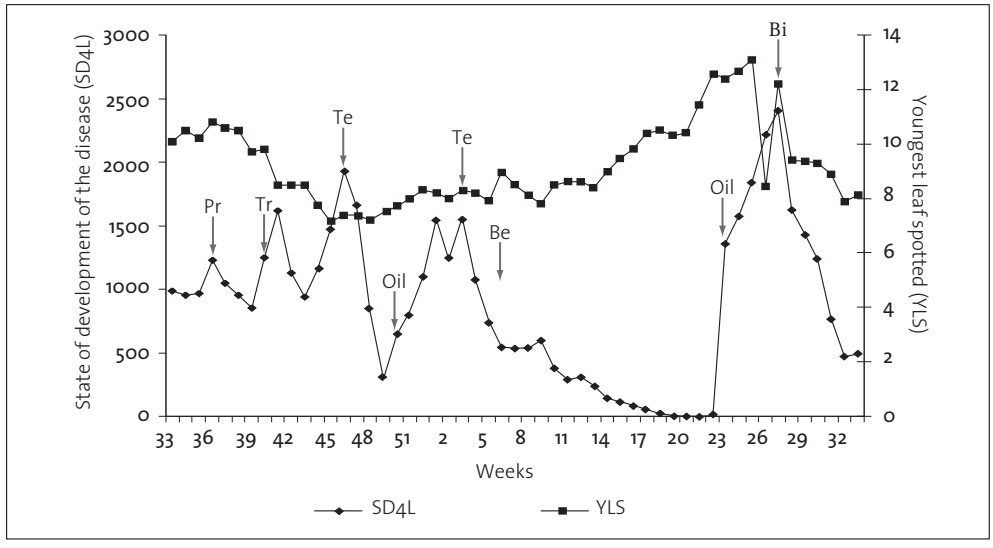

Figure 8. Black leaf streak disease control in a 'CEMSA 3/4' plantation using the bioclimatic model for timing applications in La Cuba, 1995–1996. Arrows indicate the moments of the application with tebuconazole (Te), propiconazole (Pr), mineral oil (Oil), benomyl (Be) and tridemorph (Tr). (Adapted from Perez *et al.* 2000b).

Resistance of cultivars

Different studies have been reported on the resistance of banana and plantains to *M. fijiensis* (Meredith and Lawrence 1970, Firman 1972, Fouré *et al.*1984, Fouré *et al.* 1990). Fouré *et al.* (1990) reported two different types of resistant reaction

against black leaf streak disease in *Musa*: the hypersensitive reaction observed in 'Yangambi km 5', and the partial resistance, that is expressed by the duration of the cycle of evolution of the disease and a reduction in reproduction of the pathogen. Studies done in 1995 (Hernandez and Perez, 2001), showed the reaction and components of the resistance to black leaf streak disease of various FHIA hybrids and *Musa* genotypes from the Cuba collection. Table 4 shows a significant increase in the incubation period (from leaf emergence and the appearance of the first symptoms), and in the transition period (from streak stage to necrotic spots), as well as a significant reduction in the production of pseudothecia. As a result, a reduction of the logistic rates of increment of infection (typical of partial resistance) takes place and the plants reach the flowering stage with a greater number of functional leaves.

Table 3. Comparison of the number and the cost of fungicide applications in 'Cavendish' plantations using a bioclimatic model and following a pre-determined schedule. (Adapted from Perez, 1998).

Plantations	Timing of application					
	Using a predetermined schedule			Using a bioclimatic model		
	Year	Number of treatments	Total cost (US$)	Year	Number of treatments	Total cost (US$)
La Cuba	1991	21	801.24			
	1992	23	619.66			
				1993	15	568.74
				1994	13	303.29
				1995	12	299.33
				1996	12	269.52
Limoncito	1994	22	476.19			
				1995	13	246.48
				1996	13	288.72
Quemado				1995	8	172.39
De Guines				1996	4 up to June	71.21
Menendez	1994	18	412.09			
	1995	23	518.49			
Sola				1994	11	221.56
				1995	12	282.78
				1996	11	237.31
Nueva Paz	1994	23	599.66	1996	13	326.56
Guines				1995	13	308.96
				1996	10	219.78
		(Hurricane)				

A comparison of the infection indices and the youngest leaf spotted (Stover and Dickson, 1970) are shown for FHIA hybrids plantations from four locations in Cuba, during the most favourable periods for disease development in each locality (Table 5).

Table 4. Reaction to black leaf streak disease of a group of FHIA hybrids in Cuba without fungicide protection. (Adapted from Hernandez and Perez, 2001).

Clones	Incubation period (days)		Transition period (days)		Number of functional leaves at harvest		Spot size (mm)	Number of pseudothecia
	February	June	February	June	Mother plant	First follower		
FHIA-1-1	43.5 b	28.2 a*	76.4**	75.5	9	8	17.3	15.9
FHIA-02	46.9 b	31.0 a	>150**	86.6	8	10	13.3	34.8
FHIA-03	60.4 a	24.1 b	>150**	107.7	10	8	15.5	31.6
FHIA-18	52.8 ab	28.7 a	>150**	119.0	12	9	14.3	35.0
SH 3436	35.8 c	28.0 a	84.3*	80.2	10	7	12.7	9.5
Grande naine	27.9 c	16.7 c	36.6	43.0	1	0	17.5	73.6

* Different letters indicate significant differences at probability 0.05.
** Most spots stopped developing at Fouré's stage 3 (Fouré, 1984).

Table 5. Infection index (II) and youngest leaf spotted (YLS) in FHIA hybrids and 'Cavendish' at the moment of maximum disease severity.

Cultivar	Locality and year	II (%)	YLS
FHIA-23	La Cuba, 1996	20.3	6.8
	La Cuba, 2001	16.3	7.8
	Alquízar, 2001	21.7	6.5
FHIA-18	La Cuba, 1996	2.1	11.0
	La Cuba, 2000 (Fca. Berlier)	28.2	5.6
	La Cuba, 2000 (Fca. Cozola)	23.2	6.4
	La Cuba, 2001	10.3	7.6
	Alquizar, 2001	14.2	10.6
	Baracoa, 2002	15.8	9.6
Cavendish	La Cuba, 1996	35.6	4.3
	La Cuba, 2001	-	-
	Alquizar, 2001	36.3	5.3
	Baracoa, 2002	59.8	4.3

In the last two years, there has been an increase in the severity of black leaf streak disease in experimental plots in Ciego de Avila compared with the levels observed in 1996, despite the fact that in 1996 the plots were largely surrounded by fields of 'Cavendish' bananas. Increased disease severity in Ciego de Avila has been specially marked on the 'FHIA-18' and 'FHIA-23' hybrids. The causes of the changes in the susceptibility are under study. A negative correlation has been observed between the level of K in soil and the severity of black leaf streak disease. Table 6 shows values for the concentration of K and the K/K+Ca+Mg ratio in the soil of farms with red latosolic soils planted with the cultivar 'FHIA-18' and not sprayed against black leaf streak disease, and disease severity expressed as the youngest leaf spotted.

Farms with the lowest content of K in the soil had the highest severity of black leaf streak disease (Table 6). Potassium seems to have an important role in the defence mechanisms of banana plants of the FHIA cultivars to pathogens. Peng *et al.* (1999)

reported that soils deficient in K are conducive to *Fusarium oxysporum* f.sp. *cubense* in Australia. Different stress factors have been shown to have an influence on peroxidase and phenylalanine ammonia lyase enzyme systems which are at the same time related to defence mechanism in plants to pathogens (Aguiar *et al.*, 2000).

A nationwide survey of pathogenic variability of populations of *M. fijiensis* has been undertaken based on the widespread use of FHIA hybrids in Cuba. A collection of single ascospore isolates of *M. fijiensis* from different cultivars collected at locations where FHIA hybrids have been extensively planted and from regions where they have not been introduced has been carried out. Artificial inoculations on selected hybrids under controlled conditions are in progress.

Table 6. Disease severity expressed as the youngest leaf spotted (YLS) in farms planted with 'FHIA-18' and availability of potassium in the soil, La Cuba, Ciego de Avila, in Nov 2000.

Farms	YLS	K (meq/100g soil)	K/(K+Ca+Mg)
El Berlier	5.6	0.44	1/59
Cozola	6.4	0.52	1/56
El Transformador (Cuba 3)	7.0	0.65	1/38
El Colorado (Farm 10)	8.0	0.85	1/39
La pista (Tin)	10.5	1.5	1/14
Cooperative	11.2	1.2	1/15

Conclusion

1. Black leaf streak disease has had a strong impact on the economy of growers and on banana and plantain production since the first appearance in Cuba. The costs/ha of control of black leaf streak disease increased fourfold due to the increase of the number of fungicide treatments. The annual cost of fungicides in Cuba for black leaf streak disease control in the first year after disease outbreak in Cuba reached US$2 million.

2. The area planted with 'Cavendish' cultivars and with plantains had been reduced to 15% and 18% respectively of the existing area previous to the introduction of black leaf streak disease. At the same time, the area planted with FHIA hybrids that are resistant to black leaf streak disease is steadily increasing, leading to a 19% reduction in the amounts of fungicides imported during the two first years following the arrival of black leaf streak disease.

3. The development of the disease in Cuba is highly correlated with the cumulated quantity and duration of the rain over a 14-day period, four weeks before in the case of 'Cavendish', and five weeks before in the case of plantains (AAB). These relationships can be useful for timing fungicide treatments in banana and plantains.

4. From 1993 to 1997, bioclimatic warnings were used in the main 'Cavendish' production plantations in Cuba, which allowed an optimization of control measures and a 40% reduction in the costs of protecting against black leaf streak disease.

5. Resistance of the different FHIA hybrids is expressed as a longer period of incubation, a longer period of transition from streaks to spots, and a reduction of the production of pseudothecia, all of which are typical of partial resistance. The severity of black leaf streak disease on the cultivar 'FHIA-18' is currently higher in soils with low K contents.

6. Studies are in progress to determine the potential for *M. fijiensis* populations to change pathogenicity as a result of the extensive planting of resistant hybrids.

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Management of black leaf streak disease in tropical Asia

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Abstract
In the Philippines, diseases are the major production constraint in the region. Leaf spot diseases cause significant fruit yield and quality reduction in both commercial banana plantations and in small-scale farms. Black leaf streak disease is the most important banana leaf spot disease in the region. Although eumusae leaf spot disease has been reported in India, Sri Lanka, Thailand and Malaysia, the extent of damage has not yet been established. Sigatoka disease is no longer a major concern. The damage due to black leaf streak disease affects mostly smallholders as they generally do not implement any systematized disease management programme. Disease management in commercial plantations is based on the use of fungicides together with cultural practices that reduce the inoculum of the disease and therefore its development. Changes in the cropping/production system in the commercial plantations of Southern Philippines have had an impact on the management of *Mycosphaerella* leaf spot diseases. The adoption of annual cropping reduced disease pressure and the need for year-round fungicide applications.

Resumen – Estado del manejo de la Sigatoka negra en Asia Tropical
El las Filipinas, las enfermedades representan la principal limitación en la región. Las enfermedades de las manchas foliares provocadas por el género *Mycosphaerella* causan una reducción significativa del rendimiento y calidad de la fruta tanto en las plantaciones comerciales como en pequeñas fincas bananeras. La Sigatoka negra es el problema de las manchas foliares más importante en la región. Aunque se ha reportado la presencia de *Mycosphaerella eumusae (*mancha foliar eumusae*)* en India, Sri Lanka, Tailandia y Malasia, la dimensión de los daños que ella causa aún no se ha establecido. La Sigatoka amarilla ya no representa la principal preocupación. El daño debido a la Sigatoka negra se observa principalmente al nivel de los pequeños agricultores, ya que, básicamente, ellos no implementan ningún programa de manejo sistematizado en comparación con las plantaciones comerciales. El manejo de las enfermedades empleado en las plantaciones comerciales es un programa basado en fungicidas reforzado por prácticas culturales que reduce el inóculo de la enfermedad y reduce así su desarrollo. Los cambios en los sistemas de cultivo y producción tuvieron cierto impacto sobre el manejo de la mancha foliar de Sigatoka en las plantaciones comerciales en el sur de Filipinas. La adopción del cultivo anual redujo la presión de la enfermedad y así se evita la necesidad de la aplicación de fungicidas durante todo el año.

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Résumé – Gestion de la maladie des raies noires en Asie tropicale
Aux Philippines, les maladies constituent la principale contrainte de la production. Les maladies foliaires causées par les *Mycosphaerella* réduisent le rendement et la qualité des fruits dans les plantations commerciales, ainsi que dans les petites fermes. Dans la région, la maladie des raies noires est la plus importante maladie foliaire attaquant la banana. Bien que l'ELSD (*eumusae leaf spot disease*) ait été observée en Inde, au Sri Lanka, en Thaïlande et en Malaisie, l'étendue des dégâts n'a pas encore été établie. La maladie de Sigatoka n'est plus considérée comme importante. Les dégâts dus à la maladie des raies noires affectent principalement des petits fermiers car ils ne peuvent généralement pas mettre en place de programme systématique de gestion des maladies. La gestion des maladies dans les grandes plantations est basée sur l'utilisation de fongicides ainsi que sur des pratiques culturales visant à réduire la quantité d'inoculum et donc le développement de la maladie. Des changements dans le système de production dans les plantations commerciales du sud des Philippines ont eu un impact sur la gestion des maladies foliaires causées par les *Mycosphaerella*. Le fait d'avoir adopté une culture annuelle a réduit la pression de la maladie et le recours aux fongicides tout au long de l'année.

Introduction

Banana (*Musa* spp.), a group of plants that comprises many different types of sweet dessert bananas, cooking bananas and plantains, is an important fruit crop in Asia. Bananas are grown largely by smallholder farmers, traded by local entrepreneurs and consumed locally. Thus, banana plays a major role in food security and is a source of income for the rural poor. The Philippines is the only major banana-exporting country in Asia and bananas generate important foreign exchange. In summary, banana is an important food and source of income for local farmers and for the region.

The main constraints to banana production and threats to the industry in the region are from pests and diseases. The region is the centre of origin of *Musa*, and hence many serious pests and diseases affect the crop, e.g. leaf spot diseases caused by *Mycosphaerella* spp. are responsible for a reduction in fruit yield and quality in commercial plantations and on small-scale farms.

Three species of *Mycosphaerella* are present in Asia. *M. fijiensis,* responsible for black leaf streak disease, is the most important pathogen because of its wide distribution and its virulence which gives rise to epidemics. *M. eumusae,* a newly reported pathogen, is potentially devastating and has been reported in India, Sri Lanka, Thailand and Malaysia but not, so far, in the Philippines. *M. musicola*, the causal agent of Sigatoka disease, is present in Southeast Asia but is no longer of major importance.

In commercial plantations, even if the disease does not affect yield, it can still reduce fruit quality and render the fruit unfit for export. Leaf spot diseases also cause fruits to ripen prematurely during transport to the market.

Disease management practices

Banana production systems in Southeast Asia are classified as follows (1) backyard, (2) mixed crop, (3) commercial smallholder plantation and (4) large commercial export-oriented plantation. The first three are intended for local markets and production is on a small scale. Management of leaf spot diseases varies according to the system of production.

Small-scale farming

Leaf spot disease management by small-scale banana growers ranges from minimal to none, that is there is no systematic management of the disease. The disease reduces fruit size on susceptible cultivars but the fruits are still acceptable to the local market. Local consumers are not exacting in terms of fruit quality, size and ripening characteristics, unlike the commercial export market. Thus, small-scale banana growers make a certain amount of profit even when leaf spot diseases are not controlled.

In addition, small-scale banana growers plant many varieties of banana. Figure 1 shows the various local cultivars grown in the Philippines and their proportion relative to total production. This variety of cultivars caters to the local demand for different uses or consumption of bananas. Some varieties are used cooked, processed or as fresh banana. The planting of different varieties provides genetic diversity against black leaf streak disease. Several important local varieties, e.g. 'Saba' and 'Pelipita' in the Philippines and 'Pisang kepok' in Indonesia, are resistant to *Mycosphaerella* leaf spot diseases. Hence, for banana growers who specialize in cooking bananas, leaf spot diseases are not important. 'Lakatan', 'Pisang berangan', and some 'Cavendish' varieties are susceptible to black leaf streak disease, but still produce yields that are acceptable to the local market.

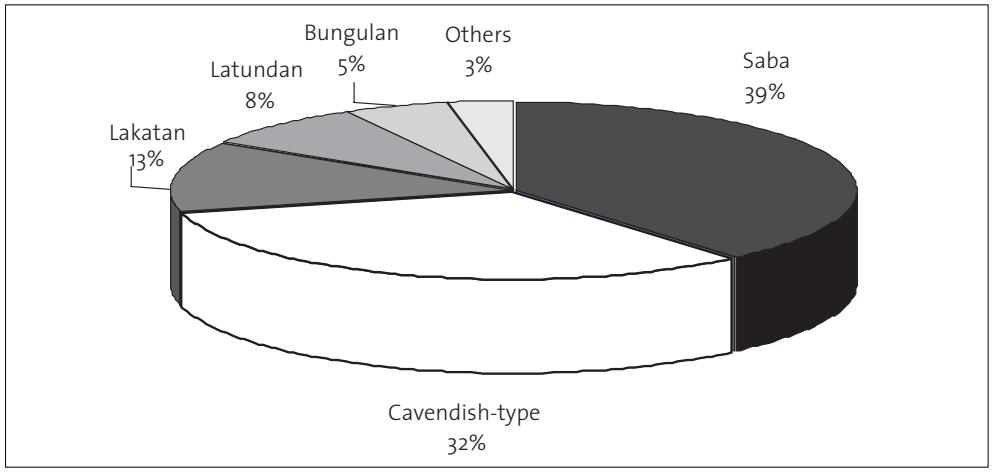

Figure 1. Most popular banana cultivars grown in the Philippines.

Chemical control is not practised by small-scale growers because of the expense. Better-off local growers may remove severely diseased leaves as a means of reducing inoculum and infection but none use fungicides. The variety of cultivars planted and the diversity of growing conditions reduce the risk of disease in comparison with large-scale monocrop commercial plantations.

Commercial plantations

A high level of disease control is required in commercial plantations for the export market. In Southeast Asia, only the Philippines export large quantities of bananas.

The high standards of fruit quality and the characteristically large-scale monocultures used in the production of banana for export require intensive disease management. Thus, the cultivation of a single banana variety 'Cavendish' and monocropping make disease management in commercial plantations very challenging. However, the value of the export market supports the use of expensive disease management practices.

The high levels of disease control achieved by a mixture of fungicides and cultural practices reduce inoculum and conditions that favour disease development. In the Philippines, fungicides may be applied up to 40 times per year (Table 1). The control programme includes contact fungicides, which are alternated or mixed with systemic fungicides applied in water or as an oil-water emulsion. The fungicides and doses used by plantation owners are approved by the local regulatory agencies. In addition, the fungicide doses are within the tolerances permitted by the importing countries. Table 2 lists the fungicides used in a typical commercial plantation in the Philippines.

Table 1. Spray programme of LADC, Davao, Philippines.

	Number of cycles				
Fungicide	**1997**	**1998**	**1999**	**2000**	**2001**
Strobilurin	0	0	4	6	6
Triazole	9	11	8	8	8
Dithane/Vondoze/Maneb	4	4	11	12	12
Daconil	11	8	7	6	5
Calixin	7	9	8	8	7
Total cycles	**31**	**32**	**38**	**40**	**38**
Oil used (L//ha/yr)	**76**	**82**	**147**	**119**	**130**

Table 2. Fungicides used by commercial growers in the Philippines.

Trade name	Chemical name	Volume/hectare
Systemic		
Bankit 25 SC	Azoxystrobin	0.4 L
Baycor 300 EC	Bitertanol	0.5 L
Bumper 25 EC	Propiconazole	0.4 L
Calixin 75 EC	Tridemorph	0.6 L
Folicur 250 EC	Tebuconazole	0.23 L
Indar 2F	Fenbuconazole	0.4 L
Siganex	Pyrazophos	0.5 L
Sico 250 EC	Propiconazole	0.4 L
Tega 075 EC	Trifloxystrobin	1.0 L
Tilt 250 EC	Propiconazole	0.4 L
Protectant		
Daconil 720 SC	Chlorothalonil	1.38 L
Dithane F448	Mancozeb	4.0 L
Dithane M-45	Mancozeb	2.5 kg
Dithane OS	Mancozeb	1.75 L
Vondozeb 42 SC	Mancozeb	4.0 L

The high dependence on the very specific fungicide propiconazole in the late 80s to early 90s resulted in a considerable increase in insensitivity of *Mycosphaerella*.

However, the loss of effectiveness was less and occurred later than in Central America. The prolonged effectiveness in the Philippines is possibly because spray programmes have always been based on the principle of rotation and/or combinations of fungicides, avoiding block or consecutive applications of the same products. The fungicide Benomyl (Benlate) was used unwisely in the late 70s and resulted in a high degree of fungicide resistance. Since then, Benlate has been withdrawn from use in the Philippines. The introduction of newer chemicals such as Azoxystrobin has provided a much-needed opportunity for fungicide control in combination with triazoles.

Commercial plantations also integrate cultural and other practices to their disease management in order to reduce conditions that favour disease development. Good drainage and irrigation practices receive attention. The removal and destruction of severely affected leaves also reduce inoculum.

Monitoring of disease severity on plants with or without a flower is regularly practiced in commercial plantation. In some plantations, early detection and quantification of symptoms are also done. The data are used to schedule fungicide treatments as well as a guide for harvest to reduce the risk of the effects of leaf spot diseases on fruit quality. Frequently, when disease is severe and reduces the numbers of functional leaves, fruits are harvested earlier to avoid the risk of premature fruit ripening during transit to the market. This also removes a source of inoculum from the plantation.

Changes in cropping/production systems have also had important effects on the management of leaf spot diseases in commercial plantations in the southern Philippines. Adoption of annual cropping reduced disease pressure and the need for year round fungicide application. In Taiwan, where annual cropping was introduced more than a decade ago, black leaf streak disease is no longer a problem. Expanded banana commercial production in the Philippines included a few thousand plantations of 'Lakatan' to supply the lucrative local market, but not controlling leaf spot diseases as intensely is providing an abundant source of inoculum for the export 'Cavendish' plantations, making control programmes more difficult. The export market has also increased considerably during the last decade (Figure 2). The increased area devoted to monoculture crops has increased the risk of epidemics.

Use of resistant varieties

As mentioned before, some popular local cultivars are resistant to black leaf streak disease. However, many of the *acuminata* dessert type bananas, e.g. 'Cavendish', 'Lakatan' and'Pisang berangan', are susceptible to black leaf streak disease.

Several of the varieties provided by INIBAP through its International *Musa* Testing Programme (IMTP) proved to be resistant to black leaf streak disease in Southeast Asia. In particular, the FHIA series are very resistant to this disease in field trials. The high yield potential and disease resistance of these varieties make them attractive to tropical Asian farmers and consumers. It is worth noting that, being the centre of origin of banana, Asian consumers in the tropics are used to eating different kinds of banana of different size, colour and taste. Moreover, Asians prepare bananas for a variety of uses. Hence, disease-resistant, high-yielding hybrids have the potential to increase productivity of bananas in tropical Asia.

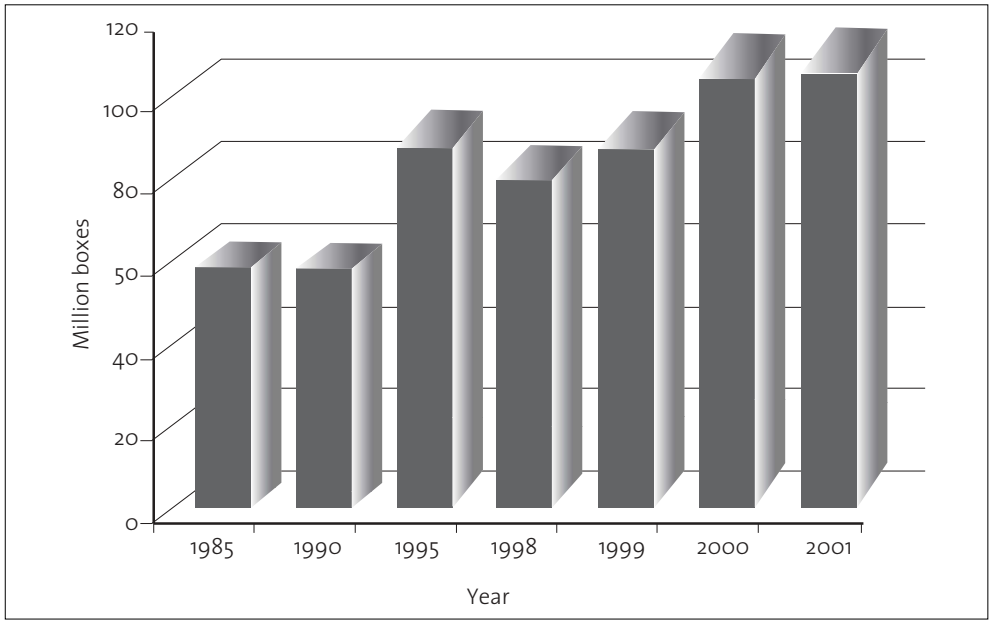

Figure 2. Number of 13-kg boxes exported by the Philippines.

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Impact of *Mycosphaerella* spp. in Brazil

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Abstract
Brazil ranks second in the world for banana production. Bananas are grown throughout the country, mainly by smallholders. Of the *Mycosphaerella* species present in Brazil, *M. musicola* (anamorph *Pseudocercospora musae)*, the causal agent of Sigatoka disease, and *M. fijiensis* (anamorph *Pseudocercospora fijiensis)*, the causal agent of black leaf streak disease, are the most serious. They can cause a 100% yield loss on susceptible varieties from the Cavendish (*Musa* cv. AAA) and Prata (*Musa* cv. AAB) groups. Sigatoka disease is present in all banana growing areas of the country. Yield loss is dependent on environmental conditions but is estimated at 50% on average. Black leaf streak disease is still confined to the north region of the country plus the State of Mato Grosso (centre-west region). It causes a 100% yield loss in dessert bananas and around a 70% yield loss in plantain, a crop which is very important as a staple food in northern Brazil. Since 1999, susceptible cultivars have been gradually replaced by resistant cultivars, e.g. 'Caipira' (AAA), 'Thap maeo' (AAB), 'FHIA-18' (AAAB) and 'Pacovan Ken' (AAAB), especially in the State of Amazonas, following the recommendations of the Brazilian agriculture research corporation (*Embrapa*). Traditional varieties have been replaced by cultivars resistant to black leaf streak disease because of the high yield losses and the lack of opportunity for growers to use fungicides. In addition there is the socio-economic impact of the ban by the Federal and State authorities on the transport of banana fruits from infected areas, designed to prevent the spread of black leaf streak disease to other banana growing regions of the country.

Resumen – Impacto de *Mycosphaerella* spp. en el banano en Brasil
La producción bananera es una actividad muy importante en Brasil, el segundo productor del mundo de este cultivo. El banano se cultiva en todo el país, principalmente por pequeños agricultores. Entre las especies de *Mycosphaerella* que se dan en Brasil, *M. musicola* (anamorpho *Pseudocercospora musae*), agente causal de la Sigatoka amarilla, y *M. fijiensis (*anamorpho *Paracercospora fijiensis*), agente causal de la Sigatoka negra, son las más serias. Ellas han sido responsables por pérdidas de rendimiento de hasta 100% en las variedades susceptibles como las que pertenecen a los grupos 'Cavendish' (*Musa* cv. AAA) y Prata (*Musa* cv. AAB). La Sigatoka amarilla se encuentra en todas las áreas productoras de banano del país causando pérdidas de rendimiento que varía de acuerdo a las condiciones ambientales prevalecientes. En

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promedio, las pérdidas debido a la Sigatoka amarilla en Brasil se estiman en un 50%. La Sigatoka negra está aún confinada a la región norte, más, el estado de Mato Grosso (región central occidental). Además de causar pérdidas de rendimiento del 100% en los bananos de postre, la Sigatoka negra también causa pérdidas de rendimiento de un 70% en plátano, muy importante para la región norte de Brasil. En esta región, especialmente en el estado de Amazonas, a partir de 1999, los cultivares susceptibles han sido reemplazados gradualmente por los cultivares resistentes, recomendados por la Corporación Brasileña de Investigación Agrícola (*Embrapa*) como: 'Caipira' (AAA), 'Thap maeo' (AAB), 'FHIA-18' (AAAB) y 'Pacovan Ken' (AAAB). El reemplazo de las variedades tradicionales fue forzado tanto por las altas pérdidas de rendimiento, como por la inhabilidad de los productores de insertar los agroquímicos como parte del sistema de producción. Además de la sostenibilidad ecológica de variedades resistentes, también se observan los impactos socioeconómicos en las acciones de las organizaciones federales y estatales que previenen el transporte de las frutas de banano de las áreas infectadas, dirigidas a proteger las regiones del país libres de las enfermedades producto de la diseminación de la Sigatoka negra.

Résumé – Impact des *Mycosphaerella* spp. au Brésil

Le Brésil est le deuxième producteur mondial de banane. Les bananiers sont cultivés dans tout le pays surtout par des petits producteurs. De toutes les espèces de *Mycosphaerella* présentes au Brésil, *M. musicola* (anamorphe *Pseudocercospora musae)*, l'agent causal de la maladie de Sigatoka, et *M. fijiensis* (anamorphe *Pseudocercospora fijiensis)*, l'agent causal de la maladie des raies noires, sont les plus sérieuses. Elles peuvent provoquer jusqu'à 100% de perte de production sur des variétés sensibles comme celles des groupes Cavendish (*Musa* cv. AAA) et Prata (*Musa* cv. AAB). La maladie de Sigatoka est présente dans toutes les régions productrices de bananes du pays. La réduction du rendement dépend des conditions environnementales mais en moyenne elle est estimée à 50%. La maladie des raies noires est encore confinée dans la région Nord du pays ainsi que dans l'état du Mato Grosso (région centre-ouest). Elle provoque des pertes de 100% chez la banane dessert et d'environ 70% chez la banane plantain, un aliment de base important dans le nord du Brésil. Depuis 1999, les cultivars sensibles ont été progressivement remplacés par des cultivars résistants, par exemple 'Caipira' (AAA), 'Thap maeo' (AAB), 'FHIA-18' (AAAB) et 'Pacovan Ken' (AAAB), particulièrement dans l'état de l'Amazone suivant les recommandations de la société brésilienne de recherche en agriculture (*Embrapa*). Étant donné les réductions importantes du rendement et l'impossibilité qu'ont les cultivateurs à utiliser des fongicides, les variétés traditionnelles ont été remplacées par des cultivars résistants à la maladie des raies noires. De plus, l'interdiction, par les autorités fédérales, de transporter les bananes provenant de zones infectées, afin de limiter la propagation de la maladie des raies noires vers d'autres régions du pays, a un impact socioéconomique certain.

Introduction

The cultivation of banana in Brazil plays an important economic and social role in large and small plantations. Smallholders grow bananas mainly as a subsistence crop. Plantains are grown as a staple food, mainly in the north and northeastern regions of the country.

Brazil is the world's second largest producer of bananas and production is estimated at six million metric tons per year. The total area cultivated with banana is about 533 000 hectares, distributed throughout the country: 8% in the south, 28% in the southeast, 8% in the center west, 32% in the northeast and 24% in the north.

Of the various phytosanitary problems that affect banana production in Brazil, the most important are those related to leaf diseases caused by *Mycosphaerella musicola* and *M. fijiensis,* wilt diseases caused by *Fusarium oxysporum* f. sp. *cubense* and *Ralstonia*

solanacearum race 2, and soft rot caused by *Erwinia carotovora*. Nematodes, mainly *Radopholus similis*, also affect the banana crop, and the weevil borer *Cosmopolites sordidus* is perhaps the most important insect pest. Due to the increasing demands by the consumer for fruits of better quality and appearance, pre and postharvest fruit diseases and damage to fruit by arthropods, e.g. thrips, have become increasingly important. Because they reduce the quantity and quality of bananas, and are difficult and expensive to control, leaf spot diseases caused by *M. musicola* and *M. fijiensis* are considered to be the most serious diseases affecting the Brazilian banana industry.

Impact of *Mycosphaerella musicola*

Sigatoka disease, caused by *M. musicola* Leach (anamorph *Pseudocercospora musae* (Zumm) Deighton) was first reported in Java, in 1902. Sigatoka disease is found in all banana growing regions except for Israel, Egypt and the Canary Islands. The disease was first reported in 1944 in the Amazon region of Brazil. Eight years later, the disease was found in the southeast region, the largest banana production region of Brazil. Today, Sigatoka disease is widespread throughout Brazil causing severe yield losses in those regions where environmental conditions favour disease development.

In Brazil, *Mycosphaerella musicola* results in an average yield loss of about 50% which may be higher in particular regions of the country. In banana orchards affected by Sigatoka disease, the number of bunches and of hands per bunch is lower and the fruits, in addition to being smaller and lighter, ripen prematurely. A high incidence of Sigatoka disease causes early decline of the banana orchard. Plant vigour declines and yield is reduced in later crop cycles (Cordeiro and Matos, 2001) and orchards have to be replanted at shorter intervals than usual.

According to Cordeiro (1990), by the end of the 80s, the cost of control using five applications per year of systemic fungicides plus mineral oil accounted for 9% of the total production cost estimated at US$1350/ha/year. In recent years, the cost has increased because of the need to increase the numbers of applications to seven per year. Today, the cost of controlling Sigatoka disease amounts to about 10% of the total production cost. In areas where fungicide control measures are not used, yield losses can exceed 80% and fruit quality is very poor (Figure 1).

Figure 1.
Damage from Sigatoka disease on 'Prata aña' in the absence of control measures.

Impact of *Mycosphaerella fijiensis*

Black leaf streak disease was first reported in 1963, in the Fiji Islands, district of Sigatoka. In America, the disease was first reported in Honduras in 1972, where it was called black Sigatoka. The disease spread from Honduras throughout the banana growing areas of Central and South America. In Brazil, black leaf streak disease was first discovered in 1998 (Pereira *et al.,* 1998; Cordeiro *et al.,* 1998), in banana orchards located in the municipalities of Tabatinga and Benjamim Constant, State of Amazonas, on the borders of Brazil, Colombia and Peru.

Distribution of black leaf streak disease in Brazil

Following the discovery of black leaf streak disease in Brazil, surveys were carried out to follow its spread. By the end of 1998, black leaf streak disease was found in the State of Acre, probably introduced accidentally from Bolivia. A survey in early 1999, revealed high levels of black leaf streak disease in Rodônia, in the northern region of the state, and Mato Grosso, in the central western region (Cordeiro *et al.,* 2000). Recent surveys in the northern region of Brazil showed that the disease had spread throughout the region, e.g. the States of Pará, Amapá, Roraima, Amazonas, Acre and Rondônia (Gasparotto *et al.* 2001a). Figure 2 shows the distribution of black leaf streak disease in northern Brazil, inclu0ding the State of Mato Grosso, and the central western region.

Phytosanitary defence strategy

The yellow band in Figure 2 corresponds to a buffer zone, and includes the States of Mato Grosso do Sul, Goiás, Tocantins and Maranhão, where a phytosanitary defence strategy has been put in place to delay as much as possible the spread of *M. fijiensis* to other banana growing areas currently free of the disease (Figure 2). Planned actions include training for technicians and banana growers with emphasis on the recognition of the symptoms of black leaf streak disease, field evaluations of resistant varieties, e.g. 'Caipira' (AAA), 'Thap Maeo' (AAB), 'FHIA-18' (AAAB), 'Pacovan Ken' (AAAB) which are recommended by Embrapa. Dependent on the agreement of growers, orchards in the buffer zone will be replanted with varieties resistant to black leaf streak disease in order to establish a barrier to prevent the spread of the pathogen. The movement of bananas from areas affected by black leaf streak disease to disease-free areas is restricted; a phytosanitary certificate of origin is required before permitting transport of fruits to other areas.

Impact of black leaf streak disease

It is not yet possible to quantify exactly the impact of black leaf streak disease on banana production in Brazil. In general, banana cultivation is characterized by a low level of technology, mainly subsistence cultivation without the use of fungicidal control measures. Nevertheless, field evaluations of fungicides to control black leaf streak disease in the Amazon region have shown that approximately

26 applications with systemic fungicide and up to 52 applications of protectant fungicides would be necessary to give adequate control of black leaf streak disease in areas where weather favours the disease (Gasparotto *et al.,* 2001b; Pereira and Gasparotto, 2001). The large numbers of fungicide applications are ten times higher than the total fungicide applied for the control of Sigatoka disease in some areas of Brazil. In areas with clear dry periods, 15 fungicide applications give sufficient control. Even in these conditions, the numbers of applications is twice that needed to control Sigatoka disease.

Figure 2. Distribution of black leaf streak disease in Brazil (April 2002).

The biggest impact of black leaf streak disease in the Amazon region is the need to change the varieties that are cultivated. According to growers of 'Prata comum' in the Amazon region, banana production fell almost to zero after the arrival of black leaf streak disease in the municipalities of Benjamim Constant and Tabatinga in the State of Amazonas. The only way to continue banana production in the area would be the use of varieties resistant to black leaf streak disease. To support the use of resistant varieties, the government should acquire micropropagated plantlets for distribution to growers. At present, however, a clear decision has yet to be made about the preferred resistant cultivar, based on information about yield performance and consumer acceptance. Since 1999, the Embrapa tissue culture laboratory has sold 1 384 003 plantlets of varieties resistant to black leaf streak disease to the states affected by the disease (Table 1).

Table 1. Varieties resistant to black leaf streak disease and number of plantlets sold to the area of incidence of the disease in Brazil.

Variety	Number of plantlets sold by 2001	Number contracted from 2002	Total number of plantlets
Thap maeo (AAB)	301 968	350 000	651 968
Caipira (AAA)	648 100	200 000	848 100
FHIA-01 (AAAB)	42 000	-	42 000
FHIA-18 (AAAB)	163 735	300 000	463 735
SH36-40 (AAAB)	128 150	-	128 150
FHIA-21 (AAAB)	9 000	-	9 000
FHIA-03 (AABB)	5 900	-	5 900
FHIA-10	3 000	-	3 000
Ouro (AA)	4 650	-	4 650
Prata zulu (ABB)	3 000	150 000	150 000
PV03-44 (AAAB)	74 500	-	74 500
PV42-85 (AAAB)	50	-	50
Total	1 384 053	1 000 000	2 384 053

Research activities

Before the discovery of black leaf streak disease in Brazil, all banana genotypes generated by Embrapa's breeding programme were sent to Costa Rica to evaluate their resistance in collaboration with CATIE, INIBAP and CORBANA. At present, genotypes have been evaluated in the states of Acre and Amazonas, in collaboration with Embrapa Acre and Embrapa Western Amazon. In addition to supporting the banana breeding programme in Brazil, the knowledge generated by researchers has also helped the phytosanitary defence strategy. One of the most important contributions to fight black leaf streak disease in Brazil has been the delivery of resistant varieties. In addition, experiments have showed that spores of *M. fijiensis* can survive for 60 days on several types of surface including clothes, fruits, wood and iron (Hanada *et al.,* 2000). That observation gave strong support to the legal actions that ban the transport and sale of fruits from affected to disease free areas.

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Poster

Fungi associated with banana foliage in South Africa

A.K.J.Surridge, A. Viljoen and F.C. Wehner

Abstract

A comprehensive investigation was conducted to determine the identity, distribution and importance of fungi associated with banana leaves in South Africa. Banana leaves were randomly collected from the five banana growing areas in the country. Spores were isolated from leaf lesions following surface sterilization and incubation in moisture chambers or taken directly collected from lesions. Single spores were then cultured on half-strength potato dextrose agar. Both molecular and morphological techniques were applied to identify the isolates. Four main diseases were found in the different banana growing areas. Yellow Sigatoka (caused by *M. musicola*), Mycosphaerella speckle (caused by *M. musae*) and Cordana leaf spot (caused by *Cordana musae*) were present in all five areas, whereas, Cladosporium speckle (caused by *Cladosporium musae*) only occurred in Levubu. Many other fungi, predominantly saprophytes and endophytes, were also isolated. The most common species include (in order of predominance) *Nigrospora sacchari, N. sphaerica* and *N. oryzae*.

Resumen - Hongos asociados con el follaje del banano en Africa del Sur

En Africa del Sur, se llevó a cabo una amplia investigación con el fin de determinar la identidad, distribución e importancia de los hongos asociados con las hojas de banano. Las hojas de banano se recolectaron al azar en cinco zonas bananeras del país. Los aislados se hicieron de las lesiones foliares después de la esterilización de su superficie, incubación en cámaras húmedas, o las esporas se recolectaron directamente de las lesiones. Luego, las esporas individuales fueron cultivadas en el agar de dextrosa de patata de fuerza media. Para identificar los aislados se emplearon técnicas tanto moleculares como morfológicas. En diferentes zonas productoras de banano se descubrieron cuatro enfermedades principales. La Sigatoka amarilla (causada por *M. musicola*), la mancha Mycosphaerella (causada por *M. musae*) y la mancha foliar Cordana (causada por *Cordana musae*) se encontraron presentes en todas las cinco áreas, mientras que la mancha por Cladosporium (causada por *Cladosporium musae*) solo se encontró en Levubu. También se aislaron muchos otros hongos, predominantemente saprofíticos y endofíticos. Las especies más comunes incluyen (en orden de predominancia) *Nigrospora sacchari, N. sphaerica* y *N. oryzae*.

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Résumé – Champignons associés au feuillage du bananier en Afrique du Sud
Une recherche détaillée a été faite afin de déterminer l'identité, la répartition et l'importance des champignons associés aux feuilles de bananier en Afrique du Sud. Des feuilles de bananiers ont été récoltées au hasard dans les cinq régions productrices du pays. Des spores ont été isolées des lésions foliaires après stérilisation de la surface foliaire et mise en incubation dans des chambres humides ou prélevées directement sur les lésions. Des spores isolées ont ensuite été cultivées sur un milieu gélifié à l'agar à demi-concentration de dextrose de pomme de terre. Des techniques moléculaires et morphologiques ont été appliquées afin d'identifier les isolats. Quatre maladies principales ont été trouvées dans les différentes régions de culture de la banane. La maladie de Sigaoka (causée par *M. musicola*), le Mycosphaerella speckle (causé par *M. musae*) et le Cordana (causé par *Cordana musae*) étaient présents dans les cinq zones, alors que le Cladosporium (causé par *Cladosporium musae*) n'a été trouvé qu'à Levubu. De nombreux autres champignons, surtout des saprophytes et des endophytes, ont également été isolés. Les espèces les plus communes sont, dans l'ordre d'importance, *Nigrospora sacchari, N. sphaerica* et *N. oryzae*.

Introduction

Among the various fungi associated with the foliage of banana plants, pathogens such as *Mycosphaerella musicola, M. fijiensis* and *M. eumusae* cause significant losses. Others, e.g. *M. musae*, *Cladosporium musae* and *Cordana musae,* can become damaging under certain climatic conditions. In addition to these pathogens, various species of endophytic fungi have also been reported on *Musa* species. The most commonly isolated are *Colletotrichum gloeosporioides*, *Nigrospora oryzae*, *Pestalotiopsis palmarum* and *Phoma* spp. (Brown *et al.*, 1998 ; Photita *et al.,* 2001). Some of these, e.g. *N. oryzae*, are also known to cause minor disease on their host plant (Ellis, 1971).

In South Africa, the fungal pathogens previously reported on banana include *M. musicola* (Van den Boom and Kuhne, 1969), *M. musae* (Brodrick, 1973) and *Cordana musae* (Roth, 1965). However, these reports were based solely on symptom sightings and not on isolation of the causal organisms. This study was conducted to determine, and update existing knowledge of, the identity of fungi associated with banana leaves in South Africa.

Materials and methods

Banana leaves were randomly collected from the five banana growing areas in South Africa during 2000-2001, namely Levubu, Tzaneen, Kiepersol, Komatipoort and southern Kwa-Zulu Natal. Samples were taken from diseased leaves incubated in moisture chambers and cultured on half-strength potato-dextrose agar. Isolates were identified morphologically. The identity of the causal organism was confirmed using diagnostic PCR and the species-specific primers of Johansen and Jeger (1993).

Results

Four leaf diseases were identified. Sigatoka disease, Mycosphaerella speckle and Cordana leaf spot were present in all five areas. Cladosporium speckle occurred only in Levubu, the most northern of the areas. *M. musicola* was the most

commonly isolated pathogen, followed by *M. musae* (Table 1). The presence of *M. musicola* and the absence of other *Mycosphaerella* pathogens was confirmed by PCR. Various saprobes and endophytes representing 23 species were also isolated from banana leaf material (Table 1). *Nigrospora oryzae* was the species isolated most frequently.

Table 1. Fungi associated with banana foliage in South Africa.

Species	Number of isolates
Alternaria alternata	21
Alternaria cf. *citri*	3
Alternaria tenuissima	4
Bipolaris cynodontis	3
Cladosporium musae	5
Colletotrichum gloeosporioides	5
Colletotrichum musae	1
Cordana musae	30
Curvularia lunata	1
Curvularia pallescens	1
Diaporthe sp.	3
Drechslera dematoidea	1
Drechslera phlei	1
Epicoccum nigrum	3
Exserohilum rostratum	1
Harpographium sp.	1
Mycosphaerella musae	30
Mycosphaerella musicola	66
Myrothecium verrucaria	1
Nigrospora oryzae	81
Nigrospora sacchari	10
Nigrospora sphaerica	25
Pestalotiopsis guepinii	9
Phoma glomerata	7
Phyllosticta sp.	1
Pithomyces sacchari	1
Selenophoma asterina	10
Selenophoma juncea	5

Discussion

M. musicola is the most common and severe pathogen of banana foliage in South Africa. It was identified morphologically and its presence was confirmed using molecular markers (Johanson and Jeger, 1993). The second most prevalent pathogen was *M. musae* which in some cases caused severe symptoms resulting in leaf death. The subtropical conditions in the banana plantations of South Africa appear to be conducive to Sigatoka disease. *Cordana musae* is considered to be a minor/secondary leaf pathogen and was most often observed infecting in conjunction with Sigatoka disease or a speckle. Its presence and that of *Cladosporium musae* was morphologically confirmed, as well as according to lesion appearance.

Most fungi isolated were strictly saprobes. The genus *Nigrospora*, particularly *N. oryzae*, was the most commonly isolated. This conforms to literature of endophytes isolated from banana leaves in Hong Kong (Brown *et al.,* 1998). All species isolated are the first recordings on banana leaves in South Africa. *Colletotrichum gloeosporioides, C. musae, N. oryzae,* and some *Curvularia, Pestalotiopsis, Phoma, Phyllosticta* species have previously been reported from *Musa* in Thailand (Photita *et al.,* 2001), Hong Kong and Northern Queensland, Australia (Brown *et al.,* 1998). However, no report could be traced referring to the presence of *Alternaria* cf. *citri, A. tenuissima, Bipolaris cynodontis, Diaporthe* sp., *Drechslera dematoidea, D. phlei, Exserohilum rostratum, Harpographium* sp., *Myrothecium verrucaria, N. sacchari, N. sphaerica, Pithomyces sacchari, Selenophoma asterina* and *S. juncea* on *Musa* species.

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Recommendations of session 1

Dispersal of *Mycosphaerella* spp.

Mycosphaerella fijiensis continues to spread to new areas. It is the dominant leaf spot pathogen in West Africa. In 2000-2002, the pathogen was identified for the first time in Madagascar, the Bahamas, the Galapagos Islands of Ecuador and in the north Queensland banana growing area where eradication is being attempted. The encroaching threat of *M. fijiensis* in the eastern Caribbean is of concern. It has been estimated that 40% of banana growers in the French Antilles would stop cultivating banana if the pathogen became established. The effects in the Windward Islands would also be significant.

Quarantine may need to be either strengthened or reinforced to prevent entry. The introduction of an appropriate monitoring system to detect any incursion of M. fijiensis *should be encouraged.*

M. eumusae is currently limited in extent throughout most of Asia, although there is some evidence that the pathogen may have reached Africa. Eumusae leaf spot disease has been observed on 'Mysore' (AAB) in Sri Lanka. As this clone is highly resistant to *M. musicola* and *M. fijiensis*, there is some cause of concern. Information suggests that Cavendish and plantain cultivars are also very susceptible. The dynamics of the disease are not fully understood. In order to prepare adequate disease control strategies, a detailed knowledge of the epidemiology of this pathogen is urgently required.

The exact distribution of M. eumusae *needs to be known. Further surveys in South and Southeast Asia, to determine where* M. musicola, M. fijiensis *and* M. eumusae *occur, are necessary.*

More information on the effect of M. eumusae *on the growth and yield of banana clones is needed.*

More laboratory research needs to be undertaken with M. eumusae *to determine its optimum growth temperature and other relevant biological information. This data would underpin epidemiological studies.*

The name of the banana clone affected, an indicator of the severity of the leaf spot and local environmental data would be useful as this may help explain distribution. IMTP trials are seen as ideal locations for assessing the reaction of different clones to the different leaf spot pathogens. The collection and diagnosis of specimens of leaf spot from IMTP trials sites needs to be continued. The cooperation and collaboration of scientists in South and Southeast Asia is viewed as essential. Identification tools should be provided to enable diagnoses to be undertaken locally.

M. eumusae was also identified from leaf spot specimens collected in 1989 and 1990 at Onne in Nigeria. Eumusae leaf spot disease has therefore been at this location for at least 13 years.

The IITA germplasm collection and clones in local banana farms in and around Onne should be surveyed for leaf spot pathogens. The results would indicate the relative competitiveness of **M. fijiensis** *and* M. eumusae *on different cultivars in West Africa. Although* M. eumusae *has not been identified from many isolates of leaf spot pathogens collected in neighbouring Cameroon, surveys of leaf spot in Nigeria and other countries in West Africa may indicate if spread has occurred*

Taxonomy

Anamorph morphology is more important than teleomorph morphology in distinguishing the *Mycosphaerella* leaf spot pathogens. It has been proposed that the anamorph stage of *M. fijiensis* be renamed Pseudocercospora fijiensis as the phylogenetic studies do not support keeping the name *Paracercospora fijiensis*.

Diagnostics

Several fungi diseases that attack leaves have been reported on *Musa* and other related species. A greater knowledge of *Mycosphaerella* pathogens/saprophytes, and of those in related genera, is a prerequisite to the development of rapid diagnostic tests to distinguish leaf spot pathogens. Diagnostic tools depend on the development of species-specific primers, such as microsatellites and ITS-sequences, tested on *Mycosphaerella* isolates from all over the world.

More taxonomic information about species of Mycosphaerella *and other related genera that either form or occur in banana leaf lesions would be beneficial.*

Diagnostic tools specific to the three main species of Mycosphaerella *pathogen on* Musa*:* M. fijiensis, M. musicola *and* M. eumusae *should be developed.*

The currently available molecular methods should be assessed for their specificity.

A manual with descriptions of symptoms and morphological characters should be produced.

Protocols for collection and analysis of samples should be developed.

*PRO*MUSA *participants should be trained on the different technologies required: collecting and sampling, single-ascosopore cultures, and molecular markers.*

Resistant cultivars

Reports of black leaf streak disease on two resistant FHIA hybrids are of concern. Stress caused by adverse growing conditions may be responsible.

Factors responsible for breakdowns need to be investigated, including the possibility that resistance is being eroded.

Session 2

Population biology and epidemiology

Introduction

Population biology and epidemiology

L. H. Jácome

Introduction

Mycosphaerella leaf spot diseases comprise one of the most important disease complex limiting banana and plantain production. Sigatoka disease is still important in some areas. In the 1990s, black leaf streak disease was spreading and is now reported from most parts of the world. Recently, a new disease, eumusae leaf spot was reported in South and Southeast Asia.

Black leaf streak disease reached most South American countries by the early 1990s and was reported in Bolivia in 1996 and Brazil in 1998. The disease was reported in the Caribbean in the Dominican Republic in 1996 and in Haiti in 2000. It threatens banana production in Puerto Rico and the Lesser Antilles. The disease was reported in the United States in 1998. In 2000, two new outbreaks of black leaf streak disease were reported in the Caribbean and Indian Ocean region. In Latin America the latest report was in the Galapagos Islands in early 2001. In April 2001, it was reported for the first time in a commercial production area near Tully in North Queensland, Australia. This illustrates the spreading capacity of this disease.

The continued spread of black leaf streak disease in the tropics within the last decade has made the disease the most economically important disease of bananas and plantains. Except in the Philippines, Sigatoka disease has been replaced by black leaf streak disease. Sigatoka disease is better adapted to cooler areas and dominates at altitudes above 1200 metres above sea level. Therefore, knowledge of the distribution and variability of the pathogens is needed to ensure the efficient introduction and sustainability of resistant hosts.

Restriction enzyme analysis has revealed unique differences in the banding pattern of DNA fragments. Genetic variability among populations of *Mycosphaerella* from different geographical regions has been identified using DNA restriction fragment

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length polymorphism (RFLP) markers. RFLPs are used as markers on physical and genetic linkage maps. To complement RFLP tests, methods based on polymerase chain reaction (PCR) could be used to obtain DNA profiles. Differentiation of the pathogens by PCR could be useful in determining their distribution, spread and prevalence. Additional work is now needed to obtain accurate information on the geographical distribution of genetic variants of *M. fijiensis*, and to test hypotheses about the origin and spread of the pathogen, and its population structure.

Given that the disease caused by *M. fijiensis* is more serious than the one caused by *M. musicola,* research is needed on the pathogenic variability of *M. fijiensis*, the distribution of its pathogenic variants and the identification of sources of resistance. Studies on genetic diversity in *M. fijiensis* using RFLP have shown high levels of polymorphism. Genetic variability was greatest in the Philippines and Papua New Guinea. Isolates from Africa, the Pacific Islands and Latin America formed genetically homologous groups that were specific to each region. Groups from the Pacific and Latin America also appeared to be related. Are these independent introductions of the pathogen? Are we dealing with the effects of genetic drift on the population structure?

Gametic disequilibrium analysis among RFLP loci has shown that genetic recombination plays an important role in the population structure of *M. fijiensis*. Gene pyramiding may not be durable. Mixing varieties or partial resistance could be more appropriate. Observations indicate that resistance to black leaf streak disease may be breaking down in some hybrids. It is not known whether this is due to more pathogenic variants or to favourable environmental conditions resulting in severe disease pressure. In addition to studying the effects of climatic conditions on the infection process, managing the inoculum using cultural practices should be considered. The fitness of fungicide-resistant *Mycosphaerella* deserves attention, as does the potential contribution of *Paracercospora fijiensis* to epidemics of black leaf streak disease in some areas.

Disease dynamics has been studied by calculating rates of disease development in relation to climatic factors. Epidemiological studies demonstrated that ascospores were the predominant inoculum of black leaf streak disease and their release was correlated with rainfall. Therefore, it should be possible to predict future rates of disease development from previous patterns of spore release in relation to temperature and rainfall. However, no consistency has been observed between ascospore release and disease development, probably because the conditions for spore release are not always conducive to infection.

There is considerable variation in disease progress curves and the relative duration of epidemics in pathosystems of *Musa-Mycosphaerella* spp. and plantation management (treated with fungicide versus untreated). For each epidemic, it is possible to determine the time of disease onset, the initial amount of disease, the rate of disease increase, the area under the disease progress curve, the shape of the curve, the maximum disease, final amount of disease and the duration of the epidemic. In tropical and semitropical climatic zones, for epidemics that are not curtailed by the harvest of an annual crop, e.g. black leaf streak disease, the progressive and regressive phases of an epidemic correspond mainly to seasonal changes in weather conditions.

At one level, a temporal analysis seeks gross comparisons between experimental treatments, e.g. fungicide spray schedules in order to evaluate strategies for disease management. At a second, more complex level, changes in specific environmental factors, pathogens or host resistance lead to changes in the epidemic that are reflected by changes in the disease progress curves. A third level of analysis corresponds to comparative epidemiology, where the purpose is to identify similarities and differences between epidemics based on the shape of the disease progress curves and to look for the elements that serve as primary determinants. Geographical populations of *Mycosphaerella*, with marked genetic differentiation, could be considered as separate epidemiological units requiring independent disease management. This suggests that studies and modelling of the epidemiology, distribution and population structure of the three *Mycosphaerella* leaf spot pathogens should be undertaken at the national, regional and global levels.

In general, there is little information on the biology, population structure and epidemiology of the *Mycosphaerella* leaf spot pathogens. As a result, areas requiring further investigation are pathogen variability, distribution of variants, sources of resistance, epidemiology and population structure. The papers and posters in this session have been selected to improve our understanding of the population biology and epidemiology of the *Mycosphaerella* pathogens of *Musa*. Aspects of the aerobiological pathway of *M. fijiensis* ascospores and conidia at small-scale and mesoscale levels are discussed. Such studies are needed to clarify the temporal and spatial patterns of the spread of leaf spot diseases within or across banana cropping sequences, and in relation to environmental and host factors. Several forecasting schemes for black leaf streak disease have been introduced and are based on those developed for Sigatoka disease in the French Antilles. The forecasting schemes combine local weather and ascospore trapping data. The value of ascospore trapping for forecasting black leaf streak disease is discussed.

Aspects of the genetic structure and evolution of *Mycosphaerella* pathogens at the global, regional and local levels are discussed. Genetic differentiation and independency of introductions of the pathogens according to the region are also described. Knowledge of the variability within *Mycosphaerella* is necessary for breeding and management of disease resistance. The usefulness of microsatellite markers for the study of fungal populations having high evolutionary rate is presented by an analysis of the introduction and spread of black leaf streak disease in Mexico. The presence of polymorphisms in chromosome length between molecular karyotypes of *M. fijiensis* is discussed. Understanding the organization of the genome could lead to the development of new strategies for disease control management.

Given that pathogens can evolve to break down host resistance, further research on the evolution of the three *Mycosphaerella* pathogens on resistant hosts is needed. As stated in the *Musa* disease fact sheet No. 8, published by INIBAP, population studies of *Mycosphaerella* pathogens of banana are required to determine whether the banana-producing regions correspond to one or several epidemiological units. The studies should use molecular markers and determine pathogenicity. Techniques are also needed to determine quickly and reliably host-pathogen interactions in controlled conditions. The variability in pathogenicity in genetically differentiated populations of the three pathogens could then be evaluated by means of a standard

set of *Musa* cultivars. Population studies should help define, for the different regions, a set of *Mycosphaerella* isolates that are representative of the variability in virulence and aggressiveness for use in resistance screening. Knowing the components of partial resistance which greatly reduce the rate of disease development in the field is also important. Finally, the evolution of the pathogen population in response to the selection pressure exerted by resistant cultivars should be evaluated if durable resistance is to be achieved.

Airborne dispersal of *Mycosphaerella fijiensis*

P. J. A. Burt

Abstract
Dispersal of *Mycosphaerella fijiensis* ascospores and conidia within, above and outside unsprayed banana plantations was studied in a series of field experiments at CATIE in Costa Rica. Laboratory experiments were also conducted in Costa Rica and the UK, to estimate the potential viability of spores dispersing through the atmosphere. Three field regimes were used to assess windborne spore dispersal on a local and mesoscale, in relation to wet and dry seasons. Spore catches were analysed in relation to the time of day of capture, temperature, rainfall and behaviour of the wind. Results showed that ascospores and conidia are windborne within infected plantations and up to several tens of kilometres away from disease sources. Laboratory studies of simulated spore release under field conditions showed that significantly fewer spores entered the air than might be expected on the basis of field surveys. The reason for this is unclear. There is also evidence that a major constraint on the airborne dispersal of viable spores is their duration of exposure to ultraviolet radiation in sunlight. A greater understanding of the microscale processes occurring on the surface of an infected banana leaf is required in order to resolve the role of the wind in the epidemiology of black leaf streak disease. A more accurate quantification of the numbers of spores undertaking long-distance dispersal (with assessments of their viability in the field) is also essential. Future research needs are discussed.

Resumen – Dispersión aérea de *Mycosphaerella fijiensis*
Se han investigado los aspectos de la vía aerobiológica de las ascosporas y conidias de *Mycosphaerella fijiensis* en experimentos en el campo en CATIE, Costa Rica. También se realizaron investigaciones en el laboratorio en Costa Rica y Reino Unido con el fin de evaluar la viabilidad potencial de la dispersión de esporas en la atmósfera. Se utilizaron tres regímenes de campo para evaluar la dispersión de esporas por el viento a escalas local y media en relación con las estaciones húmeda y seca. La captura de esporas fue analizada basándose en la hora del día de la captura, temperatura, precipitación y comportamiento del viento. Los resultados mostraron que las ascosporas y conidias se propagan por el viento dentro de las plantaciones infectadas y hasta decenas de kilómetros fuera de las fuentes de la enfermedad. Sin embargo, de los estudios de laboratorio que simularon la liberación de las esporas bajo condiciones de campo, está claro que se podría esperar que una cantidad significativamente menor de esporas penetre en el aire en base a las encuestas en el campo. La razón de este hecho no está clara. También existen

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evidencias de que la duración de su exposición a la radiación ultravioleta del sol es una de las principales limitaciones para la dispersión aérea. Se requiere un mayor entendimiento de los procesos a pequeñas escalas que ocurren en la superficie de una hoja de banano infectado con el fin de resolver con mayor profundidad el papel del viento en la epidemiología de la Sigatoka negra. También es esencial realizar una cuantificación más precisa de las cantidades de esporas que se dispersan a largas distancias (con la evaluación de su viabilidad en el campo). Se debe discutir estas futuras investigaciones.

Résumé – Dispersion aérienne de *Mycosphaerella fijiensis*
La dispersion des ascospores et des conidies de *Mycosphaerella fijiensis* a été étudiée au cours d'une série d'essais en champ au CATIE, au Costa Rica. Des essais en laboratoire ont également été effectués au CATIE et en Grande-Bretagne, afin d'estimer la viabilité potentielle des spores au cours de leur dispersion dans l'atmosphère. Trois régimes en champ ont été utilisés pour évaluer la dispersion par le vent des spores à l'échelle locale et moyenne, en relation avec la saison sèche et humide. Les spores récoltées ont été analysées en relation avec l'heure de capture, la température, la pluviométrie, et la force et la direction du vent. Les résultats ont montré que les ascospores et les conidies sont transportées par le vent au sein des plantations infectées et jusqu'à plusieurs dizaines de kilomètres des sources d'infection. Les simulations en laboratoire de la dissémination des spores en conditions naturelles ont montré qu'un nombre de spores significativement plus faible que celui attendu sur la base des mesures en champ entrait dans l'air. Les raisons de ce phénomène ne sont pas claires. Il existe également des preuves qu'une contrainte majeure à la dispersion aérienne de spores viables est la durée de leur exposition aux rayons ultraviolets du soleil. Une meilleure compréhension des processus à l'échelle microscopique qui ont lieu à la surface d'une feuille de bananier infectée est nécessaire, afin de comprendre le rôle du vent dans l'épidémiologie de la maladie des raies noires. Une quantification plus précise du nombre de spores qui sont dispersées à longue distance (avec une évaluation de leur viabilité en champ) est également essentielle. Les besoins futurs de recherches sont discutés.

Introduction

Banana and plantain are major subsistence crops for small-scale farmers in the developing world, and production is increasing worldwide. Crops are affected by a range of pests, including *Mycosphaerella musicola,* the causal agent of Sigatoka disease and *M. fijiensis,* the causal agent of black leaf streak disease. The infective agents are ascospores and conidia. Black leaf streak disease has gradually replaced Sigatoka disease in most banana growing areas and can reduce yields by up to 50% (Stover and Simmonds, 1987). Fungicides can control black leaf streak disease but they are too expensive for small-scale farmers and affect the environment. Black leaf streak disease is currently absent from most of the Caribbean. Its arrival would be disastrous for the smallholders. Quarantine regulations being strict, the risk of black leaf streak disease arriving in the Caribbean by windborne dispersion requires quantification.

Previous studies have implicated wind and water in the release of ascospores and conidia of *M. fijiensis*, but there is disagreement in the literature about their relative importance. Conidial dispersal appears to occur primarily in water, either as runoff in dew or by rain splash (Leach, 1946; Stover, 1968, 1972; Meredith *et al.*, 1973; Stover and Simmonds, 1987; Gauhl, 1989) whereas ascospores are primarily removed from diseased leaves by wind (Leach, 1946). There is also evidence that the conidia may be blown off infected leaves (Stover and Simmonds, 1987), and that ascospores

are released by water (Leach, 1941; Meredith, 1962; Meredith and Lawrence, 1970; Stover, 1970; Meredith *et al*., 1973). One thing is sure: within infected plantations, ascospores and conidia are present in the air in varying amounts (Gauhl, 1989).

Long-distance airborne dispersal is known for some fungal pathogens e.g. *Peronospora tabacina*, the causal agent of tobacco blue mould (Davies et *al*., 1985); *Cochliobolus heterostrophus,* which causes corn leaf blight (Pedgley, 1982); *Melampsora* spp., the causal agent of poplar rust; and *Hemileia vastatrix*, which causes coffee leaf rust (Burdekin, 1960; Bowden *et al*., 1971; Pedgley, 1982). There are no records of *M. fijiensis* ascospores or conidia high in the atmosphere. In the absence of such records, it is necessary to look at disease incidence, but this can be unreliable, as the infective agents may enter an area long before the disease is first observed or reported. The global pattern of Sigatoka disease suggests windborne dispersal from east to west. The pattern is less obvious for black leaf streak disease but disease records suggest that some intra-continental spread may have been windborne (Burt, 1994).

Even assuming that airborne spores travel in large enough numbers to overcome dilution in the atmosphere and that they enter an area where there are suitable hosts present, they may be affected by environmental conditions during transport. Of these, temperature and ultraviolet radiation in sunlight are probably the most significant. High temperatures destroy spore walls and denature DNA (Parnell *et al*., 1998). Ultraviolet (UV) radiation, depending on wavelength, also denatures DNA. Wavelengths below 320 nm, especially 250-270 nm (which do not reach the ground), are the most lethal (Setlow, 1974; Rotem *et al*., 1985; Chuang and Su, 1988; Rotem and Aust, 1991). UV radiation at wavelengths above 290 nm does not reach the ground, and kills spores within a few hours (Maddison and Manners, 1972; Bashi and Aylor 1983; Rotem *et al.* 1985). The ascospores of *M. fijiensis* have thin walls and are hyaline in contrast to the thick-walled spores of many windborne fungi.

It is clear from the literature that ascospores and conidia of *M. fijiensis* have the capacity to be windborne over long and short distances, and that spores are present in the air within diseased plantations. Water (dew and rain) has been implicated in short-distance dispersal, although the precise role of wind in spore dispersal is unclear.

The aerobiology of *M. fijiensis* was studied in 1992-1995 by investigating spore dispersal within a plantation; spore movement across a small experimental site; mesoscale dispersal; potential inoculum loads and spore viability. The studies were conducted at the *Centro Agronómico Tropical de Investigación y Enseñanza* (CATIE), Costa Rica, and in the UK. This paper summarizes the results.

Methods

Dispersal of spores within a plantation

Airborne spores of *M. fijiensis* were monitored at four sites using rotorod spore traps within a naturally infected plantation at CATIE; samples were taken below the canopy, at mid-canopy and above the canopy (Burt *et al*., 1997). Wind speed, temperature, relative humidity and rainfall were recorded. Spore concentrations were meas-

ured with a Burkard volumetric spore trap (Burkard Manufacturing Company, Rickmansworth, UK) at a height of 1.6 m above ground level. Disease incidence and disease development were also recorded (Burt *et al.*, 1997). No attempt was made to control black leaf streak disease by treatment with fungicide.

Small-scale dispersal of ascospores and conidia

A small-scale field investigation was undertaken between May and August 1995 in order to clarify some of the results obtained by spore trapping and to investigate the role of wind and water in spore dispersal (Rutter *et al.*, 1998). Disease development and spore movement was monitored in a small experimental plot in which 100 healthy plantain plants were arranged 2 m apart (Figure 1). Data were collected from quadrats and analysed. The numbers of ascospores and conidia in and around the plot were measured using four sets of rotorods at 0.5 m and 1.5 m above ground level for 20 minutes at 07.00 local time, the time when spore concentration is highest (Rutter and Burt, 1997). Wind, temperature, relative humidity and rainfall were also recorded. The ambient spore concentration was recorded continuously using a Burkard spore trap set at 6 m above ground level. An inoculum made up of a bunch of diseased leaves was placed in the centre of the plot (Figure 1) and the progress of the disease measured across the plot.

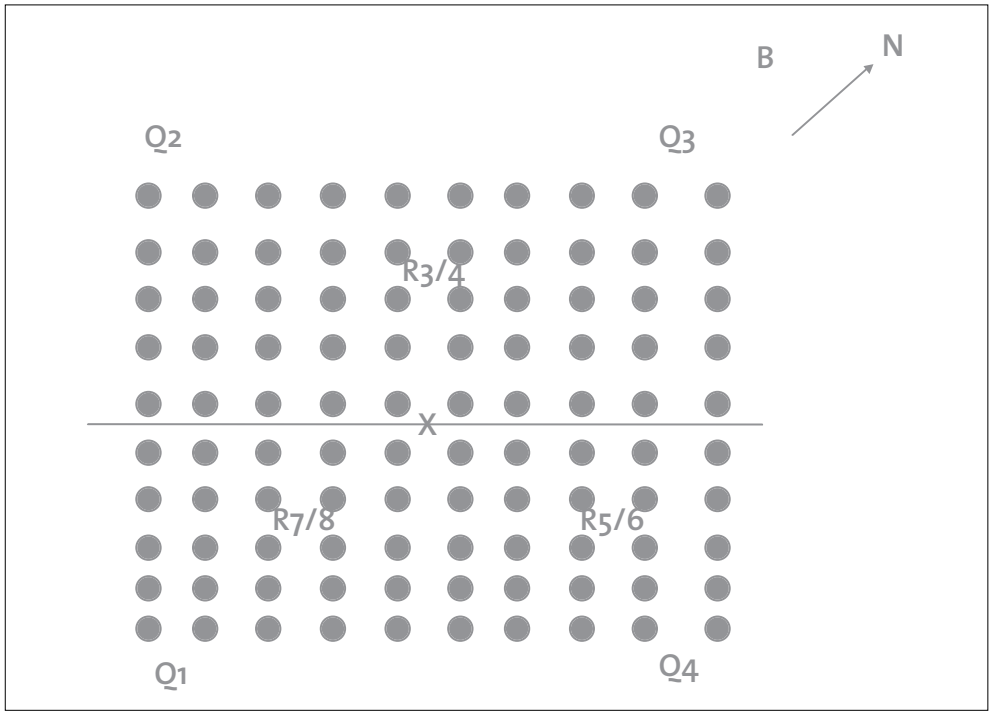

Figure 1. Small plot experimental design. Plants, shown by circles, were 2 m apart. R refers to the position and number of rotorod traps, X the location of the source of inoculum, the two rotorod traps and the weather instruments. The Burkard trap (B) was located outside the plot. Q refers to quadrats. (From Rutter *et al.*, 1998).

Mesoscale dispersal

Mesoscale dispersal of spores was investigated by sampling along a 5 km transect across the floor and up one side of a valley outside CATIE, between April and August 1995 with 3 continuously recording Burkard spore traps. Trap 1 was located in the middle of the plantation, trap 2 in the small experimental plot and trap 3 was 5 km north-north-west of the plantation, on the side of the valley at an elevation of 1000 m (Burt *et al.*, 1998). There were no obstacles between trap 3 and the other two traps and there were no sources of inoculum close to trap 3 (the nearest large source was the plantation at CATIE). Daily spore counts were analysed in relation to the speed and direction of the wind at the three sites (Burt *et al.*, 1998).

Assessment of inoculum

Disease surveys suggest that the infected plants in a plantation would act as a vast reservoir of inoculum, but trapping data suggest that, whilst both ascospores and conidia are windborne, they are not abundant in the air (particularly ascospores).

Ten banana leaves showing necrosis on at least 16% of the surface of the leaf (using the modified Stover scale to assess the levels of infection) were selected at random from the CATIE plantation during two periods: October 1993–February 1994 and April–September 1995. Each leaf was divided into three parts, each with different amounts of necrosis (Figure 2). Necrotic tissue from each part was excised, the area measured and the perithecia counted. A regression equation relating the number of perithecia to the necrotic area was calculated (Burt *et al.*, 1999).

A random selection of the leaf sections was exposed to a regime of wetting and drying under simulated field conditions (21°C and 100% relative humidity and 28°C at 60% relative humidity) in order to assess spore release (Burt *et al.*, 1999). The released spores were deposited on agar in Petri dishes and counted using a compound microscope. A second regression equation was derived, relating the number of ascospores to perithecia in infected leaf tissue (Burt *et al.*, 1999).

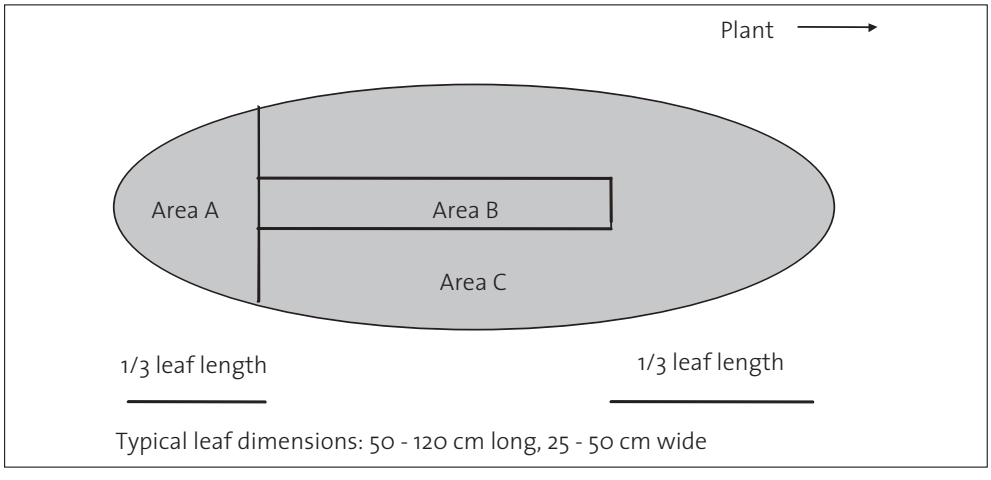

Figure 2. Leaf parts used for counts of perithecia. (From: Rutter *et al.*, 1998).

Viability of dispersed spores

Spore viability under conditions of temperatures and solar radiation likely to be encountered during atmospheric transport was investigated (Parnell *et al.*, 1998). Ascospores released from wetted leaf sections were exposed to simulated sunlight with UV wavelengths between 270 and 800 nm typical of the tropics, and incubated under a light/dark cycle at 24-26°C (Parnell *et al.* 1998). Control plants were subjected to the same regime minus the UV radiation.

Results and discussion

Dispersal of spores within a plantation

Spore numbers at all sampling heights were generally low and the majority were found at sunrise, the time of dew evaporation (Table 1). More conidia than ascospores were caught at all heights within the plantation. Spores were also present after rain in the afternoon, again in low numbers (Burt *et al.*, 1997). More conidia than ascospores were found at all heights within the plantation (Table1).

The number of spores that got into the air was much smaller than the one expected from looking at disease incidence alone. These low numbers made it impossible to investigate the relationship between spore capture and weather, especially rainfall which was low during sampling. It was also unclear whether the larger number of conidia in the air is atypical or indicates that dispersal is mainly by conidia. At a lowland site, Gauhl (1989) had found more ascospores than conidia. The different results obtained at CATIE suggest that ascospore trapping may not be a reliable method to forecast black leaf streak disease, at least at highland sites.

Table 1. Summary of spore trapping of *M. fijiensis* within an infected plantation, December 1993–February 1994.

Position relative to the canopy	Ascospores		Conidia	
	Mean spore count (min-max)	Number of samples	Mean spore count (min-max)	Number of samples
Above	1.35 (0-6)	40	5.75 (0-54)	40
Middle	1.15 (0-63)	39	6.59 (0-63)	39
	1.18 (0-5)	40	7.65 (0-44)	40
Bottom	1.20 (0-8)	40	8.52 (0-64)	40

From Burt *et al.*, 1997

Small-scale dispersal of ascospores and conidia

More conidia than ascospores were caught in the rotorod traps within and just above the plants in the small plot, but catches were again low (Figure 3). Initially, it appeared that many more ascospores than conidia were recorded in the Burkard spore trap but when the data were for sampling volume , the Burkard trap caught fewer spores overall (Rutter *et al.*, 1998). There was no evidence that spores entered the plantation from outside and caused disease.

Disease progress across the small plot was uneven; plants in separate quadrats showed different rates of disease progression (Figure 4). Wind direction was not consistent but northerly winds were the most frequent. At these times, plants in quadrat 1 were directly downwind of the inoculum source, which might account for the higher spore catches there (Figure 3). Otherwise, wind direction and the appearance of symptoms were not related (Rutter *et al.*, 1998). Southerly winds were the second most common and would have blown inoculum over quadrat 3. However neither the pattern of disease spread nor the rotorod catches reflected this. Southerly winds blowing across quadrat 1 might explain the high spore catches by rotorods in quadrat 4. Patterns of disease spread in the small plot showed no evidence of splash dispersal. Importantly, symptoms were not seen in the plants immediately surrounding the source of innoculum until the middle of June, when the upper leaves of the plants were affected. Rotorod traps 50 m downwind of the plot had no ascospores, and only a few conidia (up to 12 per sampling run) (Rutter *et al.*, 1998).

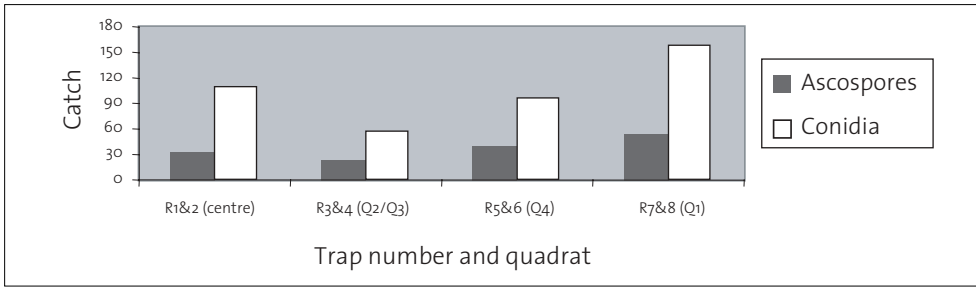

Figure 3. Total spore catch in the small plot during the sampling period 1 May-3 August 1995. (From: Rutter *et al.*, 1998).

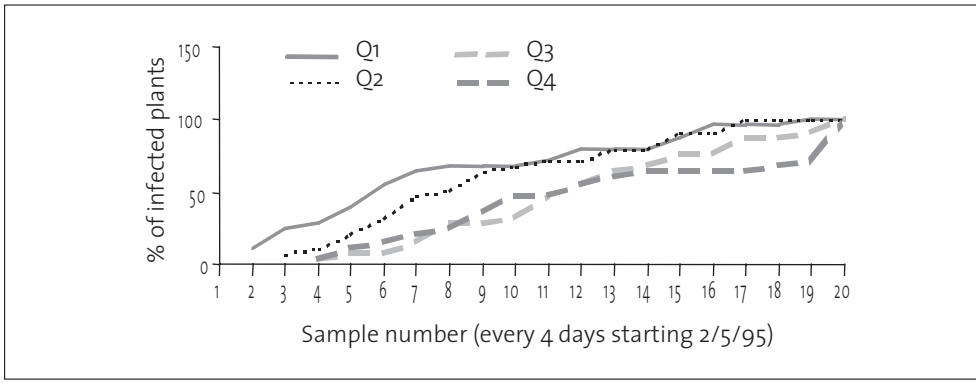

Figure 4. Appearance of disease symptoms in the quadrats of the small plot. (From: Rutter *et al.*, 1998).

Mesoscale dispersal

Ascospores and conidia of *M. musicola* and *M. fijiensis* were present in the air at all sites, usually between 0530-0830 in the morning with an abrupt cut-off at 08.30

(Burt *et al*., 1998). Many more spores of *M. fijiensis* than of *M. musicola* were caught. The analysis was therefore based on *M. fijiensis* only (Table 2). Despite the apparent relationship between spore numbers in traps 1 and 3 (Table 2) the numbers of spores caught in each trap were not statistically related to each other (Burt *et al*., 1998).

No differences in wind direction were recorded at the three trapping sites. The winds blew most frequently from north to south, or vice versa, along the valley axis, as might be expected (Burt *et al*., 1998). Winds blowing from between the north-east and south-east would have crossed over trap 3 and the large plantation at CATIE, the nearest large source. But there was no evidence that the spores were being blown directly from this source, either directly or after allowing for a lag between times of release and capture (Burt *et al*., 1998).

There were no other large sources of inoculum near trap 3. However, there are many large plantations on the Atlantic coast, 40 km east of the trapping sites. NE and SE winds could have blown spores from the plantations to the study area but the cut-off in spore captures around 0830 in the morning does not support this. It is possible that spores were descending at night at the time when the nocturnal inversion would have been breaking up.

Table 2. Mean daily spore catches of *M. fijiensis* during the sampling period of 29 April–30 August 1995.

	Mean daily catches of ascospores (standard error of the mean)	Mean daily catched of conidia (standard error of the mean)	n
Trap 1	153.5 (14.6)	72 (12.1)	116
Trap 2	124.2 (17.4)	8.5 (1..2)	127
Trap 3	144.6 (16.4)	8.2 (1..2)	118

From: Burt et al., 1998

Assessment of potential innoculum

The numbers of perithecia was related to the area of necrotic leaf tissue at different stages of disease development (Burt *et al*., 1999):

$$\ln(\text{perithecial number}) = [1.173 \times \ln(\text{necrotic area})] + 4.624$$

A second regression equation was calculated from data from the release and rewetting experiments (Burt *et al*., 1999):

$$\ln(\text{no. ascospores}) = [1.173 \times \ln(\text{necrotic area})] + 6.128$$

where necrotic area is measured in cm^2.

This gave a mean of 4.5 ascospores per perithecium, a result which does not resolve the ambiguities surrounding the number of ascospores present in each perithecium reported in the literature, with values ranging from between 1 and 27 (Stahel, 1937; Stover, 1972) and up to 160 (Meredith and Lawrence, 1970; Stover, 1970) being reported. Even if there were some errors in the counting of the released ascospores, it is clear that the number of ascospores available for release is not being represented in spore trap catches.

Ascospore viability

Exposure to UV radiation at tropical sunlight levels killed ascospores after six hours continuous exposure (Parnell *et al.*, 1998). Although spores in the air are unlikely to be exposed to continuous sunlight or temperature for these lengths of time, this gives some indication of the potential for long-distance transport of viable spores. A 12-hour transport time in winds of, for example, 10 m/s, would permit transport over distances up to 400 km. This might not preclude the transport of viable spores over that distance. In Costa Rica, however, the prevailing winds are off the sea on both coasts, so windborne transport towards the Caribbean is unlikely. This combination of unfavourable winds and viability may explain why black leaf streak disease has not reached the Caribbean in significant amounts, and why localised disease outbreaks there have not spread. Looking at spore viability in relation to distance (Burt, 1994), suggests that wind dispersal of *M. fijiensis* ascospores over longer distances is unlikely. For example, a trans-Atlantic transport time of even 5 days–which is probably the shortest period possible (Rosenberg and Burt, 1999)–is significantly longer than the time spores are likely to remain viable, even assuming that they were carried under cloudy skies all the time.

Suggestions for future research

It is clear that more research is required if the full contribution of airborne dispersal to the epidemiology of *M. fijiensis* is to be fully understood. It is clear that much more inoculum is being produced on the leaves than is entering the air. Consequently, there is a need to understand more fully the relationship between wind patterns and spore movement on all scales, but particularly updraughts in relation to spore movement inside and out of a canopy.

Detailed studies of the leaf-surface are also required, to measure runoff (the number of spores transported and their destination) and plant architecture and micrometeorology. Rather than relying on rewetting experiments, which may be prone to error, detailed histological investigations of necrotic tissue at various stages of the disease may also reveal more information about the supply of inoculum (especially the number of ascospores).

Finally, a more complex field investigation is needed to resolve whether or not spores actually travel over long distances. This should be undertaken in association with a study to determine the effect of natural environmental conditions on the viability of spores found at various distances from known sources.

Acknowledgements

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Genetic differentiation in *Mycosphaerella* leaf spot pathogens

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Abstract

Black leaf streak disease and Sigatoka disease, caused respectively by two related fungi, *Mycosphaerella fijiensis* and *M. musicola,* are the most important leaf spot diseases of bananas. Understanding the genetic structure of the populations and the evolution of these pathogens is an important aid in breeding and managing disease resistance. The population structure of each pathogen was analysed using molecular markers mainly at the global and continental scales. Features common to both were observed: 1) the centre of diversity is located in Southeast Asia and founder events accompanying the introduction of the pathogens in other regions led to a reduction of genetic diversity; 2) genetic diversity is maintained in all populations and is also present at the scale of the plant; 3) genetic recombination played an important role in the genetic structure of both pathogens; 4) genetic differentiation exists between populations from the global to the local level. The main difference between the two species had to do with the measures of genetic differentiation. Whereas the African populations of *M. fijiensis* were significantly different from the Latin American/Caribbean ones, no significant difference was observed between the African and Latin American/Caribbean populations of *M. musicola*. This suggests independent introductions of *M. fijiensis* but not of *M. musicola*. Except for this situation, the genetic differentiation observed between populations at the global and continental scales indicate an important effect of genetic drift and limited gene flow.

Resumen – Diferenciación genética en los patógenos de la mancha foliar ***Mycosphaeralla***

La enfermedades de la mancha foliar de los bananos más importantes se deben a dos hongos relacionados: *Mycosphaerella fijiensis* y *M. musicola,* los agentes causales de la enfermedad de la raya negra de la hoja y de la enfermedad de Sigatoka, respectivamente. El entendimiento, tanto de la estructura genética de la población, como de la evolución de estos patógenos proporciona información importante para brindar asistencia al mejoramiento y manejo de la resistencia a la enfermedad. La estructura de la población de ambos patógenos fue analizada utilizando marcadores moleculares a escalas global y continental. Se observaron las siguientes características comunes: 1) el centro de la diversidad está localizado en el Sudeste de Asia y los eventos de

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colonización que acompañaron la introducción de los patógenos en otras regiones han llevado a una reducción de la diversidad genética en comparación con el Sudeste de Asia; 2) la diversidad genética se mantiene en todas las poblaciones y se distribuye a escala de la planta, 3) la recombinación genética desempeña un papel importante en la estructura genética de ambos patógenos; 4) existe una diferenciación genética entre las poblaciones a escalas de global a local. La principal diferencia observada es la existencia de una diferenciación genética entre las poblaciones africanas y las poblaciones latinoamericanas y caribeñas de *M. fijiensis* pero no de *M. musicola*. Este resultado sugiere introducciones independientes de *M. fijiensis* pero no de *M. musicola*. Con excepción de la situación descrita arriba, la diferenciación genética observada en ambos patógenos entre las poblaciones a escalas global y continental indica un efecto importante de la genética y un flujo de genes bajo.

Résumé - Différenciation génétique chez les *Mycosphaerella* pathogènes

La maladie des raies noires et la maladie de Sigatoka, respectivement causées par deux champignons apparentés, *Mycosphaerella fijiensis* et *M. musicola,* sont les maladies foliaires les plus importantes chez le bananier. La compréhension de la structure génétique des populations et de l'évolution de ces pathogènes représente une aide importante pour l'amélioration et la gestion de la résistance à ces maladies. La structure de la population de chaque pathogène a été analysée en utilisant des marqueurs moléculaires, principalement à l'échelle globale et continentale. Des caractéristiques communes aux deux pathogènes ont été observées : 1) leur centre de diversité est localisé en Asie du Sud-est et des événements fondateurs accompagnant l'introduction des pathogènes dans d'autres régions ont conduit à une réduction de la diversité génétique ; 2) la diversité génétique est maintenue dans toutes les populations et est également présente à l'échelle de la plante ; 3) la recombinaison génétique a joué un rôle important dans la structure génétique des deux pathogènes ; 4) une différentiation génétique existe entre populations, du niveau global au niveau local. La principale différence entre les deux espèces concerne les niveaux de la différentiation génétique. Alors que les populations africaines de *M. fijiensis* sont significativement différentes de celles d'Amérique latine/Caraïbes, aucune différence significative n'a été observée entre les populations de *M. musicola* originaires d'Afrique et d'Amérique latine/Caraïbes. Ceci suggère des introductions indépendantes de *M. fijiensis*, mais pas de *M. musicola*. A part cette situation, la différentiation génétique observée entre les populations à l'échelle globale et continentale indique un effet important de la dérive génétique et des flux géniques limités.

Introduction

Black leaf streak disease, caused by *Mycosphaerella fijiensis,* and Sigatoka disease, caused by *M. musicola,* are the most important leaf spot diseases of bananas (Jones, 2000). The fungi are haploid and heterothallic. The anamorph and teleomorph stages are both present on infected leaves, and the ascospores produced during the sexual stage play an important role in epidemics. The first species to be described was *M. musicola,* in Java in 1902. The rapid dissemination of Sigatoka disease round the world in the 1930s, led to speculations that the spores were carried by air currents between continents: from Asia to the Pacific, from the Pacific to Australia, from Australia to Africa and from Africa to Latin America (Stover, 1962). In 1962, *M. musicola* was present in all banana-producing regions, making Sigatoka disease one of the most important plant diseases. Black leaf streak disease was reported in Fiji in 1964 and since then has been reported throughout the Pacific and Asia. The chronology of records suggests that *M. fijiensis* originated, as *M. musicola,* in Southeast Asia (Mourichon and Fullerton, 1990), which is also the centre of origin

of the host genus *Musa*. Starting in the 1970s, *M. fijiensis* spread to Africa, Latin America and the Caribbean. Being more aggressive, *M. fijiensis* replaced *M. musicola* as the dominant leaf spot pathogen in many areas. Although the distribution of both pathogens is well documented in Australia, the Pacific region, Africa, Latin America and the Caribbean, it is still not well understood in Southeast Asia.

Why and how to analyse populations of pathogens?

Knowledge of the genetic population structure and evolution of the pathogens is an important aid in breeding and managing disease resistance. The main objective of such study is to provide information on the level and distribution of variability. Molecular markers are often used to analyse population structure at different geographical scales. It should make it possible to identify potential sources of resistance, which are expected to be in areas where the diversity of pathogens and host are high. It should also ensure that the diversity of pathogens used when screening for resistance is representative of the one in the regions where resistant hosts are intended to be used. Pathogens can evolve to break down total resistance or erode partial resistance. The evolution of pathogen populations depends on mutation, recombination, genetic drift, gene flow and the selection pressure exerted by the host. It should be possible to limit and restrict the evolution of pathogenicity by varying host resistance in space and time. Such strategies should improve the durability of the types of resistance used and ensure the durability of the culture.

Another objective of pathogen population studies is to evaluate the relative importance of the evolutionary factors on the evolution of pathogens. Such information would make it possible to model and test the effect of different management strategies on the evolution of the pathogen. Analysing population structure through space allows us to evaluate the effects of genetic recombination, genetic drift and gene flow on the evolution of the pathogen. This paper reviews the results obtained at global and local scales for *M. musicola* and *M. fijiensis*. A second approach consists in studying the evolution of the pathogen in fields of resistant hosts by using molecular markers and by characterizing pathogenicity. This allows us to evaluate the effect of the selection pressure exerted by the host on the pathogen. This second approach is described in another paper (see Abadie *et al.* in this volume).

Global population structure

RFLP markers were used to study the genetic structure of the global population of *M. musicola* and of *M. fijiensis* (Carlier *et al.*, 1996; Hayden *et al.*, in prep.) Random single-locus probes were used on samples from Southeast Asia, Australia, the Pacific Islands, Africa, Latin America and the Caribbean. Features common to both pathogens were observed.

Southeast Asia has the highest level of gene diversity (Table 1) and the majority of alleles found in this region were also present in the other regions. This supports the hypothesis that the pathogens originated in Southeast Asia. Founder events accompanying the introduction of the pathogens to other regions have led to a reduction in genetic diversity in comparison with Southeast Asia. Nevertheless, genetic diversity

is maintained in all populations. Ecological conditions being favourable for disease development and banana cultivation almost year round in most growing areas, low genetic drift in large pathogen populations can maintain the high levels of genetic diversity observed. Therefore, a certain level of variability in pathogenicity might also be maintained in pathogen populations, allowing the pathogen to attack newly introduced resistant genotypes, as has been observed with *M. fijiensis* on 'Paka' and 'T8' in Rarotonga, Cook Islands (Fullerton and Olsen, 1995). The existence of specific interactions between the host and *M. fijiensis* isolates was suggested for highly resistant genotypes (Fullerton and Olsen, 1995). Variability in aggressiveness was evaluated for two *M. fijiensis* samples from Cameroon and the Philippines by inoculating five partially resistant cultivars using a leaf piece assay (El Hadrami, 2000). Variability was similar but low for both countries, however genetic diversity in the Philippines was much higher (Carlier *et al.,* 1996). Specific interactions between the isolates and the cultivar were not detected. Only susceptible hosts are cultivated in these countries, and the lack of a selection pressure being exerted by the host on the pathogens could explain the results. The potential of pathogen populations to overcome partial resistance should be analysed by following their evolution in fields of resistant hosts (see Abadie *et al*., this proceedings).

Genetic recombination plays an important role in the genetic structure of *M. musicola* and *M. fijiensis*. Genetic markers were statistically independent therefore characteristics of pathogenicity could not be related to RFLP genotypes. With regards to breeding programmes, introducing specific resistance genes in individual cultivars (pyramiding) may not be a strategy for durable resistance in banana. Mixing varieties or using partially resistant hosts might be more appropriate.

Table 1. Nei's gene diversity estimates for populations of *M. fijiensis* (Carlier *et al*., 1996) and *M. musicola* (Hayden *et al*., in prep.) from different geographical regions.

Species	Asia	Africa	Latin America and Caribbean	Australia and Pacific
M. fijiensis	0.57	0.25	0.40	0.28
M. musicola	0.41	0.27	0.21	0.33

A high level of genetic differentiation was observed between populations of *M. musicola* and *M. fijiensis* from different regions (Figure 1). The Fst parameter (Wright, 1951) estimated for all loci between pairs of populations varied between 0.14 and 0.58 for *M. fijiensis* and between 0.025 and 0.55 for *M. musicola*. But whereas the African populations of *M. fijiensis* were significantly different from the Latin American/Caribbean ones (Fst = 0.49), no significant difference was observed between the African and Latin American populations of *M. musicola* (Fst = 0.025, not significant). This suggests a separate introduction of *M. fijiensis* in each region but a common one for *M. musicola*.

On the other hand, the high levels of genetic differentiation observed between Australian and African populations of *M. musicola* (Fst = 0.47) does not support the hypothesis of Stover (1962) whereby spores of *M. musicola* were carried by air currents from Australia to Africa. In general, the high level of genetic differentiation of both pathogens at a global scale suggests occasional migration events between continents.

Long distance dissemination of the disease around the world was more likely to have occurred by movement of infected plant material, as proposed by Mourichon and Fullerton (1990).

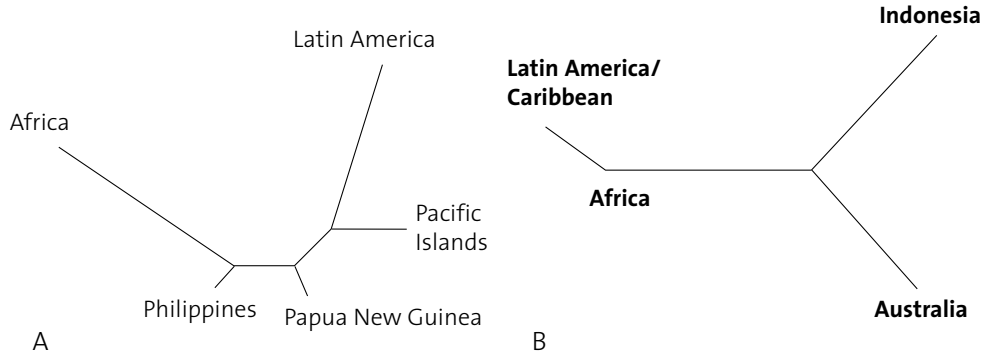

Figure 1. Global population structure of *M. fijiensis* (Carlier *et al.,* 1996) and *M. musicola* (Hayden et *al.,* in prep.). Additive trees constructed from estimates of Wright's Fst among pairs of geographical populations.

Continental and local population structures

Population structures at a continental scale were studied in Australia for *M. musicola* (Hayden, 2000). Collections of isolates from twelve sites along the east coast were analysed using 15 RFLP markers. The level of gene diversity (Nei, 1978), varied between 0.14 and 0.37. On a plant, the pathogen isolated from a given lesion would often be a haplotype not found in the other lesions, meaning that diversity is also present at a fine scale. Low to high levels of genetic differentiation were observed between populations (Fst = 0.04-0.45). There was no apparent correlation between genetic and geographical distances as high levels of genetic differentiation were observed between neighbouring populations and low levels were observed in populations separated by long distances.

The population structure of *M. fijiensis* was analysed in Africa, Latin America and the Caribbean (Rivas *et al.*, subm.). Samples from different countries were characterized using CAPS (Cleaved Amplified Polymorphic Sequence) markers (Zapater *et al.*, subm.). The results obtained for both continents were similar. The value of gene diversity varied between 0.19 and 0.38 for Africa, and 0.16 and 0.36 for the Latin America/Caribbean region. The low levels detected in some populations suggest that founder effects occurred during the spread of the disease on both continents. In the Latin America and Caribbean region, the highest levels are observed in populations from Honduras and Costa Rica, supporting the suggestion that the pathogen entered the continent in this area. In one locality in Cameroon, the values of gene diversity estimated at the scale of the field and of the plant are similar suggesting, as with *M. musicola*, that diversity is distributed at a fine scale.

Important levels of genetic differentiation were detected between most of the populations (Fst = 0.04-0.45 for Africa and 0.01-0.56 for Latin America/Caribbean).

There is sufficient differentiation between populations in the Caribbean islands to support the hypothesis that there was more than one introduction from Latin America. In Cameroon, genetic differentiation was detected between localities 300 km apart but not between localities 200 km apart.

Finally, the genetic structure of *M. fijiensis* was studied in the Australasia/ Pacific region using RFLP markers (Hayden *et al.,* subm.). Genetic differentiation was detected between the Torres Straits Islands, Pacific Islands and Papua New Guinea. At a local scale, there was no differentiation between two sites in the small Mer island.

The levels of genetic differentiation observed at the continental scale for both pathogens suggest an important effect of genetic drift on population structure and limited gene flow. Thus, spread of the diseases within continents could be due to the movement of infected plants and/or very restricted ascospore dispersal. As populations are probably not in genetic equilibrium, gene flow resulting from ascospore dispersal may be underestimated. However, preliminary results from an epidemiological study of black leaf streak disease suggests that dispersal of the pathogen is more restricted than previously thought. The results suggest a dispersal gradient of about 25 m from an inoculum source (Abadie *et al.,* this proceedings).

Conclusion and perspectives

The population structures of *M. musicola* and *M. fijiensis* are now better known at different geographical scales. However, at a regional scale few samples from Southeast Asia have been analysed. A new pathogen, *Mycosphaerella eumusae,* was recently discovered and detected mainly in Southeast Asia (Carlier *et al*., 2000). Southeast Asia is not only the centre of origin of all three pathogens but also of the host genus *Musa*. The distribution of the pathogens and their population structure should now be determined in detail for this region. Host-pathogen interactions could differ for each pathogen. One hypothesis to explain the continued presence of the three pathogens in Southeast Asia is the high diversity of host species. The hypothesis could be tested by surveying the fungal species in relation to host diversity. If host-pathogen interactions differ, the resistance genes introduced to produce new varieties could be more or less efficient depending on the pathogen they are exposed to. Their utilization should take into account the distribution of pathogen species. Zones of co-evolution for the three pathogens could be localized in Southeast Asia. This area is a potential source of resistance, therefore a study of pathogen populations in natural systems should provide us with information to complement evaluations of the relative importance of the different evolutionary forces.

The results to date suggest that genetic drift has an important effect on the structure of pathogen populations and that gene flow is limited. The limit of ascospore dispersal should be estimated indirectly at a country scale using genetic models such as the isolation by distance model (Rousset, 1997). However, we can already predict that the improvement of quarantine measures at the continental scale might limit the risk of introducing the disease to new areas, and limit the

exchange between existing pathogen populations from different countries. At a country and local level, geographical obstacles could also limit exchange between populations from different fields by playing on gene flow. Such a measure could have an impact on the durability of the resistances and of the management strategies.

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Development and application of molecular markers in *Mycosphaerella* populations in Colombia

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Abstract
In order to design effective strategies against the *Mycosphaerella* banana pathogens *M. fijiensis* and *M. musicola*, it is essential to have information on genetic diversity and population composition. Information to understand the population dynamics of these banana pathogens was based on microsatellite markers. The present study reports tests on 48 primer pairs designed for *M. musicola*, of which 26 proved to be polymorphic and four were transferable to *M. fijiensis*. Based on microsatellites, a comparison was made of the genetic variability in *M. fijiensis* and *M. musicola* populations from Colombia, Costa Rica and Venezuela. Dendograms for each species were based on the Dice similarity algorithm and grouped with the UPGMA clustering method. With the exception of a few isolates, most clusters coincided with the geographical locations (sampling sites).

Resumen – Desarrollo y aplicación de los marcadores moleculares en las poblaciones de *Mycosphaerella* en Colombia
Con el fin de diseñar estrategias eficaces contra los patógenos de *Mycosphaerella, M. fijiensis* y *M. musicola,* en el banano, es esencial disponer de la información sobre la diversidad genética y composición de sus poblaciones. La información, con ayuda de la cual se entienden las dinámicas de las poblaciones de estos patógenos de banano, se basa en los marcadores de microsatélites. El estudio actual presenta los informes de las pruebas de 48 pares de iniciadores diseñados para *M. musicola*, de los cuales 26 resultaron ser polimórficos y cuatro transferibles a *M. fijiensis*. Basándose en los microsatélites, se hizo la comparación de la variabilidad genética en las poblaciones de *M. fijiensis* y *M. musicola* procedentes de Colombia, Costa Rica y Venezuela. Los dendogramas para cada especie se basaron en el algoritmo de similitud de Dice y se agruparon con el método de análisis de conglomerados UPGMA. Con excepción de unos pocos aislados, la mayoría de los conglomerados coincidieron con las localizaciones geográficas (sitios de muestreo).

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Résumé - Développement et application des marqueurs moléculaires chez des populations de *Mycosphaerella* en Colombie
Afin de développer des stratégies efficaces contre les *Mycosphaerella* pathogènes du bananier, *M. fijiensis* et *M. musicola*, il est essentiel d'avoir des informations sur la diversité génétique et la composition des populations. Les informations visant à comprendre la dynamique des populations de ces pathogènes du bananier ont été basées sur les marqueurs microsatellites. Cette étude présente les tests effectués avec 48 paires d'amorces conçues pour *M. musicola*, dont 26 se sont avérées polymorphes et quatre ont été transférables à *M. fijiensis*. En se basant sur les microsatellites, une comparaison de la variabilité génétique a été faite chez des populations de *M. fijiensis* et *M. musicola* originaires de Colombie, du Costa Rica et du Venezuela. Les dendrogrammes pour chaque espèce ont été basés sur l'algorithme de similarité de Dice et groupés avec la méthode d'agrégation UPGMA. A l'exception de quelques isolats, la plupart des agrégats coïncidaient avec les localisations géographiques (lieux de collecte).

Introduction

Mycosphaerella fijiensis and *M. musicola*, the two most severe fungal pathogens of plantain and banana, are the major cause of economic losses in commercial plantations and in numerous smallholdings. Both pathogens spread around the world through demographic events such as founder effects. Population bottlenecks have been caused by increased doses of fungicides, the introduction of partly resistant host varieties, and isolation by distance and geographical barriers between populations.

M. musicola was first reported from Java in 1902 (Mourichon *et al.,* 1997), and from the Sigatoka Valley, Fiji in 1912 (Leach, 1941), where it caused an epidemic. About 50 years later a more aggressive pathogen, *M. fijiensis*, was detected in the same region (Leach, 1964). Both pathogens rapidly colonized the South Pacific Islands, Asia, Africa and America (Stover, 1976). *M. fijiensis* has been reported from sites where *M. musicola* was formerly present, suggesting a gradual displacement of *M. musicola* to higher altitudes (inter-Andean valley populations). Therefore, the dynamics of population structure of both pathogens is in some way interdependent and most likely to be influenced by parameters common to both.

Direct comparison of the populations of both pathogens would help understand local and regional genetic diversity and differentiation, and the influence of environmental pressures on the spread of the disease to new sites. It could also help predict the behaviour of new epidemics.

Molecular markers have become important tools for the investigation of the genetic composition of fungal populations (Groppe and Boller, 1997; Bucheli *et al.,* 2001). Restriction Fragment Length polymorphism (RFLP) markers were developed for the *M. fijiensis* genome, and used to characterize populations of *M. fijiensis* at a regional and global scale (Carlier *et al.,* 1994, 1996; Müller *et al.,* 1997). More recently, simple sequence repeat (SSR) markers have been established for *M. fijiensis* (Neu *et al.,* 1999) and *M. musicola* (Molina *et al.,* 2001) which, together with other PCR-based DNA profiling methods, provide a new method to compare the genetic diversity of both pathogens.

In the present study, dendograms based on Dice similarity algorithms were obtained for each species. With the exception of a few isolates that grouped outside the main clusters, the majority of individuals grouped according to their geographical location.

Materials and methods

Sampling sites and fungal material

Eighty isolates of *Mycosphaerella fijiensis* and 64 of *M. musicola* were collected from 12 locations. Isolates from Colombia were from two adjacent locations on the Caribbean coast (Santa Marta), and from three sites in the inner valleys of the Andes, Caldas, La Mesa and Cachipay. Isolates from Costa Rica and Venezuela were included for comparison. Costa Rican isolates were collected in "Valle Central" and those from Venezuela were collected in Mérida, a mid-altitude region located in the eastern branch of the Andes. There are two types of banana and plantain crop management in these areas (i) extensive banana plantations that are typical of the Colombian Atlantic coast and (ii) smallholdings that are characteristic of higher altitudes such as "Valle Central" in Costa Rica and the inner Andean valleys of Venezuela and Colombia (Price 1999).

DNA isolation

Infected plantains and banana leaves with advanced stages of lesion development were transferred to a humid chamber to allow ascospores to discharge onto 1.5% water agar. Single ascospores were identified microscopically, transferred to V8 medium and incubated for 12 days at 25°C. DNA was isolated from mycelium using a FastDNA Kit, Bio 101. DNA preparations were further purified by phenol:chloroform extraction (24:1, v/v) and ethanol precipitation. After washing with 70% ethanol, the final pellets were dissolved in an appropriate volume of 10 mM Tris-HCl, 1 mM EDTA, pH 8.

SSR design for *M. musicola*

The single ascospore culture *MmCol-LM9.5.1* (collected from plantations severely affected by Sigatoka disease in a mid-altitude region of Colombia) was used as source material for the construction of a genomic library. Fungal DNA was isolated according to Weising *et al.* (1991) and purified by cesium chloride gradient centrifugation. Microsatellite enriched libraries were constructed and screened according to Fischer and Bachmann (1998). Detailed information about methodology, primer sequences and genebank accession numbers for the 26 polymorphic *M. musicola* primers can be found in Molina *et al.* (2001).

Microsatellite analysis

PCR amplifications were performed in a Perkin Elmer 9700 thermocycler in 10 µl reactions containing 5 ng of genomic DNA template, 0.5 µM of each forward and

reverse primer, following PCR conditions according to Neu *et al*. (1999) and Molina *et al*. (2001). Products were separated on 6% sequencing gels and autoradiographed (Sambrook *et al*., 1989).

For microsatellite allelic data, an initial matrix containing allele sizes was constructed, from which a 0/1 matrix was also derived. Dice similarity coefficient matrices and their corresponding dendrograms, grouped by the UPGMA agglomerative method, were calculated with the NTSYS software package (Rohlf, 1993).

Results

SSR design for *M. musícola*

Out of 1029 colonies screened, 205 yielded a positive hybridization signal (enrichment efficiency: 19.9%). Sixty-four clones were sequenced, and primers could be designed for 48 clones. Primer functionality was tested on a set of 24 template DNAs comprising 18 *M. musicola* isolates from a single field in Colombia, four isolates from Costa Rica and two from Mexico. The original clone served as a positive control. Primer transferability was tested with three *M. fijiensis* isolates: Mf-Col-LD9.1 (Colombia), Mf-Mex-015 (Mexico), Mf-PNG-294 (Papua New-Guinea). A total of 48 primer pairs were tested and 26 yielded single polymorphic bands of the expected size. The characteristics are summarized in Table 1.

Successful cross-priming with *M. fijiensis* DNA was observed at four loci. The availability of polymorphic microsatellite markers specific for *M. musicola* makes it possible to study the population structure of the pathogen in areas infested with Sigatoka disease, and to compare the pathogens using the same marker system.

SSR typing

SSR markers were used to type all isolates from both species, nine for *M. fijiensis* and eleven for *M. musicola,* showing high levels of polymorphism (Neu *et al*., 1999; Molina *et al*., 2001). Although the markers proved to be highly informative for the species for which they were developed, they were of limited value when transferred to other species. For example, *M. fijiensis* marker Mf-SSR-061 resulted in monomorphic patterns when used for typing *M. musicola* populations. The same was true for *M. musicola* marker Mm-SSR-024 in *M. fijiensis* isolates. An average of 3.4 alleles were observed for *M. fijiensis* and 4.0 alleles for *M. musicola*. Polymorphic loci were 80.0% and 90.9% for *M. fijiensis* and *M. musicola* respectively.

Cluster analysis based on Dice similarity index

Dendograms based on Dice similarity indexes were produced for each species. The *M. musicola* tree was based on 42 SSR markers. Isolates grouped into six clusters (I to VI in Figure 1), which mostly paralleled the geographic locations. For example, in cluster I all isolates belonged to Cachipay and La Mesa, Colombia. These two locations are close to each other, have similar weather conditions and similar crop management practices. Cluster II mostly contained isolates from La Mesa, with some

Table 1. Characteristics of microsatellites cloned from *Mycosphaerella musicola.*

Locus	Repeat of cloned allele	Primer sequences (5' – 3')
Mm SSR 01	$(CA)_9$ G $(CA)_{11}$	TAGTTGCAACCGAACAGG/ CTCCGTAGGTATGATGGTGT
Mm SSR 03	$(GA)_4$ $(GC)_2$ $(GA)_{32}$	CTCCGTAGGTATGATGGTGT/ GCTTCGTCAAGACCCTTAC
Mm SSR 05	$((GT)_4$ $(C))_3$	CCTCTTACGAAGTCTGTGGT/ TATCTCGGGAGACCAGACTA
Mm SSR 06	$(GA)_8$	CGAACAGGACGAAAGAATAG/ GTTTGTTCCAGTTCGTCAAG
Mm SSR 07	$(CA)_{50}$	ACGAGGTTTCAGAAGCAATA/ TCTTTCACCGAAGAAACCT
Mm SSR 09	$((GT)_6$ AT $(GT)_3$ GCn$(GT)_5$ (GC)	AGGGACGAACAAAACAGAG/ CCATGGTTTTCAAGCATATT
Mm SSR 10	$(CA)_{30}$	GAGAGCATGAAAAGTGGAAA/ CGTGACACTCGTCAGTTACA
Mm SSR 14	$(CA)_7$ CAA $(CA)_{19}$	ATTTGGTGAATGGGGTAAG/ ACAGAGGGAAGCAAGTTTTT
Mm SSR 15	$(CA)_{27}$	CTACTGAGGCAGTCGCTAAC/ GGAGAGGTGGAAAAAGAAGT
Mm SSR 16	$(GA)_6$ AAA $(GA)_{17}$	CCATCTGCCTTGAGATAGTC/ GAATTTATTCCAGCGAAGC
Mm SSR 18	$(GA)_n$	ATCTGATTCGTATGGTGGAG/ TTGCTACTACTGGTGCTTCTC
Mm SSR 21	$(CTT)_9$	GTCGACCTCCATGACACTC/ TGCATGCAATCTGTAACCT
Mm SSR 22	$(GAA)_9$	CCAAAGCTTGAGTTGCTATT/ ACAACTTTTTGAGGAAAATGTAA
Mm SSR 23	$(CTT)_{27}$	CGACCTAGTCGAGGATGATA/ CGAAGACTTCTGAAAGGTCA
Mm SSR 24	$(GAA)_2$ GG $(GAA)_3$GG $(GAA)_{12}$	TCAAGAGGAGGAGAAGTTGA/ GGTTCTGATCAAGAGGAGGA
Mm SSR 26	$(CAA)_8$	ATATCTCTTCGTGTTTTGCG/ AAGTGTGGTCACAGCAAGTT
Mm SSR 30	$(CA)_{28}$	TGATGTTAAGTTGACGGACA/ CTAAGCCAAACCTCAATCAG
Mm SSR 31	$(AC)_{27}$	AACCACATCTTCGATCAGG/ CACATGGAATATCCTTGGTC
Mm SSR 34	$(CA)_{19}$	CTCGCTGCCTGATTATTCT/ AGATGGCATCGCTTCAC
Mm SSR 35	$(CA)_4$ AA $(CA)_{26}$	TAACAATGTCCCTGAGAAGC/ GCCTTATCTGGAAAGTATCGT
Mm SSR 36	$(CA)_{13}$	ATTCCAGGTACGGCTACAC/ ATTCAGATCTGGTCTGGTTG
Mm SSR 38	$((GT)_n$ $(CG))_3$	GAGAGTGAGATCGGTAGCAA/ CGGGATTAAGGTCTACCAA
Mm SSR 39	$(CA)_{19}$	TGCGAATTCCATTGATATG/ CGTGTGCTGACGAGAGAT
Mm SSR 41	$(GT)_{14}$	GGTGAGGTCGTTATTGTTGT/ GCTTTAGAGGTTTCGTTCTTC
Mm SSR 44	$(CA)_9$ $(CT)_{14}$	CCTCACTCTCGCTCATACA/ AGAATGGACGAAAAACACTG
Mm SSR 46	$(CT)_6$ $(GT)_{38}$	CGTGGACCTATTGTCAACTC/ TGGGTTACATTTACGAGAGAA

isolates from Costa Rica and one from Venezuela. For clusters III and IV, all isolates were from Venezuela with each cluster having isolates from a single location. Cluster III represents isolates from Mérida and cluster IV represents isolates from Santa Rosa. Isolates from clusters V and VI were respectively from Costa Rica and Colombia.

The *M. fijiensis* tree, based on 42 polymorphic SSR markers, shows four clusters (Figure 2). Cluster I comprises, almost exclusively, Santa Marta isolates, with a few isolates from Costa Rica and one from Caldas (Colombia). Clusters II and IV mostly comprise isolates from different locations in Costa Rica, with some isolates from

Colombia. Cluster III comprises 15 isolates from Caldas (Colombia), two isolates from Costa Rica and four from Santa Marta (Colombia). The 80 isolates used represent 51 haplotypes. One particular haplotype was found in 10 isolates from Santa Marta (Colombia).

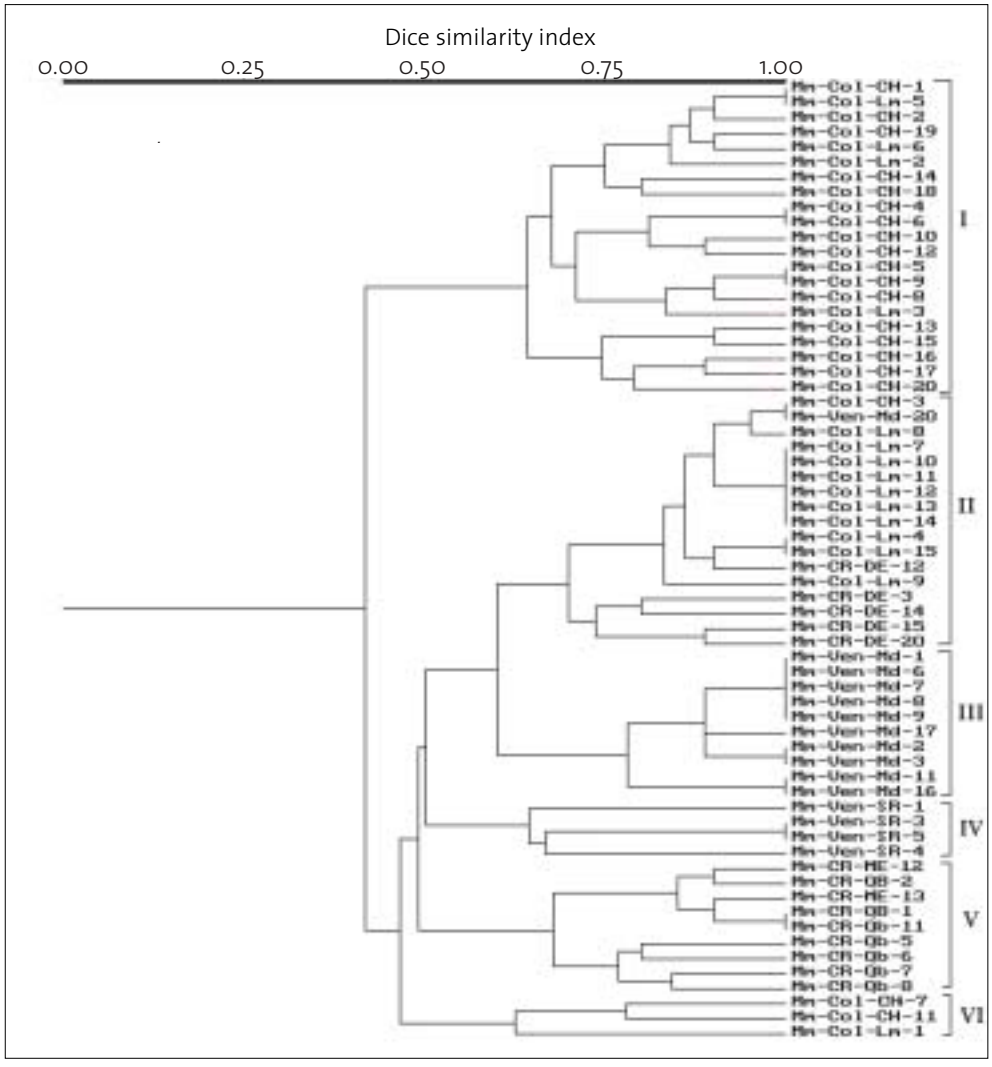

Figure 1. Dendogram for *Mycosphaerella musicola* populations, based on Dice similarity indexes. Colombian populations are Mm-Col-CH: Clachipay and Mm-Col-LM: La Mesa; Venezuelan populations are Mm-Ven-Md: Merida and Mm-Ven-SR: Santa Rosa; Costa Rican populations are Mm-CR-DE: el Descanso, Mm-CR-ME: Ma. Eugenia and Mm-CR-QB: Quebrador.

Discussion

In the *M. fijiensis* and *M. musicola* dendograms, most clusters correspond to the original sampling sites and show a correlation between clusters and discrete populations. Studies of genetic diversity at a worldwide level (Carlier *et al.*, 1996) and a

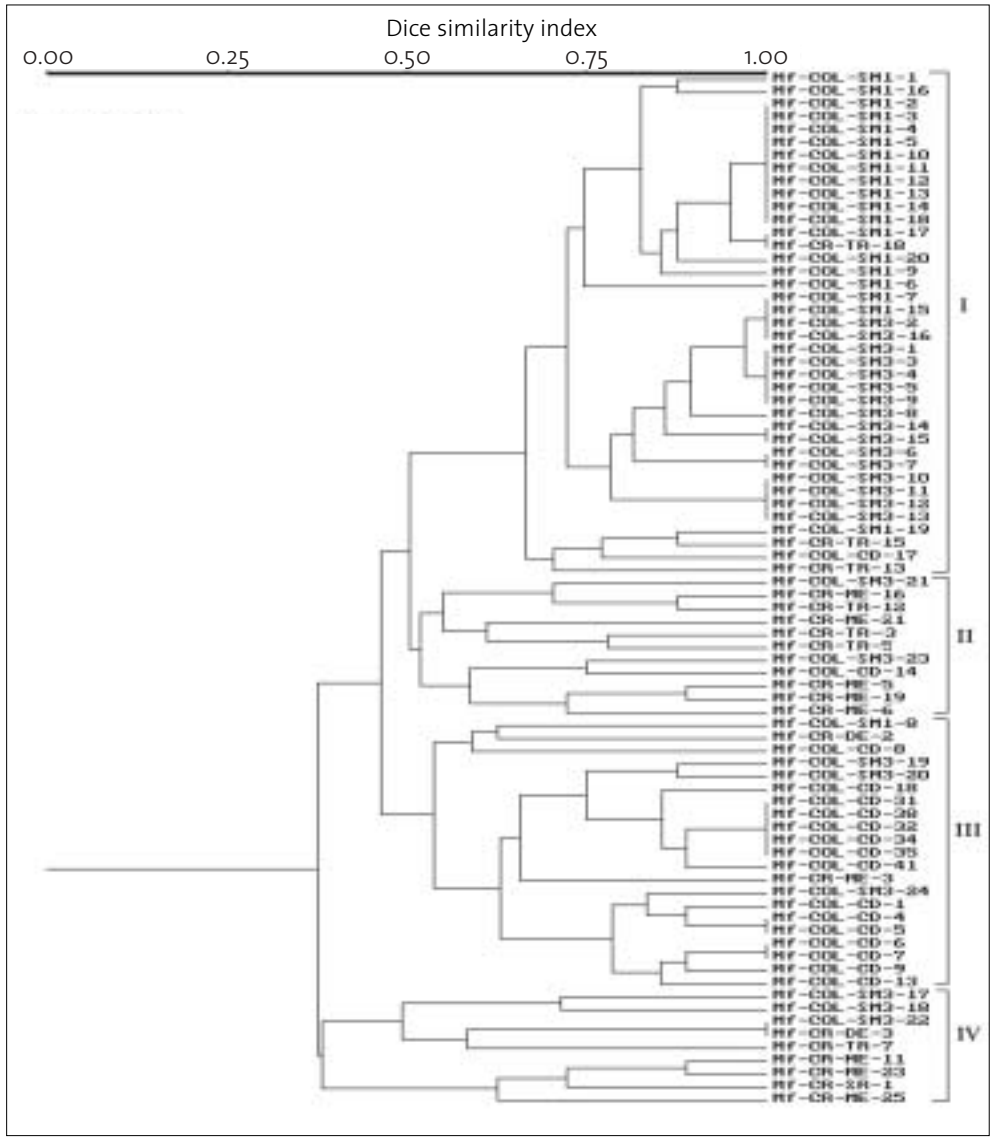

Figure 2. Dendogram for *Mycosphaerella fijiensis* populations, based on Dice similarity indexes. Colombian populations are Mf-Col-SM: Santa Marta and Mf-Col-CD: Caldas; Costa Rican populations are Mf-CR-DE: el Descanso, Mf-CR-ME: Ma. Eugenia, Mf-CR-TR: Trsissia and Mf-CR-SR: San Rafael.

regional level in Africa (Müller *et al.*, 1997) suggest that in most situations colonization by *M. fijiensis* involves founder effects, a few individuals from an original population representing the haplotypic pool of a derived population.

In the *M. musicola* tree, all but one of the Venezuelan isolates are grouped in two discrete clusters (III and IV). This is particularly true for the isolates from Mérida, which show very similar to identical DNA profiles. Only one Venezuelan isolate (Mm-Ven-Md-20) is in a separate group, with Colombian isolates from La Mesa (cluster II). *M.*

musicola populations are mainly confined to mid-altitude regions of the Andes where bananas are grown in smallholdings. These smallholdings are separated by deep valleys and high mountains which are geographical barriers to gene flow. This may explain the separation of Colombian and Venezuelan populations in the *M. musicola* tree, despite being located in the same mountain range.

In the *M. fijiensis* tree, it is important to highlight that the majority of isolates from the Atlantic coast (Santa Marta) are grouped in cluster I, whereas isolates from the mid-Andean region (Caldas) are in cluster III. Both regions are isolated from each other by physical distance and geographical barriers that prevent gene flow between populations. In addition, populations from the Atlantic coast are constantly under environmental pressure (high dosages of fungicides) whereas Andean populations are rarely treated with chemicals, keeping those areas as reservoirs of genetic diversity.

The colonization dynamics of *M. fijiensis* for Central America has been documented (Unión de Países Exportadores de Banano, 1994). Colonization started from Honduras and continued southwards to Costa Rica and Colombia. Black leaf streak disease was first reported in 1981 in Urabá, Colombia, where it was confined for four years, until a first outbreak occurred along the shores of the Atrato River and then spread to the Atlantic coast and mid-Andean regions. Forty four isolates from Santa Marta (Atlantic Coast) corresponded to only 23 haplotypes, whereas 16 isolates from Caldas (mid-Andes) represented 10 haplotypes. This could be an indication of higher genetic diversity in the Caldas populations. In the Atlantic coast of Colombia, the genetic consequences of the founder effect could have been enhanced by the pressure exerted by high doses of fungicide and strict regulations on the transport of plant material between populations.

M. fijiensis and *M. musicola* followed the same route, that is from Central America to Colombia. In cluster II of each phenogram (Figure 1 and 2), Costa Rican isolates are found in the same group with Colombian isolates. The genetic similarity of these two distant populations is consistent with the fact that Central American populations are ancestral and that the similarity cannot be explained by gene flow.

Acknowledgements
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Poster

An electrophoretic karyotype for *Mycosphaerella fijiensis*

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Abstract

In view of the current problems caused by black leaf streak disease in banana production, a knowledge and understanding of the genetics and organization of the genome of *Mycosphaerella fijiensis* could lead to the development of new control strategies. Regarding the former, mycelium was obtained from isolates of *M. fijiensis* from three sites in Mexico (Veracruz, Colima and Chiapas) in order to estimate the size of the genome by using the CHEF (Contour clamped Homogeneous Electric Field) system. Different conditions of pulse field electrophoresis enabled the separation of *M. fijiensis* chromosomes and a preliminary estimate of the karyotype of each isolate was obtained. Isolates from Colima and Chiapas had bands corresponding to at least 10 chromosomes in the size range 0.71 to 2.2 Mb. The Veracruz isolate had at least 14 chromosomes in a size range of 0.67 to 5.6 Mb. Genome size calculated for the Veracruz isolate was at least 28 Mb, which is comparable to that of some ascomycete fungi. Attempts were made to estimate the genome size of the Colima and Veracruz isolates. Differences in the principal band suggested the presence of polymorphisms in chromosome length between the isolates studied, as reported for other species of fungi.

Resumen – Cariotipo molecular de *Mycosphaerella fijiensis*

Ante la problemática actual ocasionada por la Sigatoka negra en la producción de banano, el conocimiento y la comprensión de la genética y la organización del genoma de *Mycosphaerella fijiensis* podrían conducir al desarrollo de nuevas estrategias para su control. Considerando lo anterior, se propuso obtener el cariotipo molecular de tres aislados de *M. fijiensis* por medio del sistema CHEF (*Contour clamped Homogeneous Electric Field*), así como estimar su tamaño genómico. Para ello, se utilizó el micelio de aislados procedentes de tres diferentes lugares de México (Veracruz, Colima y Chiapas). Se ensayaron diferentes condiciones de electroforesis de campo pulsante que permitieron separar los cromosomas de *M. fijiensis*. Se obtuvo una estimación preliminar del cariotipo de cada aislado. En los aislados de Colima y Chiapas se observaron bandas correspondientes a por lo menos 10 cromosomas, en un rango de tamaño entre 0.71 y 2.2 Mb. En

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el aislado de Veracruz se observaron por lo menos 14 cromosomas en un rango de tamaño entre 0.67 y 5.6 Mb. El tamaño del genoma calculado para el aislado de Veracruz es de al menos 28 Mb lo cual es comparable con algunos hongos ascomicetos reportados en la literatura. Se pretende realizar experimentos para estimar el tamaño genómico de los aislados de Colima y Veracruz. Diferencias observadas en el patrón de bandeo sugieren la existencia de polimorfismos en la longitud de los cromosomas entre los aislados estudiados, lo cual también ha sido reportado en otras especies de hongos.

Résumé – Un karyotype électrophorétique pour *Mycosphaerella fijiensis*
Du fait des problèmes posés à l'heure actuelle par la maladie des raies noires pour la production de bananes, la connaissance et la compréhension de la génétique et de l'organisation du génome de *M. fijiensis* pourrait conduire au développement de nouvelles stratégies de contrôle. En ce qui concerne ce dernier point, du mycelium a été obtenu à partir d'isolats de *M. fijiensis* provenant de trois sites au Mexique (Veracruz, Colima et Chiapas) afin d'estimer la taille du génome en utilisant le système CHEF (*Contour clamped Homogeneous Electric Field*). Des conditions différentes d'électrophorèse en champ pulsé ont permis la séparation des chromosomes de *M. fijiensis* et une estimation préliminaire du karyotype de chaque isolat a été obtenue. Les isolats provenant du Colima et du Chiapas avaient des bandes correspondant à au moins 10 chromosomes dont la taille variait entre 0,71 et 2,2 Mb. L'isolat de Veracruz avait au moins 14 chromosomes dont la taille variait entre 0,67 et 5,6 Mb. La taille du génome calculée pour l'isolat provenant de Veracruz était d'au moins 28 Mb, ce qui est comparable à celle de certains champignons ascomycètes. Des essais ont été réalisés pour estimer la taille du génome des isolats provenant de Colima et de Veracruz. Les différences observées pour la bande principale suggèrent la présence de polymorphismes dans la longueur des chromosomes entre les isolats étudiés, comme cela a été rapporté chez d'autres espèces de champignons.

Introduction

The chromosomes of many fungi are too small to be identified by cytological methods therefore the detailed karyotype and genome size of most fungal species are unknown. But starting with the successful separation of *Saccaromyces cerevisiae* chromosomes (Schwartz and Cantor, 1984), Pulsed Field Gel Electrophoresis (PFGE) has been used to obtain the molecular karyotypes of important fungi, including pathogenic fungi. New technologies and electrophoresis apparatus have since been developed, resulting in improved techniques for separating large DNA fragments and chromosomes.

The CHEF (contour-clamped homogeneous electric field), electrophoresis system (Chu *et al.*, 1986), has been used to separate fungal chromosomes and estimate genome size. Karyotyping procedures using PFGE generally involve the production of protoplasts or sphaeroplasts. However a different technique (McCluskey *et al.*, 1990) permits the preparation of chromosome-sized DNA without the need for such laborious procedures.

Even though it affects *Musa* worldwide, there are no data on the karyotype or genome size of *Mycosphaerella fijiensis*. Knowing and understanding the genetics and genome organization of fungal pathogens could lead to the development of new strategies in controlling black leaf streak disease. This paper describes the use of PFGE to obtain the molecular karyotype of *M. fijiensis* and estimates of genome size.

Methods

Leaves displaying symptoms of black leaf streak disease were collected from Veracruz, Colima and Chiapas in Mexico. Fungal isolates were obtained from single ascospores and grown on PDA medium. The identity of isolates was verified by the morphology of mycelium, Koch´s postulates and PCR (Johanson, 1997; Neu *et al.*, 1999). For the preparation of agarose plugs, isolates were grown in liquid shake culture (PDB medium) for 6 days at 26ºC and 100 rpm. The mycelium was separated by centrifugation, ground, washed with buffer, mixed with melted agarose at 45°C and transferred to plug moulds. Plugs were incubated in proteinase K at 60°C for 48 h, and washed and stored in 0.5 M EDTA at 4°C. Empirical methods were tried to optimize the resolution of chromosomes of different size ranges, using PFGE with CHEF DR II and CHEF DR III. Finally, genome size was estimated by adding the values assigned to each band resolved in pulsed field gels.

Results and discussion

We obtained a preliminary estimation of the karyotype of each isolate. For the Veracruz isolate, 12 bands were resolved, representing 14 chromosomes in a size range of 0.67 to 5.6 Mb (Figures 1 and 2). At least 10 chromosomes were resolved in the Colima and Chiapas isolates, in a size range of 0.71 to 2.2 Mb. The estimated genome size for the Veracruz isolate is at least 28 Mb (Figure 3), which is comparable to that reported for other ascomycetes (Cooley and Caten, 1991; McDonald and Martinez, 1991). The genome sizes of the other two isolates are to be determined in further experiments. Differences observed in banding patterns suggest the existence of chromosome length polymorphisms, which has been reported for other fungi. A broader study with different and well characterized haplotypes of *M. fijiensis* is now underway at the *Centro de Investigación Científica de Yucatán*.

Conclusion

This is the first estimate of the karyotype and genome size of *M. fijiensis*, a useful tool for constructing a physical and genetic map or for calculating the required size of a genomic library

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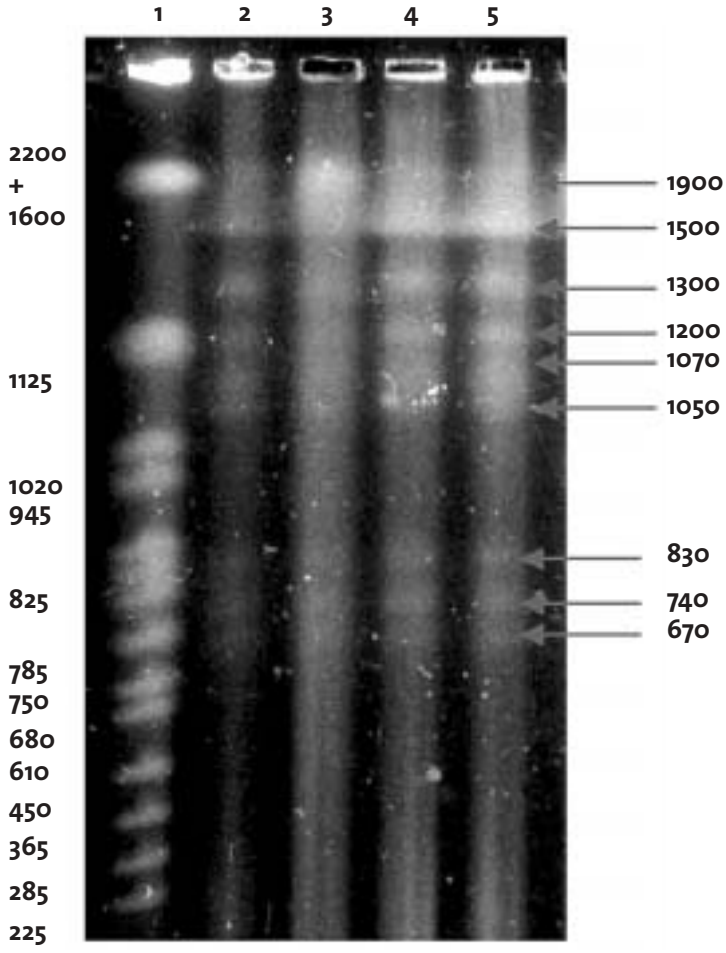

Figure 1. CHEF gel stained with SYBR Green showing separation of medium size chromosomes. Lane 1: *S. cerevisiae* chromosome size standards. Lanes 2-5: *M. fijiensis* isolate from Veracruz. Numbers on the left give the siez of the standards. Numbers on the right correspond to the nine bands resolved. Numbers in bold represent comigrating bands.

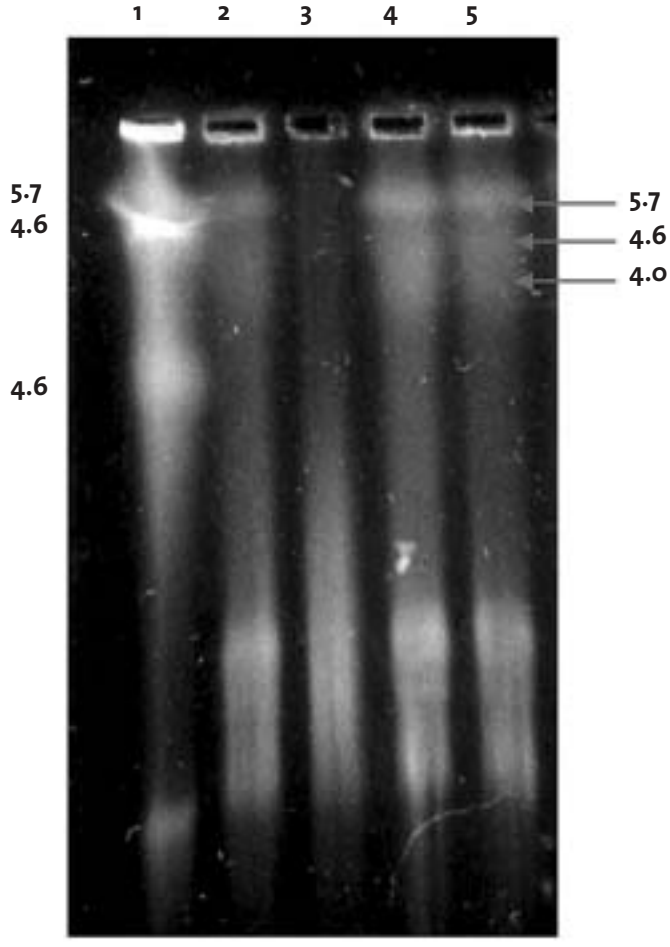

Figure 2. CHEF gel stained with SYBR Green showing separation of largest chromosomes. Lane 1: *S. pombe* chromosome size standards. Lanes 2-5: *M. fijiensis* isolate form Veracruz. Numbers on the left give the size of the standards. Numbers on the right correspond to the three bands resolved. Numbers in bold represent comigrating bands.

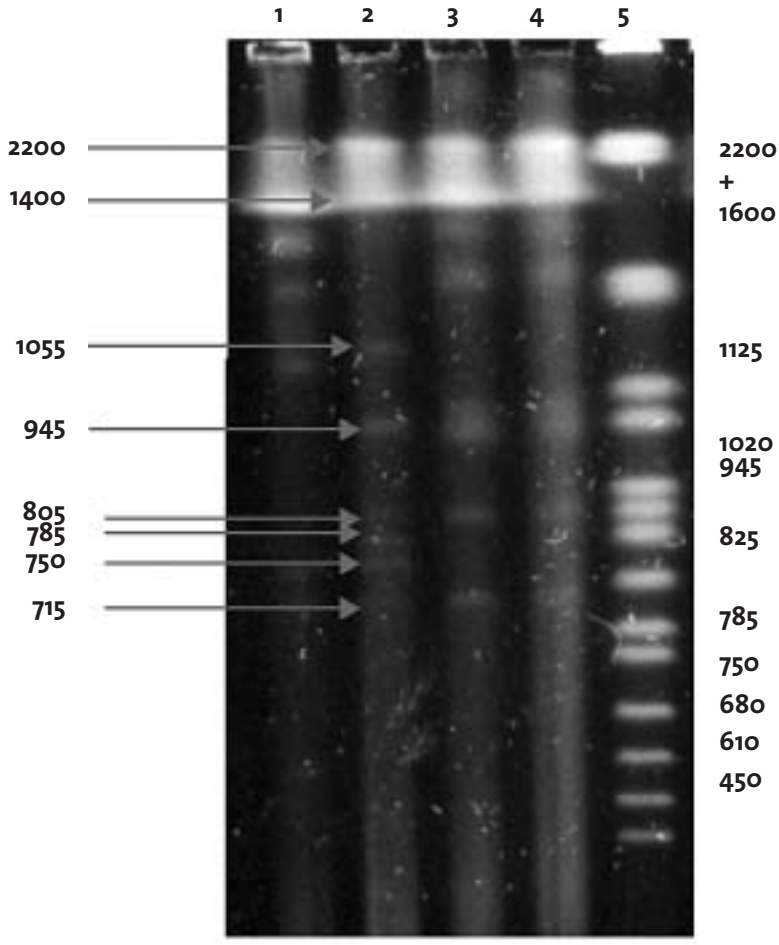

Figure 3. CHEF gel stained with SYBR Green showing separation of largest chromosomes. Lanes 1-4: *M. fijiensis* isolates from Veracruz, Colima, Chiapas #1 and Chiapas #2 respectively. Lane 5: *S. cerevisiae* chromosome size standards. Numbers on the right give the size of the standards. Numbers on the left correspond to the eight bands resolved. Numbers in bold represent comigrating bands.

Recommendations of session 2

Information on the epidemiology and population structure of the main *Mycosphaerella* species (*M. fijiensis*, *M. musicola* and *M. eumusae)* at the national, regional and international levels are needed to better understand the distribution and the spread of the pathogens, to anticipate the evolution of pathogen populations and to define resistance management strategies. Such studies are particularly necessary in Asia, the centre of diversity of the three pathogens and where little research has so far been conducted.

Distribution of *Mycosphaerella* spp.

Knowing the name of the banana clones affected, the severity of the leaf spot diseases and the local environmental conditions would help explain the distribution of *M. fijiensis, M. musicola* and *M. eumusae*. IMTP trials are seen as ideal locations for assessing the reaction of the banana clones to various leaf spot pathogens.

The collection and diagnosis of specimens from IMTP trials sites should be continued. Identification tools should be provided to enable diagnoses to be undertaken locally.

The exact distribution of M. eumusae *needs to be known.*

Further surveys in South and Southeast Asia are necessary to determine where M. musicola, M. fijiensis *and* M. eumusae *occur. The cooperation and collaboration of scientists in South and Southeast Asia is viewed as essential. The INIBAP regional office for Asia and the Pacific should strengthen and facilitate any exchange between Asian partners and the rest of the PRO*MUSA *community.*

National and international collections

The creation of national collections of strains of *Mycosphaerella* pathogens is of special relevance to the understanding of population structure. The collections must be based on single-spore cultures with an *in vitro* characterization of the anamorph stage (*in vitro* sporulation of conidia). Diagnostic tools would help the development of collections of *Mycosphaerella* isolates. It has been recommended to provide the participants with a protocol to sample, establish and maintain the collections (see the "Diagnostics" section in session 1 recommendations).

A reliable, rapid test to distinguish M. musicola, M. fijiensis, M. eumusae *and possible other* Mycosphaerella *pathogens/saprophytes needs to be developed. Information on how to distinguish the three pathogens on morphological characteristics also needs to be produced and circulated to banana scientists. INIBAP was asked to address this need.*

The establishment of a national collection should be promoted and facilitated through the organization of a training course; especially for those countries that develop breeding programmes, but also in places where banana resistant hybrids are used on an industrial scale, and where the high diversity of Musaceas has likely produced a similar diversity of pathogens.

It was recommended to develop an international core collection of M. fijiensis, M. musicola *and* M. eumusae. *The different strains should be conserved as fungal mycelia and DNA. CIRAD was suggested as host of the international collection, using a similar mechanism as INIBAP developed with KULeuven regarding* Musa *germplasm. INIBAP was asked to address this needs in collaboration with CIRAD.*

Genetic population structure

The study of the genetic population structures of *Mycosphaerella* pathogens is already ongoing at the national, regional and international levels. However, the number of countries involved at the national level should be increased to refine regional and international studies.

The sampling protocols should be standardized and widely distributed. INIBAP and CIRAD agreed to work together in the preparation of this information which should include several detailed illustrations of the different pathogens and their anamorph stages. This information should also be part of the IMTP guidelines.

More molecular markers, such as SSR and CAPS, should be developed to improve the understanding of the different populations structures.

Pathogenicity characterization

In vitro and *in vivo* inoculation systems exist to evaluate the pathogenicity of the various *Mycosphaerella* strains. The different pathogens and their relation to their host need to be compared under controlled conditions using these methods.

The methodologies that currently exist should be standardized. The in vitro *inoculation on leaf fragment developed at CIRAD should be distributed together with the methodology to isolate, cultivate and produce the inoculum of the different pathogens. INIBAP and CIRAD have been requested to compile in a technical document, all the different information already published on these methods.*

Dispersal of *Mycosphaerella* spp.

More research is necessary to understand spore dispersal.

Disease incidence data should be collected from the field and the scientific literature.

Laboratory methods to understand the mechanism of spore release, and spore survival in the atmosphere should be developed.

The potential for windborne dispersal suspected from laboratory studies should be verified and assessed at the plantation level (as opposed to dispersal through other means, such as infected planting material).

Session 3

Host-pathogen interactions

Introduction

Banana–*Mycosphaerella fijiensis* interactions

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Abstract

Using standard testing procedures, banana genotypes were classified as 1) highly resistant cultivars characterized by an early blockage of leaf infection (incompatible interactions), 2) partially resistant cultivars exhibiting a slow rate of symptom development (compatible reactions) and 3) susceptible cultivars, characterized by rapid development of necrotic lesions (compatible reaction).

Most information on incompatible reactions comes from observations of early necrosis of stomatal guard cells and the deposit of electron-dense compounds around the penetration sites of *M. fijiensis* on the cultivar 'Yangambi km5'. Such rapid death of a few host cells, associated with the blockage of the progression of the infecting agent is usually defined as a hypersensitive reaction. Such a reaction often operates within a gene-for-gene relationship and as a consequence the resulting resistance may be unstable.

As regards compatible interactions, cytological studies showed that *M. fijiensis* behaves first as a biotrophic parasite which colonizes exclusively the intercellular spaces without the formation of haustoria. Two main mechanisms have been investigated to explain the slow development of a lesion in partially resistant genotypes: preformed antifungal compounds and tolerance to putative toxin(s) produced by *M. fijiensis*.

The mechanisms will be presented in relation to their possible use as early screening markers for selecting banana genotypes for durable resistance to *M. fijiensis*.

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Resumen - Interacciones banano – *Mycosphaerella fijiensis*
Utilizando los procedimientos de evaluación estándar, los genotipos de banano fueron clasificados en tres categorías: 1) cultivares altamente resistentes caracterizados por un bloqueo temprano de la infección foliar (interacciones incompatibles), 2) cultivares parcialmente resistentes que exhiben una evolución lenta de los síntomas (reacciones compatibles), y 3) cultivares susceptibles, caracterizados por un desarrollo rápido de las lesiones necróticas (reacción compatible).
La mayor parte de la información sobre las reacciones incompatibles proviene de los estudios del cultivar 'Yangambi km5'. Se observaron la necrosis de las células de guarda estomatales y los depósitos de los compuestos con alta densidad de electrones alrededor de los sitios de penetración de *M. fijiensis*. La muerte tan rápida de unas cuantas células hospedantes asociada con el bloqueo de la progresión del agente infectante se define usualmente como una reacción hipersensible. Esta reacción a menudo opera dentro de una relación de gen por gen y podría convertir la resistencia en inestable.
Con las reacciones compatibles, los estudios citológicos revelaron que *M. fijiensis* se comporta primero como un parásito biotrófico que coloniza exclusivamente los espacios intercelulares sin formar los haustorios. Dos mecanismos principales podrían estar involucrados en el desarrollo lento de las lesiones observado en los genotipos resistentes parcialmente: compuestos antifungosos sintetizados de manera constitutiva o tolerancia a la(s) toxina(s) putativa(s) producidas por *M. fijiensis*.
Estos mecanismos se presentarán en relación con su posible utilidad como marcadores de cribado temprano en la selección de los genotipos de banano con respecto a la resistencia duradera a *M. fijiensis*.

Résumé - Interactions bananier–*Mycosphaerella fijiensis*
En utilisant des procédures de test standard, des génotypes de bananier ont été classés en : 1) cultivars hautement résistants caractérisés par un blocage rapide de l'infection foliaire (interactions incompatibles), 2) cultivars partiellement résistants montrant un développement lent des symptômes (réactions compatibles) et 3) cultivars susceptibles caractérisés par un développement rapide de lésions nécrotiques (réaction compatible).
L'essentiel des informations sur les réactions incompatibles provient d'observations de nécrose précoce des cellules de garde des stomates et du dépôt de composés denses en électrons autour des sites de pénétration de *M. fijiensis* chez le cultivar 'Yangambi km5'. La mort aussi rapide d'un petit nombre de cellules hôtes, associée avec le blocage de la progression de l'agent infectieux, est habituellement définie comme une réaction hypersensible. Une telle réaction se produit souvent dans le cadre d'une relation gène pour gène et, en conséquence, la résistance qui en résulte peut être instable.
Pour ce qui concerne les interactions compatibles, les études cytologiques ont montré que *M. fijiensis* se comporte d'abord comme un parasite biotrophique qui colonise exclusivement les espaces intercellulaires sans formation d'haustoria. Deux mécanismes principaux ont été étudiés pour expliquer le développement lent des lésions chez les génotypes partiellement résistants : des composés antifongiques préformés et la tolérance à une(des) toxine(s) putative(s) produite(s) par *M. fijiensis*.
Les mécanismes sont présentés en relation avec leur utilisation possible comme marqueurs lors de criblage précoce pour sélectionner des génotypes de bananiers possédant une résistance durable à *M. fijiensis*.

Introduction

Black leaf streak disease is the most devastating disease of banana and plantain worldwide. The fungus induces foliar leaf streaks which, in highly susceptible cultivars, leads to the total collapse of the plant.

Just as host plants evolved several defence mechanisms, pathogens have ways to evade or suppress these defence mechanisms. The response of the host and the

pathogen are crucial to the outcome of infection. A knowledge of the interactions is increasingly important for the rational selection of genotypes resistant to plant pathogens. The interactions between banana and *Mycosphaerella fijiensis* remained unknown for a long time.

Although the field performance of an accession is the ultimate reference for evaluating its resistance, the method is not suitable for the study of host pathogen interactions. Nevertheless, the reaction to black leaf streak disease of about 50 *Musa* species belonging to various genetic groups was studied under natural infection conditions (Fouré *et al.,* 1990). The study led to the grouping of the banana genotypes in three categories: 1) highly resistant (HR) cultivars characterized by an early blockage of leaf infection (incompatible interactions); 2) partially resistant (PR) cultivars exhibiting slow rates of symptom development (compatible interactions); and 3) susceptible (S) cultivars characterized by a rapid development of necrotic lesions (compatible interactions). Later, banana-*M. fijiensis*-interactions were studied under controlled conditions of inoculation (Mourichon *et al.*, 1987) which reproduced the behaviour in the field of three reference cultivars: 'Yangambi km5' (AAA; HR), 'Fougamou' (ABB; PR) and 'Grande naine' (AAA; S).

These preliminary results, which were presented at the International workshop held in San José in 1989, were the start of investigations into banana-*M. fijiensis* interactions. They began with the microscopic events that take place in banana tissues and were followed by the analysis of the biochemical processes that culminate in the expression of resistance or susceptibility.

Host-pathogen interactions

Cytological studies of the interactions between *M. fijiensis* and the three reference cultivars 'Yangambi km5', 'Fougamou' and 'Grande naine' revealed that *M. fijiensis* enters banana leaves by the stomata.

In compatible interactions ('Grande naine' and 'Fougamou' inoculated with *M. fijiensis,* strain 049 HND from Honduras), the pathogen colonized exclusively the intercellular spaces between mesophyll cells, without forming haustoria. There was a long period of biotrophy before the observation of the first cytological alterations to the mesophyll cells. Hyphae were observed between living cells ahead of the necrotic zone, a faster growth rate of hyphae being the main difference between susceptible ('Grande naine') and partially resistant ('Fougamou') cultivars (Beveraggi, 1992; Beveraggi *et al.*, 1995).

In contrast, early necrosis of stomata guard cells and appositions around the penetration sites were observed with incompatible interactions ('Yangambi km5' inoculated with *M. fijiensis* strain 049HND) (Beveraggi *et al.*, 1995).

The behaviour of partially and highly resistant genotypes of banana can be linked to major groups of plant-parasite interactions. The rapid death of only a few host cells, associated with the blockage of the progression of the infecting agent in the highly resistant cultivar 'Yangambi km5', is usually defined as an hypersensitive reaction. These often operate within a gene-for-gene relationship giving rise to resistance that is unstable. In comparison, the partial resistance of the reference cultivar 'Fougamou', for example, is usually considered polygenic and durable.

Incompatible interactions: Highly resistant cultivars

The hypersensitive reaction operating within a gene-for-gene relationship is generally explained, either by the presence of a specific avirulence factor (or elicitor) or by the coordinated action of non-specific elicitor(s) and a specific suppressor (de Wit, 1992; Atkinson, 1993). In banana–*M. fijiensis* interactions, there is no experimental evidence of a gene-for-gene relationship because it is difficult to study the genetics of triploid genotypes such as 'Yangambi km5'. But that such a relationship exists is supported by laboratory tests in which isolates of *M. fijiensis* overcame the resistance of 'Yangambi km5' (Fullerton and Olsen, 1995).

Riveros and Lepoivre (1994) did preliminary experiments to identify the elicitors that induce resistance. Intercellular fluids (IF) from leaves of 'Yangambi km5' (incompatible) and 'Grande naine' (compatible) inoculated with 049HND, and crude eliciting fractions (CEF) prepared from germinating spores of virulent and avirulent *M. fijiensis* isolates, elicited necrosis and appositions in banana cultivars (Riveros and Lepoivre, 1994).

Regardless of the eliciting preparation (IFs or CEFs prepared with avirulent or virulent *M. fijiensis* isolates), the reaction was more intense and quicker in 'Yangambi km5' than in the susceptible 'Grande naine'. The behaviour of 'Yangambi km5' cannot be explained by a race-specific eliciting activity in the IFs or the CEFs. However, the eliciting activity present in the IFs of 'Yangambi km5' inoculated with the avirulent strain 049 HND appeared to be higher than that in the compatible relationship between 'Grande naine' and the same isolate. Thus, we speculate that 'Yangambi km5' could have a higher sensitivity to the elicitor(s) but could also have a host-mediated effect on the release, production or stability of specific elicitor(s) produced by the fungal isolates. Such host-mediated effects have been reported in soybean tissues where plant enzymes are responsible for the release of elicitors from hyphal walls of *Phytophthora megasperma* (Boller, 1987).

A wide range of fungal compounds have been implicated as elicitors of HR: polysaccharides (Sharp *et al.*, 1984), glycoproteins (Schaffrath *et al.*, 1995), peptides (de Wit *et al.*, 1985) and hydrolytic enzymes (Boller, 1987). In banana–*M. fijiensis* interactions, polysaccharide compounds may be involved in eliciting activity (Riveros, unpublished data).

Evidence of a hypersensitive-like-reaction to *M. fijiensis* represents a first step towards a better characterization of that reaction. Independently of the mechanisms of resistance, there is the problem of the durability of resistance. Durability is of outmost importance because breakdown of resistance for a staple crop such as plantain would have dramatic effects. Because of the difficulties inherent in improving triploid bananas, breeding for resistance to black leaf streak disease often did not take into account host-pathogen interactions. The existence of *M. fijiensis* isolates able to overcome resistant 'Yangambi km5' in laboratory tests (Fullerton and Olsen, 1995) shows that highly resistant parents should not be used without appropriate management procedures, such as mixtures of cultivars, choice of the "right gene combination" and co-ordinated regional deployment of genes.

Compatible reaction: partial resistant cultivars

With partially resistant cultivars (compatible interactions), cytological studies showed that *M. fijiensis* behaves at first as a biotrophic parasite that colonizes exclusively the intercellular spaces (Beveraggi *et al.* 1995). Two possible mechanisms have been investigated to explain slow lesion development in partially resistant genotypes: preformed antifungal compounds and tolerance to putative toxin(s) produced by *M. fijiensis*.

Constitutively synthesised antifungal compounds

Many antimicrobial compounds produced by plants play an important role in the response to infection by cellular pathogens. Defence compounds may be classified into phytoanticipins, which are constitutive, and phytoalexins, which are synthesised in response to microorganisms. The two groups of secondary metabolites include a wide range of chemical families. However, phytoanticipins are primarily involved in non-host rather than varietal resistance.

For 'Fougamou', histological analysis revealed the presence, in mesophyll layers, of many specialized cells containing vacuoles rich in polyphenol. The contents of the vacuoles were released into the intercellular spaces. The contents had a high affinity for fungal cell walls and their presence around hyphae seemed to be correlated with the slow growth of mycelium in parenchyma tissues (Beverragi *et al.*, 1992, 1995). Gire (1994) identified soluble phenols in the leaf tissues of several banana cultivars with different levels of partial resistance. He also observed a close correlation between flavane (protoanthocyanidins) content and the level of partial resistance. However, a study conducted on a larger number of genotypes suggested that the role of these constitutive compounds in partial resistance is restricted to a limited number of cultivars (El Hadrami, 1997).

The role of toxins in pathogenesis

Pathogen toxins could constitute an alternative technique for rapidly screening resistant banana genotypes as *in vitro* plant tissues or young plants. The symptoms of black leaf streak disease suggest a possible involvement of phytotoxic compounds. Such compounds were found in culture filtrates of *M. fijiensis* (Molina and Krausz, 1989; Lepoivre and Acuna, 1989; Upadhyay *et al.*, 1990; Strobel *et al.*, 1993). Stierle *et al.* (1991) reported that 2,4,8-tetrahydroxytetralone and juglone were the most abundant and most phytotoxic compounds.

If toxins are involved in the development of black leaf streak disease, it may be possible to use them to identify resistant genotypes. During the previous workshop at San José, the use of *M. fijiensis* toxins for screening had four major limitations: 1) a lack of quantitative and sensitive bioassays to measure the effects of *M. fijiensis* metabolites on banana genotypes; 2) insufficient characterization of the variability in toxin production of *M. fijiensis;* 3) a lack of experimental evidence for the role of the metabolites in the disease; and 4) the assurance that the susceptibility and/or resistance of cultured tissues reflected the reaction of the whole plant.

Bioassay to quantify the effect of *M. fijiensis* metabolites

A set of bioassays was developed to quantify the toxic effects of the metabolites obtained from *M. fijiensis* culture filtrates. The induction of necrosis by a leaf puncture bioassay on detached banana leaves, or the injection of ethyl acetate crude extract (EaCE) into the leaves are easy to perform but neither method is sensitive (injections of 250 ppm EaCE are required for 'Grande naine') or quantitative.

The electrolyte leakage assay represented a quantitative but rather insensitive assessment of the toxicity of *M. fijiensis* metabolites. The test did not distinguish between cultivars. The most sensitive and accurate toxin assay was based on the measurement of chlorophyll fluorescence. The vitality index seemed to be the most sensitive method for early assessment of the effects of EaCE and a specific indicator of photosynthetic activity.

Purification of the EaCE revealed the presence of different fractions with similar properties to the crude extracts. Juglone, a purified metabolite previously shown to be present in extracts of *M. fijiensis* culture filtrates, was identified in the extracts of all the strains analyzed. Injection of juglone into banana leaf tissues gave similar results to EaCE for ranking cultivars (Etame, unpublished data).

Chloroplasts as target site of juglone

The involvement of the photosynthetic apparatus in reaction to EaCE and juglone is in agreement with observation of light-dependent toxicity irrespective of the bioassay. The observation of swelling chloroplasts as the first abnormality observed by electron microscopy of EaCE-treated leaves also fits this pattern.

Busogoro (unpublished data) developed a bioassay using isolated chloroplasts and measuring their capacity to reduce 2,6- dichlorophenoindolphenol (DCPIP) as a marker of the Hill reaction, which expresses electron transport from water to any electron acceptor by intact chloroplasts when exposed to light (Allen and Holmes, 1986).

Juglone inhibited the Hill reaction in suspensions of banana chloroplasts. In addition, 'Fougamou' chloroplasts appeared to be less affected by juglone than 'Grande Naine' chloroplasts. These results suggest that chloroplasts are one of the primary action sites of juglone.

The role of toxins in banana-*M. fijiensis* interactions

The electrolyte leakage assay and chlorophyll fluorescence were used to compare the sensitivity to EaCE of different banana cultivars with their behaviour in the field (highly resistant, sensitive or partially resistant) as scored using the rank of the youngest leaf spotted with necrotic lesions.

These toxin assays confirmed that the incompatible interactions of 'Yangambi km5' were not related to resistance to EaCE. The toxicity of EaCE preparations was independent of the virulence of the strain (unpublished data). Mechanisms of resistance in highly resistant cultivars were definitely not related to the action of these toxic metabolites.

Considering just the susceptible 'Grande naine' and the partially resistant 'Fougamou' cultivars, susceptibility to EaCE was correlated with sensitivity to infection, suggesting that slow lesion development is associated with a lower sensitivity to *M. fijiensis* toxins.

Such quantitative assessment is difficult to interpret because the concentrations of toxin(s) that were used in the bioassay could exceed the *in vivo* concentration and affect the mode of action of EaCE, hence affecting the rating of the cultivars.

The hypothesis that *M. fijiensis* metabolites have a secondary role as determinants of pathogenicity agrees with cytological studies, which showed no evidence of an early effect of toxic compounds in the long period of biotrophy before observing the first cytological alterations in the mesophyll cells.

Selection of banana tissues resistant to juglone

The work was done with 'Three hand planty', a genotype susceptible to black leaf streak disease, and juglone for which an embryo cell suspension was available. Juglone was toxic to embryogenic cell suspensions and somatic embryos of the cultivar. Necrosis of all cell suspensions and somatic embryos was quickly observed at 50 ppm or more of juglone, with the exception of some somatic embryos that continued development after treatment with 50 ppm of juglone.

The plants regenerated from the surviving embryos showed a higher resistance to juglone: 250 ppm was required to induce necrosis in the leaf puncture bioassay with selected plantlets in comparison with 100 ppm for non selected plants. However, the selected plants did not show higher resistance to black leaf streak disease than the mother Three hand planty' genotype following inoculation with *M. fijiensis* (El Hadrami, unpublished data).

Daub (1986) advised caution when using metabolites from pathogens to screen tissue cultures for resistance. Nevertheless, fungal toxins have been proposed to screen banana *in vitro* (Strobel *et al.*, 1993). There have been claims that resistant material has been produced by selecting callus of banana that survived increasing concentrations of *M. fijiensis* toxins (Okole and Shulz, 1993). Our results confirm the possibility of selecting banana plants resistant to *M. fijiensis* metabolites but this approach did not result in higher resistance to black leaf streak disease.

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Efficiency and durability of partial resistance against black leaf streak disease

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Abstract

Black leaf streak disease caused by *Mycosphaerella fijiensis* is the most destructive leaf disease of bananas and plantains. Genetic improvement for resistance appears as the most appropriate tool to control the disease. As a high level of diversity is maintained in pathogen populations, breeders prefer working with partial resistance, which is thought to be durable, instead of total resistance. Our aim is to evaluate the efficiency and durability of partial resistance. To achieve this objective, three complementary approaches were undertaken:

1) Partial resistance was characterized by measuring various variables over the life cycle of the fungus under field and controlled conditions. The evaluation of 13 partially resistant varieties revealed the existence of several components acting at various stages of the infectious cycle.

2) The efficiency of two resistant varieties which differ for two resistance components (infection efficacy, ascospores production) were studied. No difference in disease dispersal and incidence was observed between resistant varieties during the first year whereas small differences in disease severity, increasing over time, were measured. These results could be explained by differences in endogeneous inoculum production. Experiments are conduced to measure endogeneous inoculum in each field to confirm this hypothesis.

3) The durability of resistance is being studied by analyzing the evolution of pathogen populations. Molecular characterization using CAPS markers was used on populations isolated after 6 and 25 months of cultivation. No significant difference between the populations taken from susceptible and resistant bananas was observed after 6 months. Pathogenicity variability was undergone to assess an eventual selective effect of hosts.

Resumen – Eficacia y durabilidad de la resistencia parcial contra la enfermedad de la raya negra de la hoja

La enfermedad de la raya negra de la hoja causada por *Mycosphaerella fijiensis* es la enfermedad foliar más destructiva de bananos y plátanos. El mejoramiento genético con respecto a la resistencia parece convertirse en la medida de control más apropiada. Debido a un alto nivel de diversidad que se mantiene en las poblaciones del patógeno, los programas de mejoramiento se basan en la resistencia parcial que se considera hasta más durable que la resistencia total. El propósito de estos estudios consiste en evaluar la eficacia y durabilidad de la resistencia parcial

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para ayudar a los programas de mejoramiento. Para lograr este objetivo, se examinaron tres enfoques complementarios:
1) La caracterización de la resistencia parcial se estudió a través de la medición de diferentes parámetros del ciclo de vida del hongo en condiciones de campo y controladas. La evaluación de 13 variedades diferentes con resistencia parcial reveló la existencia de varios componentes de resistencia que actúan en diferentes etapas del ciclo de infección.
2) La eficacia de dos variedades resistentes en las cuales los dos componentes de resistencia (la eficacia de la infección y la producción de ascosporas) se estudiaron. Durante el primer año no se observaron diferencias entre las dos variedades en cuanto a dispersión espacial o su incidencia. Sin embargo, las mediciones indicaron bajas diferencias en la severidad de la enfermedad, la cual aumentó en los años siguientes. Las diferencias en la producción del inóculo endógeno podrían explicar los resultados arriba mencionados. Varios experimentos que se están llevando a cabo actualmente para estudiar el inóculo endógeno podrían confirmar la hipótesis.
3) Se está estudiando la durabilidad de la enfermedad a través de la evolución de las poblaciones del patógeno. La caracterización molecular con los marcadores CAPS se utilizó en las poblaciones aisladas después de 6 y 25 meses de cultivo. Después de seis meses no se observaron diferencias significativas entre las dos poblaciones provenientes de los bananos susceptibles y resistentes. Se estudió la variabilidad en el poder patógeno para evaluar un eventual efecto selectivo de los hospedantes.

Résumé – Efficacité et durabilité de la résistance partielle contre la maladie des raies noires
La maladie des raies noires, causée par *Mycosphaerella fijiensis*, est la maladie foliaire la plus destructrice chez les bananiers et les plantains. L'amélioration génétique pour la résistance apparaît comme le moyen le mieux approprié pour contrôler la maladie. Comme un niveau élevé de diversité est maintenu dans les populations de pathogènes, les sélectionneurs préfèrent travailler avec une résistance partielle, qui est considérée comme étant plus durable, plutôt qu'avec une résistance totale. Trois approches complémentaires ont été suivies pour atteindre cet objectif :
1) La résistance partielle a été caractérisée en mesurant différentes variables pendant le cycle vital du champignon au champ et en conditions contrôlées. L'évaluation de 13 variétés partiellement résistantes a révélé l'existence de plusieurs composants agissant à des stades différents du cycle infectieux.
2) L'efficience de deux variétés résistantes qui différaient pour leurs composants de résistance (efficacité de l'infection, production d'ascospores) a été étudiée. Aucune différence dans la dispersion et l'incidence de la maladie n'a été observée entre les variétés résistantes pendant la première année, alors que de petites différences ont été notées pour la sévérité de la maladie, qui augmentait avec le temps. Ces résultats pourraient être expliqués par des différences dans la production d'inoculum endogène. Des essais sont en cours pour mesurer l'inoculum endogène dans chaque champ pour confirmer cette hypothèse.
3) La durabilité de la résistance a été étudiée en analysant l'évolution des populations de pathogènes. La caractérisation moléculaire avec des marqueurs CAPS a été utilisée sur des populations isolées après 6 et 25 mois de culture. Aucune différence significative n'a été trouvée entre des populations prélevées sur des bananiers résistants et sensibles. La variabilité de la pathogénicité a été étudiée pour évaluer la possibilité d'un effet sélectif des hôtes.

Introduction

Black leaf streak disease, caused by the ascomycete fungus *Mycosphaerella fijiensis*, is the most important foliar disease of banana worldwide (Jones, 2000). Indeed, the main varieties cultivated industrially and by smallholders, and belonging to the Cavendish and plantains groups, are very susceptible to black

leaf streak disease. Chemical control strategies based on biological forecasting system were first developed in industrial plantations (Fouré, 1988). Although efficient, chemical control negatively affects the environment and human health, and is too expensive for poor smallholders. Managing black leaf streak disease must integrate the use of resistant varieties.

Two types of resistance against black leaf streak disease have been described (Fouré, 1992). High resistance, characterized by a blockage of symptom expression and an absence of sporulation, and partial resistance, characterized by moderate disease expression and a normal but slow development of symptoms up to necrosis.

High resistance is believed to be similar to hypersensitivity and under the control of a mono or oligogenic system which may be easily circumvented by pathogens (Fouré *et al.,* 2000). For example, the high resistance of 'Paka' is no longer effective in the Cook Islands and studies of pathogenic variability showed that it was overcome by some virulent strains after 8 years of cultivation (Fullerton and Olsen, 1995).

Analysis of population genetic structure of *M. fijiensis* at various geographical scales revealed high levels of gene diversity and genetic differentiation (Carlier *et al.,* 1996 and in this volume). Such structures suggest a high adaptation potential of the pathogen, hence the use in the banana breeding programmes of CARBAP and CIRAD of partial resistance instead of high resistance.

Partial resistance is a complex character which could include several components corresponding to different stages of pathogen infectious cycle (Young, 1996). Until recently, only one variable, youngest leaf spotted (YLS), was used to evaluate partial resistance of bananas in screening trials. Such evaluation was useful to characterise germplasm but not sufficient to identify the components of partial resistance. For example, sporulation, which could have an important effect in the case of a polycyclic epidemic like black leaf streak disease, is not measured. Identifying the components coming into play at various stages of the infectious cycle can be conducted by inoculation under controlled conditions.

The efficiency of the components of partial resistance can only be evaluated in the framework of an epidemiological study in a field of one variety. Pathogen populations could evolve and erode resistance depending on the evolutionary forces at work. Strategies based on the evolutionary potential of the pathogen could improve the durability of resistance. To define such strategies, the relative importance of the evolutionary forces acting on the pathogen. The effect of genetic recombination, genetic drift and gene flow on pathogen evolution can be evaluated by analyzing population structure (Carlier *et al,* 1996 and this volume).

Resistant hosts exert a selection pressure on the pathogens. The evolution of the pathogen can be studied in fields of resistant hosts by using molecular markers and characterizing its pathogenicity. The durability of partial resistance will be studied by following over time the evolution of pathogens in fields of resistant hosts.

The objectives of this study are to identify components of partial resistance to black leaf streak disease in bananas, and to evaluate their efficiency and durability in controlling the disease. A three-step experimental approach was developed: 1) characterization of partial resistance of various cultivars under controlled

conditions, 2) epidemiological study using one variety to evaluate the efficiency of the components of partial resistance, and 3) analysis of the pathogen population over time to evaluate the durability of partial resistance in relation to the selection pressure exerted by the host.

Components of partial resistance

To characterize partial resistance, variables corresponding to the different stages of an infectious cycle were estimated and compared among various partially resistant varieties. Evaluations of resistance were conducted in Cameroon on 7 partially resistant and susceptible cultivars under field conditions and on 10 cultivars under controlled conditions. In the latter, pieces of banana leaf maintained on a culture media were inoculated (El Hadrami *et al.*, 1998). Fifteen strains were used.

The infectious cycle was dissected in eight stages (Figure 1) including incubation period; spore efficacy (number of lesions); size and growth rate of lesions; asexual and sexual latency period; asexual and sexual sporulation capacity.

Many variables were significantly different between resistant varieties under field and controlled conditions. For example, the lesions were significantly smaller in 'Zebrina' and 'Pisang Ceylan' than in other resistant varieties (Figures 2a and 2b). The number of lesions and the incubation period also varied significantly with the highest number of lesions found on 'Pisang Berlin' and 'Pisang Ceylan' and the

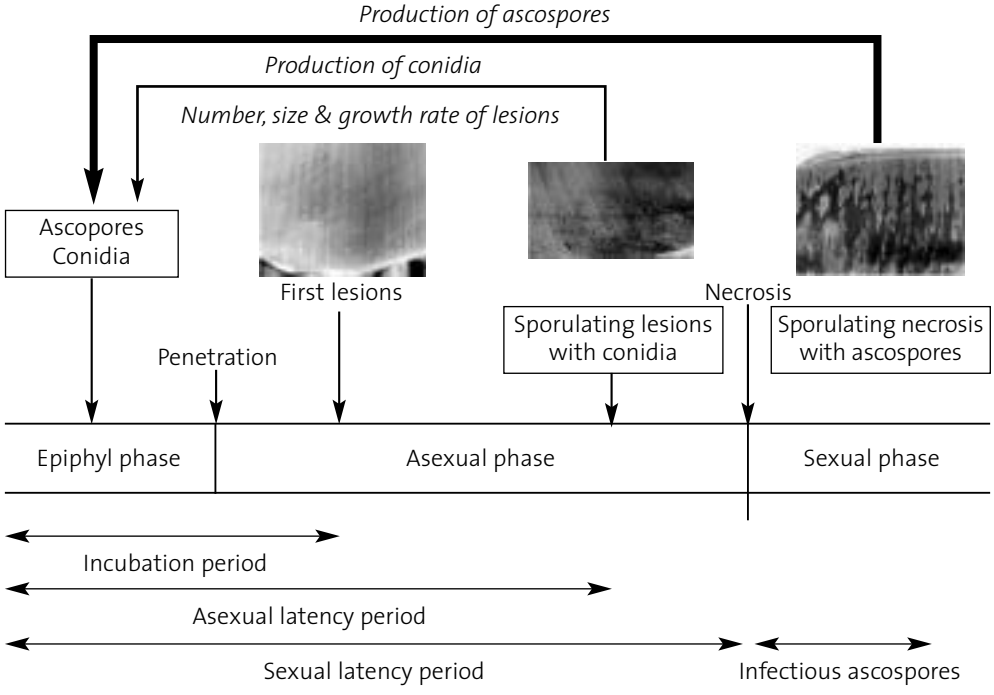

Figure 1. Infectious cycle of *Mycosphaerella fijiensis* (from El Hadrami, 2000). The components of partial resistance being characterized are in italics.

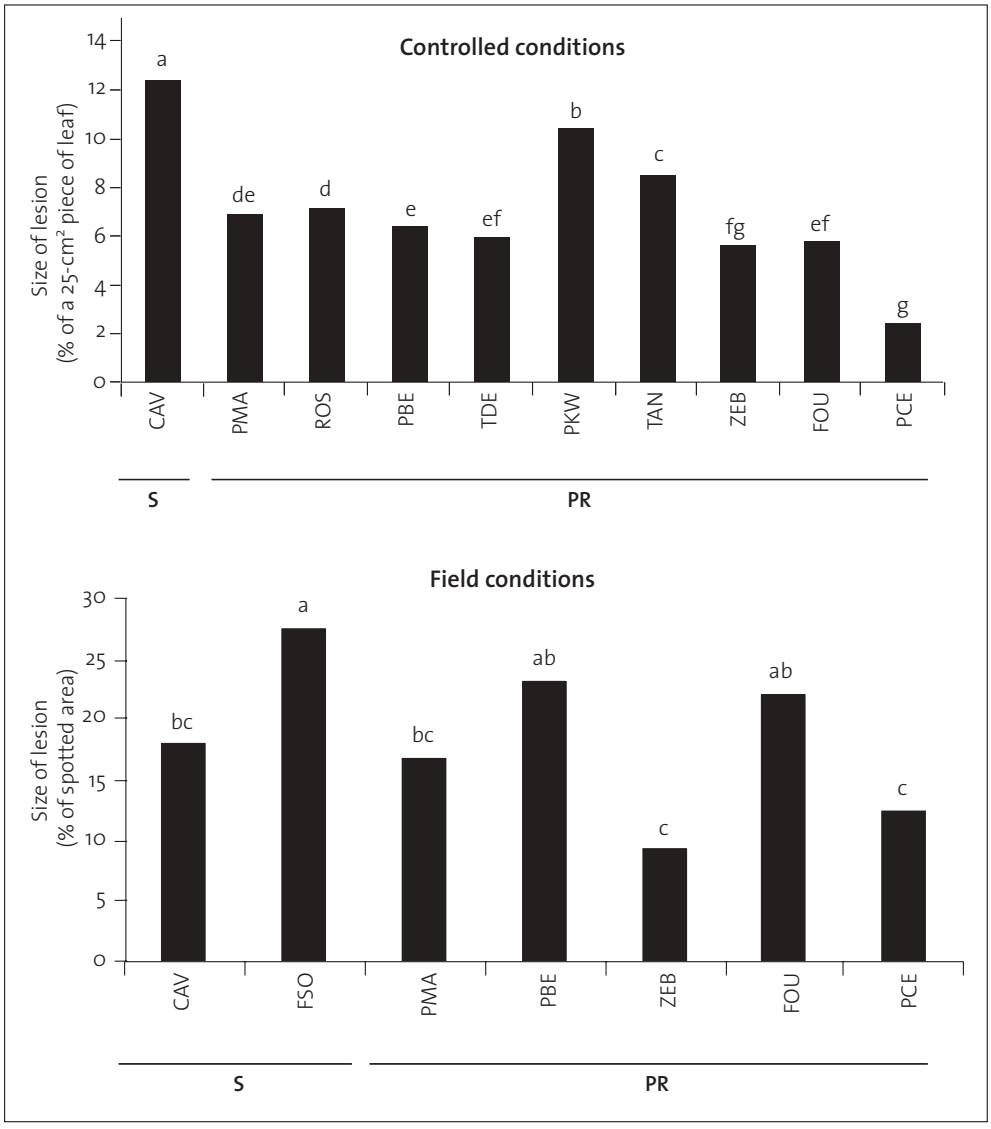

Figure 2. Size of lesions in partially resistant (PR) and susceptible (S) varieties under (a) controlled conditions and under (b) field conditions. (Cav: Cavendish (AAA), FSO: French sombre (AAB), PMA: Pisang madu (AAcv), ROS: Rose d'Ekona, PBE: Pisang Berlin (AAcv), TDE: Thong det (AAcv), PKW: Pisang klutuk wulung (BBw), TAN (BBw), ZEB: Zébrina (AAw), FOU: Fougamou (ABB), PCE: Pisang C eylan (ABB)).

longest incubation period observed on 'Fougamou' and 'Tani' (data not shown). No difference was observed in the production of asexual spores whereas significant differences were observed in the production of sexual spores (Figure 3). 'Pisang madu' and 'Zebrina' produced 3 to 8 times less than the other three partially resistant varieties, including 'Pisang Berlin'. Thus, although 'Pisang madu' and 'Pisang Berlin' behave similarly in field trials as regards YLS, 'Pisang madu' produced four times less perithecia/cm² of necrosis than 'Pisang Berlin'.

This first step allowed us to identify components of partial resistance which act at different stages of the infectious cycle : early stage (e.g. small lesions on 'Zebrina' and 'Pisang Ceylan'), intermediate stages (e.g. long incubation period on 'Fougamou' and 'Tani') or end of the cycle (e.g. low production of sexual spores on 'Pisang madu' and 'Zebrina').

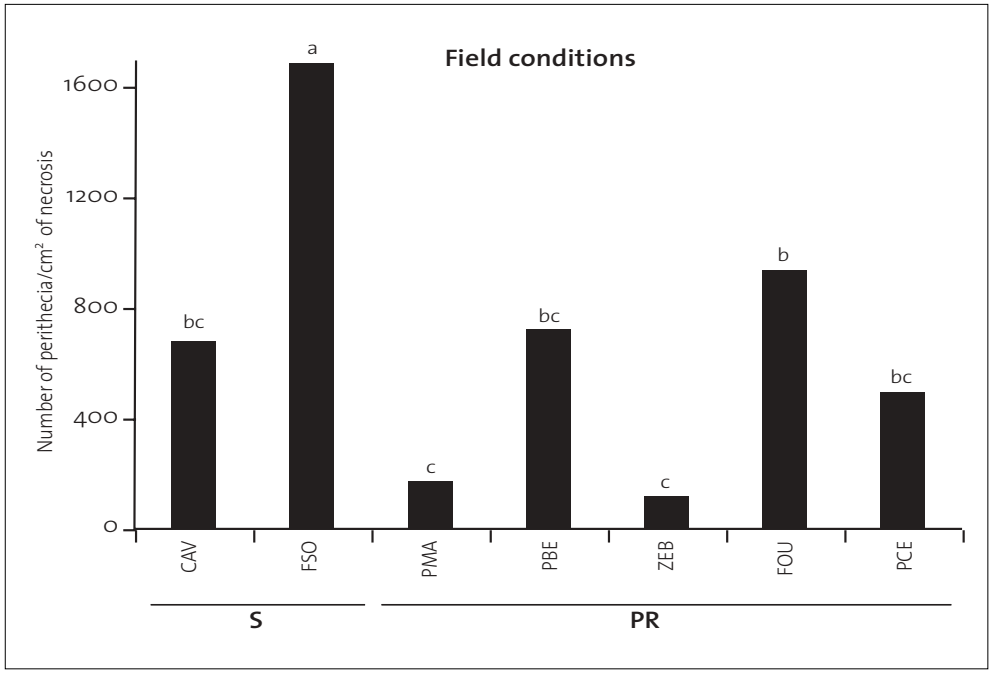

Figure 3. Production of sexual spores in five partially resistant (PR) and two susceptible (S) varieties under field conditions.(Cav: Cavendish (AAA), FSO: French sombre (AAB), PMA: Pisang madu (AAcv), PBE: Pisang Berlin (AAcv), ZEB: Zébrina (AAw), FOU: Fougamou (ABB), PCE: Pisang Ceylan (ABB)).

Efficiency of components

An epidemiological study was conducted on two varieties to evaluate the efficiency of two components of partial resistance: spore efficacy and production of sexual spores. 'Pisang madu', 'Pisang berlin' and a susceptible control ('Grande naine') were cultivated in three rectangular plots containing 150 bananas/plot. Measures were taken over 3 cropping cycles.

During the first year, no difference in disease dispersal and disease incidence was observed between the 3 plots. No spatial auto-correlation was measured at plot scale (Moran index not significant) and disease incidence was similar between plots (data not shown).

Disease severity (visual quantification of percentage of spotted surface per plant) was significantly different in resistant varieties (about 20 % for 'Pisang Berlin') and susceptible varieties (an average of 40%) (Table 1). This result shows the efficient role of partial resistance in controlling black leaf streak disease. Significant

differences were also observed between the two resistant varieties, differences which increased over time due to the declining disease severity on 'Pisang madu' (Table 1). Production of sexual spores, on the other hand, was stable over the three cropping cycles. This result could be due to differences in components of resistance or an increase in resistance 'Pisang madu' over time. To test the first hypothesis, secondary inoculum was measured by using spore trap plants in each resistant plot. The precision of this method needs to be improved to be able to show eventual differences in auto-inoculum production.

Table 1. Efficacy of partial resistance expressed as disease severity (% of spotted area/banana) on susceptible (S) and partially resistant (PR) varieties over three cropping cycles.

Varieties	Disease severity (% of spotted area)		
	1st cycle	2nd cycle	3rd cycle
Grande naine (S)	37.5 a	39 a	36 a
Pisang Berlin (PR)	22.7 b	18.6 b	17.2 b
Pisang madu (PR)	17.5 c	7.1 c	0.8 c

Durability of components partial resistance

The population structures of about 50 isolates of *M. fijiensis* taken from resistant ('Pisang madu') and susceptible ('Grande naine') after 6 and 25 months of cultivation were analysed using molecular markers and a pathogenic test to evaluate the relative importance of genetic drift and selection by the host.

Seven CAPS (cleaved amplified polymorphism sequences) neutrals markers were used and the pathogenicity of the isolates was measured by inoculating leaf pieces of 'Pisang madu' and 'Grande naine' maintained on a culture media (El Hadrami *et al.*, 1998).

No significant difference in genetic differentiation was detected between the samples isolated from resistant and susceptible bananas. The estimate of Wright's Fst parameter over all loci between pairs of populations was low and ranged from 0.007 to 0.0432. This absence of differentiation between populations suggests a low genetic drift effect during colonisation and/or important gene flow between fields.

No clear evidence of differences in aggressiveness were observed between the two pathogens after 6 months of cultivation. Samples isolated from resistant and susceptible bananas seemed to have the same pathogenic behaviour. Further analyses will be done on populations after 25 months of cultivation.

Conclusion and perspectives

This study revealed the existence of various components of partial resistance under controlled and field conditions. Then, the efficiency of two components of resistance (spore efficacy, production of sexual spores) on disease control were tentatively evaluated. A selective effect of partial resistance components on pathogen population was not detected but aggressiveness of isolates were evaluated only after six months of cultivation. We are analysing pathogens population after

two years of cultivation. However, the absence of genetic differentiation between fields containing different varieties could be the result of high gene flow. High gene flow can counteract changes in gene frequency as a result of selection by the host. To clarify this point, spore dispersal needs to be specified directly in epidemiological studies or indirectly ing population genetic models.

Computer models can now simulate epidemics using parameters corresponding to the different stages of the pathogen's infectious cycle. The effect and the importance of the components of partial resistance can then be tested by comparing simulated and observed epidemics. Computer models can help in designing future experiments and in defining spatio-temporal management strategies of resistant varieties at different scales (from plot to regional scale) by testing different scenarios.

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Poster

Early evaluation of black leaf streak resistance by using mycelial suspensions of *Mycosphaerella fijiensis*

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Abstract

A standardized method for the early evaluation of resistance to black leaf streak on *in vitro Musa* plants was developed using mycelial suspensions of *Mycosphaerella fijiensis.* Seven cultivars: 'FHIA-18', 'FHIA-01', 'FHIA-21', 'Grande naine', 'Yangambi', 'Calcutta 4' and 'Niyarma yik' were tested in a greenhouse. Inoculum was adjusted to 10^5 cfu/ml and applied to the lower surfaces of the first three open leaves. Plants were evaluated 15 days after inoculation and at 15-day intervals until 60 days. A standardized scale of leaf symptoms ensured consistency between evaluators. All cultivars except 'Yangambi', showed a similar response to *M. fijiensis* in natural conditions. Partial resistance expressed in FHIA cultivars was characterized by a slow rate of symptom development with 'Calcutta 4' the slowest. 'Grande naine' and 'Niyarma yik' gave a susceptible reaction and their symptoms were more severe. Artificial inoculation of *in vitro* plants with mycelial suspensions was an easy, rapid and practicable method to determine resistance to *M. fijiensis*. An inoculum adjusted to an appropriate concentration gave uniform symptoms on the inoculated leaf. The method has promise for the evaluation of *in vitro* plants in breeding programmes.

Resumen - Evaluación temprana de la resistencia a la raya negra de la hoja mediante el uso de la suspensión del micelio de *Mycosphaerella fijiensis*

Se describen los métodos de normalización para la evaluación temprana de la resistencia a la raya negra de la hoja en las plantas *in vitro* de *Musa* mediante el uso de la suspensión del micelio

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de *Mycosphaerella fijiensis*. Siete cultivares: 'FHIA-18', 'FHIA-01', 'FHIA-21', 'Grande naine', 'Yangambi', 'Calcutta 4' y 'Niyarma Yik' fueron utilizados para la prueba de inoculación en el invernadero. La concentración del inóculo fue ajustada a 10^5 ufc/ml y las primeras tres hojas en abrirse fueron inoculadas en la superficie inferior. El período de evaluación empezó a los 15 días y termino 60 días después de la inoculación. En la evaluación de las hojas se utilizó una escala (unidad experimental) que permitió evitar la confusión del evaluador y describir el desarrollo de los síntomas de mejor manera. Con excepción del 'Yangambí', los cultivares mostraron un comportamiento similar contra los patógenos en condiciones naturales. La resistencia parcial expresada en los cultivares de la FHIA se caracterizó por una lenta evolución de los síntomas. El cultivar 'Calcutta 4' mostró el tiempo más lento de desarrollo de los síntomas. Los cultivares 'Grande naine' y 'Niyarma yik' mantuvieron reacciones susceptibles y sus síntomas alcanzaron grados mayores de afectación. La inoculación artificial de las plantas *in vitro* utilizando la suspensión de micelio resultó ser un método fácil, rápido y factible para conocer la expresión de la resistencia de las plantas contra *M. fijiensis*. La utilización del inóculo con una concentración ajustable permitió obtener síntomas homogéneos y uniformes de la hoja inoculada. El mismo representa una herramienta útil para la evaluación de las plantas *in vitro* en los programas de mejoramiento.

Résumé – Evaluation précoce de la résistance à la maladie des raies noires au moyen de suspensions mycéliennes de *Mycosphaerella fijiensis*
Une méthode standardisée d'évaluation précoce de la résistance à la maladie des raies noires sur des vitroplants de *Musa* a été développée en utilisant des suspensions mycéliennes de *Mycosphaerella fijiensis*. Sept cultivars, 'FHIA-18', 'FHIA-01', 'FHIA-21', 'Grande naine', 'Yangambi', 'Calcutta 4' et 'Niyarma yik' ont été étudiés en serre. L'inoculum a été ajusté à 10^5 cfu/ml et appliqué sur la surface inférieure des trois premières feuilles ouvertes. Les plantes ont été évaluées 15 jours après l'inoculation, puis à intervalles de 15 jours jusqu'à 60 jours. Une échelle standardisée de symptômes foliaires a permis d'assurer la consistance entre évaluateurs. Tous les cultivars sauf 'Yangambi' ont montré une réponse similaire à *M. fijiensis* en conditions naturelles. La résistance partielle exprimée par les cultivars FHIA a été caractérisée par un faible taux de développement des symptômes, qui était le plus bas chez 'Calcutta 4'. 'Grande naine' et 'Niyarma yik' ont donné une réaction susceptible et leurs symptômes étaient plus sévères. L'inoculation artificielle de vitroplants avec des suspensions mycéliennes s'est avérée une méthode simple, rapide et praticable pour déterminer la résistance à *M. fijiensis*. Un inoculum ajusté à une concentration appropriée a donné des symptômes uniformes sur la feuille inoculée. Cette méthode est prometteuse pour l'évaluation de vitroplants dans des programmes d'amélioration.

Introduction

Screening for resistance to black leaf streak in field condition is time-consuming and expensive. Early evaluation in controlled conditions is an important requirement to increase success and evaluate feasibility. Stover (1987) recommended the use of standard varieties and the development of standardized methods for preparing inoculum and measuring disease response in controlled environments.

Mourichon *et al*. (1987) developed a greenhouse method for artificial inoculation with *Mycosphaerella fijiensis* conidia and mycelium onto three reference cultivars with different levels of resistance ('Grande naine', 'Fougamou' and 'Yangambi km $5^{1/2}$'). Romero and Sutton (1997) used suspensions of conidia to evaluate the reaction of four *Musa* genotypes at three temperatures with isolates from different geographical regions. Jones (1995) used hyphal fragments of *M. musicola* for the rapid assessment of different *Musa* spp., and Balint-Kurti *et*

al. (2001) developed inoculation techniques using transgenic strains of *M. fijiensis* expressing GFP by using conidia and mycelia.

The development of artificial inoculation techniques is also necessary to improve and simplify selection procedures in breeding programmes. Even though ascospores and conidia are commonly used for artificial inoculation, mycelial fragments may provide an alternative for evaluating disease development in controlled conditions.

The objective of the work was to prepare a standardized method for the rapid evaluation of resistance to black leaf streak disease in *in vitro Musa* plants using mycelial suspensions of *M. fijiensis*, and its application in breeding programmes.

Material and methods

Plant material

In vitro plants of 'FHIA-18' (AAAB), 'FHIA-01' (AAAB), 'FHIA-21' (AAAB), 'Grande naine' (AAA), 'Yangambi' (AAA), 'Calcutta 4' (AA) and 'Niyarma yik' (AA) were inoculated in a greenhouse. Plants were acclimatized for eight weeks on a substrate comprising 50% casting, 30% compost and 20% zeolite. Plants were grown in plastic pots 100 cm in diameter.

Preparation of mycelial suspension

The pathogenic monoascosporic strain CC-IBP-1 (isolated from 'Grande naine') was used. Colonies of *M. fijiensis* were grown on potato dextrose agar (PDA) at 28°C for 14 days. Pieces fragments were transferred to an Erlenmeyer flask containing 200 ml of V8 liquid medium (200 ml V8 juice, 0.3 g $CaCO_3$, 800 ml water, pH 6.0). The flask was incubated at 28°C in a shaker (130 rpm) for 15 days. The mycelium was then blended and 1 ml was transferred to a Petri dish containing PDA to obtain homogeneous growth over the surface of the dish. The culture was incubated in the dark at 28°C for 15 days. Two 1-cm^2 discs of mycelium were transferred to a 1-litre flask containing 400 ml of liquid V8, and shaken at 130 rpm at 28° C for 15 days. The mycelium (10 g in 900 ml of sterile distilled water) was blended and filtered through two layers of gauze to remove large fragments of hyphae. The concentration was determined with a haemacytometer and adjusted to 10^5 cfu/ml. Gelatin at 1% was added to the final inoculum.

Inoculation

Plants 20 cm in height and with four active leaves were selected for inoculation. There were 15 plants of each cultivar.

The first three open leaves of each plant were inoculated on the under surface using a brush. Leaves were marked on the upper side. The plants were allowed to dry for 2 h, and the relative humidity maintained at 90-100% for the first three days by spraying continuously with water. Afterwards, the humidity was saturated during the night but spraying was suspended during the day. Sunlight was used.

Evaluation

Inoculated leaves were examined every 15 days starting on the 15th day after inoculation and ending on the 60th. Symptoms corresponded approximately to the descriptions of Fullerton and Olsen (1995) for *in vitro* plants inoculated with conidia. Table 1 describes the scale used to evaluate symptom development and the classification of genotypes according to the stage of symptom development.

Table 1. Stages of symptom development in *in vitro Musa* plants inoculated with mycelial suspensions of *Mycosphaerella fijiensis* in the greenhouse.

Stage	Description
0	Leaf symptoms mostly absent.
1	Reddish flecks on lower leaf surface. No symptoms on the upper surface.
2	Regular or irregular reddish circular spots on the lower leaf surface. No symptoms on the upper surface.
3	Regular or diffuse light brown circular spots oin the upper leaf surface.
4	Black or brown circular spots, possibly with a yellow halo or chlorosis of adjacent tissues, on the upper leaf surface. Areas of green tissue sometimes present.
5	Black spots with dry centre of grey colour. Leaf completely necrotic, sometimes hanging down.

Classification of genotypes according to symptom development

Resistant: stages 0-1

Partially resistant: stages 2-3

Susceptible: stages 4-5

In vitro plants of 'Grande naine' and 'FHIA-21', obtained from IBP breeding programmes (by mutagenesis), were evaluated as described above. Figure 1 describes the work schedule for the method.

Results and discussion

As described by Mourichon *et al.* (1987), symptoms developed on the leaves of *in vitro* plants artificially inoculated with mycelial suspensions of *M. fijiensis*. The appearance of symptoms was similar to those observed on suckers in the field. The necrotic spots were slightly circular, possibly because young plants derived from tissue culture have limited vein development and black leaf streak lesions tend to be spherical (Mourichon *et al.*, 2000). The majority of lesions were often observed on the same leaf at the same stage of development. All cultivars, except 'Yangambi', responded to *M. fijiensis* in a similar manner to that observed in natural conditions (Table 2).

The reaction of 'Yangambi' was characterized by the presence of symptoms after the first 15 days, symptoms which reached stages 2 and 3 in 30-45 days similar to the behaviour of susceptible genotypes. Some leaves had necrotic spots. Mourichon *et al.* (1987) had reported a hypersensitive reaction for *in vitro* plants of 'Yangambi' inoculated with conidia. In contrast, Fullerton and Olsen (1995) reported a typical susceptible response in 'Yangambi' when *in vitro* plants were inoculated with the conidia of a virulent strain of *M. fijiensis*.

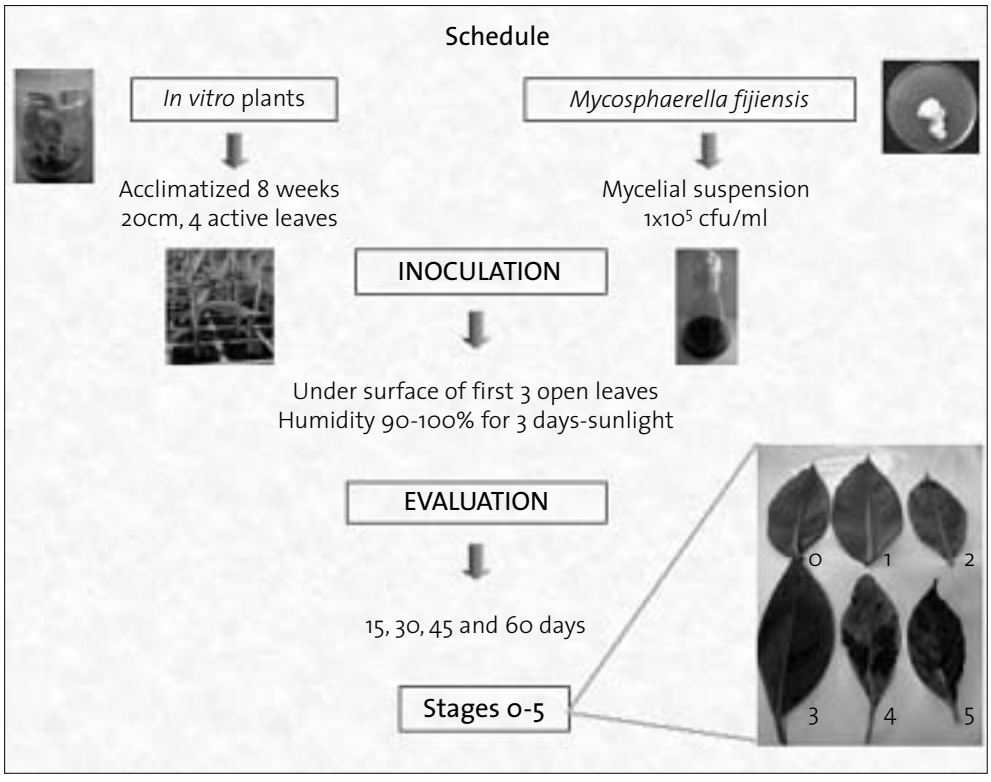

Figure 1. Schedule of the method for artificial inoculation of *in vitro* plants of *Musa* with mycelial suspensions of *Mycosphaerella fijiensis* in the greenhouse.

Table 2. Reaction of seven *Musa* cultivars to artificial inoculation with mycelial suspensions of *Mycosphaerella fijiensis* in the greenhouse.

Cultivars	Reaction in the field	Symptom stage			
		15 d*	30 d	45 d	60 d
Grande naine	Susceptible	1	1	2	4
Niyarma yik	Susceptible	1	1	1	4
FHIA-01	Partially resistant	0	0	1	2
FHIA-18	Partially resistant	0	0	1	2
FHIA-21	Partially resistant	0	1	1	2
Calcutta 4	Resistant	0	0	1	1
Yangambi	Resistant	1	2	3	3

*d = days after inoculation.

The partial resistance expressed by FHIA cultivars was characterized by a slow development of symptoms. Only after 60 days were reddish spots seen on the upper surface of the leaves and the majority of leaves remained free of symptoms. Romero and Sutton (1997) reported similar results when they examined the

response of 'FHIA-01' and 'FHIA-02' inoculated with conidia. They pointed out that although the mechanism of resistance to black leaf streak is not known, a low density of stomata, and increased production of cuticular wax, phytoalexin, suberin and lignin, or resistance to phytotoxins may be associated with partial resistance.

'Calcutta 4' showed the slowest rate of symptom development. Most leaves had stage 1 symptoms although stages 2 and 3 were observed on a few leaves. 'Grande naine' and 'Niyarma yik' reacted, as expected. They were susceptible and the symptoms developed to the fullest extent. On 'Grand naine', symptoms were mostly at stages 4 and 5 whereas they were mostly at stages 3 and 4 on 'Niyarma yik'. High humidity in the greenhouse caused the leaves of 'Calcutta 4', 'Niyarma yik', 'Yangambi' and 'FHIA-21' to senesce rapidly.

As for the *in vitro* plants from the IBP mutagenesis breeding programmes, differences in response were found between lines of the same genotype ('FHIA-21') and between 'Grande naine' and the control (Table 3).

Table 3. Reaction of *in vitro* plants obtained from IBP mutagenesis breeding programmes to artificial inoculation with mycelial suspensions of *Mycosphaerella fijiensis* in the greenhouse.

Cultivar	Symptom stage
'**Grande naine**'	
Line-GN-A1	4
'**FHIA- 21**'	
Line-F21-A	3
Line-F21-B	3
Line-F21-C	2
Line-F21-D	1
Line-F21-E	2
Control	
'Grande naine'	5
'FHIA-21'	2

Conclusion

Artificial inoculation of *in vitro* plants with mycelial suspensions was easy, rapid and practical for determining the expression of resistance of plants to *M. fijiensis*. Its use in breeding programs could reduce time and work.

The standardized scale of symptoms was useful in order to ensure consistency between evaluators and to improve the description of symptom development. The use of inoculum with an adjusting concentration resulted in homogeneous and uniform symptoms on inoculated leaves. There was no interference from saprophytic fungi. Large quantities of mycelial fragments were produced in a short time and could solve the problem of loss of conidial production *in vitro*, which occurs with some isolates of *M. fijiensis*. An additional advantage is that mycelial fragments can be produced at different periods of the year. The method may have uses in other studies on host-pathogen interactions.

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Recommendations of session 3

Several cases of an unexpected level of susceptibility to black leaf streak disease have been reported. Although different reasons have been offered to explain the phenomenon (poor nutrition, environmental stress), the problem of the erosion of resistance cannot be ignored and requires a precise characterization of the pathogen population. A greater understanding of the mechanisms involved in plant-pathogen interactions continues to be needed to ensure the long term success of breeding programmes. The current programmes based on *a priori* hypothesis have shown their limits.

Mechanisms of pathogenicity

Other pathosystems (such as *Magnaporthe grisea*) have shown the powerful nature of the genetic approach to identify, without any *a priori,* the pathogenicity factors. These approaches include the study of gene expression during infection (differential display, DNA chip, SSH, etc.), production of pathogenicity mutants, comparative genomic and gene function validation techniques. A technique for the transformation of *M. fijiensis* is already available at the Boyce Thompson Institute (USA). The genetics and physical mapping of *M. fijiensis* genome in Mexico, in collaboration with PRI, Netherlands, should speed up the expected progress of these experimental approaches.

It was recommended to develop genetic and molecular biology tools for M. fijiensis *in collaboration with groups working on* M. graminicola.

It was recommended launching a genomic initiative to access to genomic tools (EST collection, physical map, genome sequence) and set up a genomic-wide comparison of M. fijiensis *to* M. graminocola

Mechanisms involved in partial resistance

We recommend studying differences in resistance and susceptibility in hosts which have a similar genetic background. Future work should concentrate on segregating populations to evaluate critically possible mechanisms of resistance.

The genetic approach recommended to study pathogenicity factors should also be adopted to identify the mechanisms of partial resistance (patterns of genes expression during infection of resistant cultivars). These genetic studies should be accompanied by a dissection of the infection cycle to identify the components of resistance.

Vertical resistance

It was considered that characterization of the different resistance genes represents a prerequisite to evaluate several strategies of gene deployment (pyramiding, mixture, multilines). Detection and identification of resistance genes in host plants relies on the availability of different isolates of the pathogen exhibiting different virulence phenotypes.

It was recommended to collect pathogenic isolates on resistant cultivars for evaluation [genetic (to evaluate the genetic control of pathogenicity in resistant cultivars) and controlled inoculation on resistant cultivars (to evaluate the genetic control of resistance in banana)]. In this respect genetic crossings between isolates showing different behaviors on resistant cultivars are recommended to identify the genetic controls of virulence on resistant cultivars, thus leading to a better identification of genes for resistance.

It was recommended to develop marker-assisted breeding (both for partial resistance and resistance genes). This will be facilitated by the activities of CIRAD on the genetic map of banana.

Session 4

Genetic improvement for the management of resistance

Introduction

Genetic improvement for a sustainable management of resistance

K. Craenen[1] and R. Ortiz[2]

Abstract

In the 1990s, innovative cross-breeding and classic genetic analysis of segregation ratios allowed advances in the understanding of host plant response to black leaf streak disease. Partial resistance owing to a recessive major gene (bs_1) coupled with at least two additive minor genes (bsr_i) appears to be durable because this genetic system slows disease development in the host plant. As a consequence, resistant hybrids show more healthy leaves, i.e. greater photosynthetic leaf area, than their susceptible full-sibs, which may partially account for their high yield. Although other breeding approaches such as genetic transformation, mutagenesis and somaclonal variation are advocated to develop new resistance to *Mycosphaerella* leaf spot diseases in *Musa*, farmers today are only adopting the research products from the so-called conventional breeding, i.e. tetraploid or triploid resistant hybrids from interspecific interploidy crosses. Recent findings on pathogenicity with molecular and cellular biology tools are providing new knowledge on host plant – pathogen interactions, which may result in science-led approaches for deploying resistance against sigatoka diseases within a holistic integrated disease management framework. For example, cultivar mixtures and gene pyramiding may be alternatives for potential durable resistance to sigatoka diseases of plantain and banana.

Resumen - Mejoramiento genético para un manejo sostenible de la resistencia

El cruzamiento innovador y el análisis genético clásico de las proporciones de segregación en las poblaciones resultantes de estos permitió avanzar en el entendimiento de la respuesta de la planta hospedante a la Sigatoka negra en *Musa* en la década de los 90. La resistencia parcial debido a un gen principal recesivo (bs_1) acoplado al menos a dos genes secundarios aditivos (bsr_i) parece ser duradera, debido a que este sistema genético retarda el desarrollo de la enfermedad en la planta hospedante. Como consecuencia, los híbridos resistentes muestran mayor cantidad de hojas sanas, es decir, una mayor área foliar fotosintética que sus hermanos completos susceptibles, lo que puede explicar su alto rendimiento. Aunque se afirma que otros enfoques de mejoramiento como la transformación genética, mutagénesis y variación somaclonal desarrollan una nueva resistencia a las enfermedades de las manchas foliares causadas por *Mycosphaerella* en *Musa*, actualmente los agricultores están adoptando solo los productos de investigaciones del tal

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llamado mejoramiento "convencional", es decir, híbridos tetraploides o triploides resistentes de los cruces interespecíficos, interploidia. Los recientes descubrimientos sobre la patogenecidad con la ayuda de las herramientas de la biología molecular y celular están proporcionando nuevos conocimientos sobre las interacciones planta hospedante-patógeno, que pueden resultar en enfoques guiados por la ciencia para desarrollar resistencia contra las enfermedades de la Sigatoka en el marco holístico de manejo integrado de la enfermedad. Por ejemplo, las mezclas de los cultivares y la construcción piramidal de genes podrían representar alternativas para una resistencia duradera potencial a las enfermedades de Sigatoka de bananos y plátanos.

Résumé – L'amélioration génétique pour une gestion durable de la résistance
Dans les années 1990, des croisements novateurs et l'analyse génétique classique des ratios de ségrégation ont permis de mieux comprendre la réaction des plantes-hôtes à la maladie des raies noires. Une résistance partielle due à un gène récessif principal (bs_1) couplée à au moins deux gènes mineurs additifs (bsr_i) semble durable étant donné que le système génétique ralentit le développement de la maladie dans la plante-hôte. Par conséquent, les hybrides résistants ont plus de feuilles fonctionnelles, c.-à-d. une plus grande surface photosynthétique, que leurs parents susceptibles ce qui expliquerait en partie leur rendement élevé. Même si d'autres méthodes d'amélioration, comme la transformation génétique, la mutagénèse et la variation somaclonale sont prônées pour mettre au point de nouvelles résistances aux maladies foliaires causées par *Mycosphaerella* spp., de nos jours les fermiers adoptent seulement les produits issus des méthodes traditionnelles d'amélioration, c.-à-d. des hybrides tétraploïdes ou triploïdes résistants issus de croisements interploïdes interspécifiques. Des recherches récentes sur la pathogénicité en utilisant des outils de la biologie cellulaire et moléculaire nous renseignent sur les interactions plante-pathogène qui pourraient mener à des méthodes scientifiques pour déployer la résistance aux cercosporioses dans un cadre global de lutte intégrée aux maladies. Par exemple, l'assortiment de cultivars et le cumul des gènes (*gene pyramiding*) pourraient être des options pour créer une résistance durable aux cercosporioses qui affectent les bananiers.

Dedication. To Dirk R. Vuylsteke (1958-2000) the 'father' of genetic-led *Musa* improvement, with whom we learned some of the issues discussed in this article. Dirk himself, his many articles and book chapters and research products ensuing from his breeding work will be always a source of inspiration to us and the new generation of plantain and banana breeders worldwide. We miss him greatly but Dirk will always live among us and those who share his humanitarian view of improving the livelihoods of the rural poor, particularly in Africa.

Breeding for disease resistance

Knowledge about the kind of disease, its effects and epidemics should be acquired before launching an efficient plant breeding program for disease resistance (Simmonds and Smartt, 1999). Throughout the breeding process information may be gathered on the kinds of resistance and the genetics of the host-plant reaction. At the same time research products, i.e., resistant germplasm from this approach, are made available for eco-friendly and sustainable disease control. The breeding strategy for durable host plant resistance needs to consider the populations and their genetic diversity (for both plant host and pathogen) and reliable screening methods.

Interaction between the product of a resistance gene *R* in the plant host and the avirulence (*avr*) gene encoded by a given pathogen isolate, results in the specific recognition of the pathogen (Flor, 1971). If *R* or *avr* is absent, the pathogen continues

colonising the plant host, reproduces and ultimately causes the disease (Holub, 2001). However, when *R* matches *avr*, the plant host recognises the pathogen, and a series of intracellular signal events occur in the plant host. The most common genetic interpretation for such an interaction claims that *R* products are receptors for *avr*-encoded ligands, and that this recognition often leads to rapid, localized, cell death of those penetrated by the pathogen, i.e. hypersensitivity.

Recent reports suggest that *R* genes are usually organized as clusters in plant genomes (Fluhr, 2001; Leister *et al.,* 1998), which provides a comparative advantage for pyramiding specific resistance genes that may protect an individual plant host against many pathogen isolates (Dangl and Holub, 1997). Likewise, pyramiding will benefit the plant host because it may have a genetic reservoir from which new specific resistance may evolve. Advances in molecular breeding can assist in monitoring and accelerating the introgression of *R* genes into the host plant (Rommens and Kishore, 2000).

The crop

Bananas and plantains are giant perennial herbs that thrive in the humid tropics and subtropics. The edible cultivars, in order of decreasing numerical importance, are triploid, diploid or tetraploid, and belong to the *Eumusa* series of the genus *Musa*. The warm, humid conditions required for banana and plantain also favour the development of *Mycosphaerella fijiensis*, the causal agent of black leaf streak disease. All plantain cultivars and most triploid bananas are susceptible to black leaf streak disease. In the long-term, identification of resistant landraces or resistance breeding are generally considered as the most appropriate strategies to control the disease (Craenen *et al.,* 2000).

The disease and pathogen

Black leaf streak disease has become a major constraint to expanding the cultivation of edible *Musa*. The causal pathogen of black leaf streak disease, *Mycosphaerella fijiensis* Morelet, is a fungus that attacks the leaves. The fungal spores are disseminated by wind and infect the leaves as they unroll. The disease develops faster where humidity and rainfall are high. It has spread rapidly to all major banana and plantain growing areas and the spread is still continuing. Chemical control strategies exist, but are environmentally unsound and socio-economically inappropriate, particularly within the framework of the resource-poor smallholders that grow the crop in Africa (Craenen *et al.,* 2000).

Diversity and pathogenecity

Isolates of *M. fijiensis* from different geographical origins were assayed with restriction fragment length polymorphisms (RFLP) (Carlier *et al.,* 1994, 1996). Australasia and Southeast Asia isolates showed the greatest variation, suggesting the pathogen originated there. Genetically homogenous groups with low variation were observed in Africa, the Pacific Islands, and Latin America. These results indicate a

few introductions of small size in each of these regions from Southeast Asia (Carlier *et al.,* 1994). Genetic distances between regions were high but the isolates from the Pacific Islands and Latin America seem related, suggesting that the introductions to Latin America came from the Pacific Islands. Founder effects accompanied the introductions to Latin America, some Pacific Islands and Africa (Carlier *et al.,* 1999), thereby reducing genetic diversity in these regions.

Recent analysis with microsatellite polymorphisms revealed variation in the fingerprint patterns of *M. fijiensis* populations from Nigeria (Muller *et al.,* 1995, 1997). Microsatellite (oligonucleotide) fingerprinting appears to be a reliable technique for assessing genetic variation among individuals as well as for defining clusters of related genotypes, i.e. detecting intraspecific variation even on a microgeographical scale.

Fullerton and Olsen (1993) evaluated the pathogenic diversity within populations of *M. fijiensis*. They used as differential hosts most of the standard cultivars currently being used in the International *Musa* Testing Programme. A wide host response to the whole range of strains was reported. The most susceptible across all isolates were 'Grande naine' and 'SF 215', while 'Calcutta 4', the widest source of alleles resistant to black leaf streak disease, was susceptible to some strains, particularly those collected in the Pacific Islands and Papua New Guinea. However, the host reaction to the pathogen must be tested with adult plants and, as their results suggest, for probably more than one year in order to detect strains present at low frequency. Perhaps, resistant germplasm needs to be tested over several years in locations with very virulent strains in order to evaluate the durability of resistance.

The data in Fullerton and Olsen (1993) were re-analysed using simple linear regression models to determine the stability of resistance to black leaf streak disease in *Musa acuminata,* and using principal component analysis to study the pattern of strain and genotype variation in the pathogen-host plant interaction (Ortiz *et al.,* 2000). 'Tuu Gia' was regarded as having non-specific resistance to the 33 strains of *M. fijiensis* used in the experiment. Furthermore, it seems that its resistance does not break down even under the pressure of high virulent strains. In contrast, the resistance of 'Calcutta 4' was very unstable and may break down in environments where highly virulent strains have evolved.

Principal component analysis revealed that isolates of *M. fijiensis* from Papua New Guinea were the most virulent, and that 'Calcutta 4' accounted for most of the genotype x strain interaction. Strains collected from the same country could be clustered together (e.g. Nigeria or most of the Pacific Islands' isolates), or had a continuous virulence distribution (e.g. Papua New Guinea), or were completely distinct (e.g. Central America). The latter suggests a different geographical origin for the introduced *M. fijiensis* or a change in virulence genes in the strains from Central America as a result of the intensive use of fungicides to control black leaf streak disease.

Incidence and severity of black leaf streak disease

Quantification of black leaf streak disease is necessary to evaluate resistance and to determine yield loss, the importance of black leaf streak disease in particular areas, and the efficacy of control measures (Craenen, 1998). Fouré (1985) described in detail the experimental methods to characterize the different host responses. A high level of

resistance to black leaf streak disease is characterized by hypersensitivity. Different levels of partial resistance, ranging from strong partial resistance to susceptibility have been observed in *Musa* germplasm (Fouré, 1994).

Damage resulting from disease can be evaluated accurately only by measuring its incidence and severity (Gauhl *et al.,* 1994; Jones, 1994). Severity of the disease relates to the intensity of damage to individual plants, while incidence deals with the percentage of plants affected in a population.

The incidence and severity of black leaf streak disease on *Musa* can be assessed in the laboratory or in the field. The laboratory method involves determining ascospore and conidia production with the aid of a microscope, but this method is tedious, very time-consuming and not very accurate.

Field evaluation under conditions of natural infection is the most common and preferred method to assess incidence and severity of black leaf streak disease (Craenen, 1997). The nature and amount of lesions and the rate of their development on the leaves is observed in the field. This method does not require a full understanding of host-pathogen interactions, nor plant population systems and is therefore suitable for field workers trained in symptom recognition.

Although field-screening methods are relatively simple, they are also time-consuming and influenced by environmental factors, such as weather and soil, which affect symptom expression. Therefore, it is recommended to compare the test plants with reference cultivars and to gather a large number of observations to validate the results.

Laboratory evaluation

The production of ascospores can be estimated by taking leaf samples with pseudothecia from the same plant at different times, as described in Stover (1976). For conidiophores, leaves at stage 2 of symptom development are collected in the field (Fouré, 1982). However, results can vary widely from leaf to leaf; spots on each leaf can differ enormously in number of spore-producing organs, and subjective errors are the source of wrong results. Not being very valuable, this method is not described further.

An inoculation technique using leaf pieces under controlled conditions has proven to be very simple and has many advantages (El Hadrami *et al*., 1998a, b). This technique enables the production of the sexual and asexual phases of *M. fijiensis* and is very useful to study host-parasite interactions (El Hadrami *et al*., 2000). The infection patterns and symptom evolution were the same as those observed in the field, and the leaf piece assay allowed the expression of resistant phenotypes. Furthermore, this method may allow the epidemiological appraisal of partial resistance and the variability of virulence in *M. fijiensis*.

Field evaluation

Field evaluation of disease severity requires knowledge not only of the stages of symptom development and the percentage of leaf area spotted, but also of the different stages of unrolling of the leaf (Craenen, 2001). Disease severity is evaluated by recording the percentage of the leaf area that is spotted using a 7-category scale (modified from Stover

and Dickson, 1970), which ranges from 0 (no symptoms) to 6 (51-100% leaf area with symptoms) (Craenen 1997, 1998).

Epidemiology

In order to gain insight into the epidemiology of the disease, it is necessary to quantify the amount of inoculum in an area. At the IITA High Rainfall Station in Onne, Nigeria, spore-trapping and weather data were recorded for three years. This research indicated that spore concentrations in the air were lower during the dry season and higher in the rainy season. Ascospores were more frequent than conidiospores. During the dry season, ascospore concentration was only three times as high as conidiospore concentration, whereas during the rainy season ascospore concentration was found to be 40 times higher (IITA, 1995).

Measuring the different characteristics of disease development on parents and their hybrids in epidemiological studies led to the identification of different types of host response and to the classification of *Musa* germplasm into different categories according to the response to the disease. The different characteristics to be recorded assume that *M. fijiensis* infects first the unfolded (> 10 cm) cigar leaf of the host plant (Fullerton, 1994; Jones,1994). These characteristics are:

- Incubation time: calculated as the number of days between cigar leaf emergence (Brun's stage 2) and the appearance of the initial chlorotic fleck symptoms (i.e. depigmentation spot) relating to symptom stage 1 of Fouré's scale. Brun's stage 2 refers to an upright cigar leaf, still strongly rolled and free from the petiole of the preceding leaf, but not reaching its full length. At the first stage of symptom development only minute yellowish specks (< 1 mm in length) are seen on the lower (abaxial) surface of the leaf. They are not visible in translucent light.

- Evolution time: calculated as the number of days between first symptoms (Fouré's stage 1) and the occurrence of mature lesions (Fouré's stage 6). At this last stage of symptom development, the centre of the spot dries out and fades into a clear gray. Often a black ring, surrounded by a yellow halo, encircles the gray centre. Necrotic spots remain visible after the leaf has dried up completely.

- Disease development time: defined as the number of days elapsing between Brun's stage 2 of leaf emergence and Fouré's stage 6 of symptoms, i.e. incubation time plus evolution time.

- Lifetime of the leaf: recorded as the number of days between leaf emergence (Brun's stage 2) and leaf death.

- Youngest leaf spotted (YLS) at flowering: recorded as the first leaf (counting downwards from the first top unfurled leaf) that shows spots (equal to or more than 10) with a necrotic dry center (Vakili, 1968).

The higher the YLS the more fully functional leaves on the plant, and hence, greater resistance to the fungus (Craenen and Ortiz, 1997). The YLS score correlates significantly with disease development time and other parameters to assess host plant response to black leaf streak disease (Craenen, 1994). The YLS is also very easy to score, heritable, and after scoring the level of host response can be defined, thereby allowing grouping

of genotypes. Four distinct levels of host response to black leaf streak disease were defined by Ortiz and Vuylsteke (1994) as follows:

susceptible (< 8 leaves without spots before or at flowering),
less susceptible (8–10),
partially resistant (> 10)
and highly resistant (none)

These epidemiological characteristics do not only depend on the amount of inoculum present, but also on climatological factors that may affect the development of the disease. Hence, it is essential to monitor environmental factors. Daily readings from a weather station near to the experimental site are required. If there is no weather station available, daily readings can be taken (if possible early in the morning) for the following factors:

– rainfall (with a simple rain gauge);
– minimum and maximum temperature (with a minimum/maximum thermometer);
– relative humidity (with a hygrometer).

Temperature and relative humidity can also be recorded continuously with a mechanical hygrothermograph.

Variability may be introduced in data sets from different locations due to environmental or genetic causes (Ortiz *et al.,* 1993). To minimise these effects the host response was re-defined as the ratio between the YLS and the total number of standing leaves (NSL) at the time of scoring. The **index of non-spotted leaves** (INSL) to assess the host response to black leaf streak disease is calculated as follows:

INSL = 100 – 100 x [(NSL – YLS + 1)/NSL]

The INSL is the proportion of standing leaves without typical late stage symptoms of the disease (i.e. spots with a necrotic centre). This index provides an estimation of the available photosynthetic leaf area prior to fruit filling.

Early screening

Breeding resistance requires methods able to discriminate resistant and susceptible genotypes at different stages of plant development (Leproive *et al.,* 1993). *In vitro* selection or field assessment using young plant materials are among some of the early screening methods.

Inoculating *Musa* leaf tissue with a crude extract of *M. fijiensis* was suggested as an early screening method (Hernández 1995). The crude extract screening method allows a rapid (48 hours in greenhouse plants and 72 hours in callus tissue) identification of host-plant resistance. For example, leaf tissue of susceptible cultivars such as 'Grande naine' or 'Currare' showed the highest levels of phenolic compounds while resistant germplasm (e.g. 'Yangambi km5', 'Calcutta 4' or 'Saba') showed low phenol content, after being inoculated with a crude extract of *M. fijiensis*.

Pino (1997) indicated that 120 hours after inoculating *in vitro* plants with *M. fijiensis* toxins, lesions in susceptible banana cultivar 'Grande naine' were larger than in putative resistant mutants of 'Grande naine' (24.12 – 49.22 mm^2 vs. 1.07 mm – 4.07 mm^2, respectively). Similarly, Okole and Schulz (1997) reported as promising an *in vitro* selection technique using microsections (or callus cultures) of banana and plantain using

a double selection system. The selection system uses in the first stage raw filtrate concentrations (10-100 mg/L) of the fungus, and then a specific purified fungal toxin (2,4,8-trihydroxyltetralone or 2,4,8-tht) isolated from *M. fijiensis*. This toxin plays an important role in the development of necrotic leaf symptoms that causes host-specific reactions depending on their concentration at different pathogenesis stages in tissue culture materials (Hoss, 1998). A *Musa* genotype resistant to black leaf streak disease with increased 2,4,8-tht content, produced the hypersensitive reaction and elicited postinfectional defense reactions in the host plant, which led to its incompatibility with the pathogen. A susceptible *Musa* genotype showed toxic doses of 2,4,8-tht but only after the establishment of a compatible interaction, which first helped the biotrophic nutrition of the pathogen, and acted as a virulence factor at the necrotrophic phase of pathogenesis.

Toxin-resistant plantlets of two Cavendish cultivars ('Williams' and 'Petite naine') and of 'Horn plantain' were regenerated using the above-mentioned method (Okole *et al.*, 2000). Rooted plants were further transferred to soil infected with suspensions of *M. fijiensis* spores (0.3 g/ml). About 11 to 19% of the plantlets resistant to the toxin were resistant to *M. fijiensis* in this culture chamber test, which reproduces the symptoms of black leaf streak disease. However, the plants that withstood the toxin injection to their tissues and the double selection procedure have not yet been field-tested.

As pointed out by Harelimana *et al.* (1996), screening with toxins to select resistant germplasm has two major limitations. First, the lack of experimental evidence on the role of toxins in disease development, and second, the susceptibility or resistance of the cultured tissues do not reflect those of the adult plant because of the mode of action of the toxin. It has not been demonstrated that toxins of *M. fijiensis* participate in the initiation of infection or in the hypersensitive reaction of highly resistant adult plants. Nonetheless, toxins could play a secondary role in pathogenicity, e.g. in disease development in partially resistant cultivars. Research showed that chloroplasts could be a precocious site of action of the toxins, suggesting that *in vitro* heterotrophic *Musa* tissues may not be suitable for early screening.

Another early screening method for host response consists in using natural inoculum on young *Musa* plants (3-month-old micropropagated plants). This method confirmed the resistance of plantain hybrids and the susceptibility of their female plantain parent to this disease (Mobambo *et al.*, 1994). Furthermore, the characteristics associated with disease development in these young plants were similar to those observed on adult plants of the same genotypes (Mobambo *et al.*, 1997). This method was also faster, cheaper, less labour intensive and required less field space than screening of adult plants. Highly susceptible germplasm can be rejected at a precocious stage with this early screening method, thus reducing the sample size (and associated) costs for testing adult plants in the field.

Host plant response to black leaf streak disease

Genetics of resistance

In plantains and bananas, resistance to black leaf streak disease is genetically controlled. Genetic analysis has been carried out on diploid and tetraploid progenies

obtained from triploid (plantain) x diploid crosses (Vuylsteke *et al.,* 1993). The diploid male parent was the resistant true-breeding line 'Calcutta 4' (Ortiz *et al.,* 1998b). Thus, the populations produced can be considered to be genetically equivalent to test-crosses for the host response to this disease. Resistance to black leaf streak disease is mainly the result of the interaction of three independent alleles: a recessive allele at a major locus (*bs1*) and the alleles of at least two independent minor, modifying genes with additive effects (*bsri*) (Ortiz and Vuylsteke, 1994). These genes have a strong dosage effect at the tetraploid level that results in higher levels of resistance in tetraploid than in diploid hybrids. Resistance genes are present in the genome of susceptible plantains, but their expression is masked by the dominant effect of the major gene for susceptibility (Ortiz and Vuylsteke, 1994).

These results were further confirmed by investigating tetrasomic segregration in a cross between a resistant and a susceptible tetraploid hybrid (Ortiz, 2000). The resistant maternal genotype was a nulliplex for the major resistance locus and the paternal susceptible genotype was a duplex for the corresponding host response. Both parents were balanced diallelic for the two minor modifier loci with additive effects. The segregating tetraploid population from this cross showed a tri-modal frequency distribution in the population, which was not significantly different to the expected ratio (1.7 resistant : 1 susceptible) from the early genetic model defined by Ortiz and Vuylsteke (1994).

Using the gene-for-gene hypothesis (Flor, 1971), a host-plant resistance system based on recessive alleles is difficult to overcome by the pathogen as this requires a mutation to the dominant allele of the virulence locus (Ortiz and Vuylsteke, 1994). Since such mutations are rare (Simmonds, 1979), resistance based on recessive alleles may prove to be durable.

Resistance genotypes are expressed phenotypically in the plants. Highly resistant plants exhibit the longest incubation time and leaf life span as well as a hypersensitive reaction to black leaf streak disease (Craenen and Ortiz, 1998). This extremely resistant polygenic response (Ortiz and Vuylsteke, 1994) blocks disease development at an early stage, thereby impeding the occurrence of mature necrotic lesions in the leaves. In contrast, susceptible cultivars have a short incubation time, evolution time and disease development time. This indicates that after infection, disease symptoms evolve quickly into necrotic spots, resulting in extensive leaf death and defoliation.

Results from multilocational trials in Africa showed that all partially resistant hybrids had a homeostatic host response to black leaf streak disease (Ortiz *et al.,* 1997). Also some of them achieved high and stable yields across environments due to their resistance (Ortiz and Vuylsteke, 1995), even under low organic matter inputs (Ortiz *et al.,* 1995).

Mechanisms of resistance

A sample of 20 euploid *Musa* hybrids of various ploidy, exhibiting a range of resistant and susceptible responses, were used to investigate the role of stomatal density, stomatal length and the thickness of epicuticular wax in resistance to *M. fijiensis* (Craenen *et al.,* 1997). The female parents of these hybrids were susceptible plantains, while the male parent was a wild, non-edible resistant banana ('Calcutta 4'). Stomatal length was

negatively correlated with the initial development (incubation time) of black leaf streak disease in the leaves of young diploids but not in those of polyploid hybrids. Stomatal density on the abaxial surface of young leaves was negatively correlated with incubation time only in polyploids. Incubation time was positively correlated with the accumulation of epicuticular wax in both diploid and polyploid hybrids. Although the resistant male parent lacked epicuticular wax, derived hybrids possessed epicuticular wax of various thickness which enhanced resistance. Hence, the two minor additive modifier genes (bsr_i) which enhanced resistance may control decreased stomatal density and increased leaf waxiness. Both characteristics may be two resistance mechanisms that lengthen the incubation time of the disease in the leaves.

Craenen and Ortiz (1997) determined the role of the major gene for resistance (bs_1) in a sample of euploid hybrids from triploid-diploid crosses of two French plantains and a diploid wild banana, and with a known genotype for the bs_1 locus. Their host response was assessed in the humid forest zone of Nigeria. Analysis of frequency distribution in each segregating population showed that almost all the traits displayed a normal distribution across ploidy level. This suggests that additive gene action plays an important role in the host plant response to the fungus. However, the environment and the genotype x environment interaction significantly affected the host response, which explains the low reproducibility of all traits. Intrafamily variation was larger than interfamily variation, and most of the genetic variation in each family depended on individual genotypes, regardless of their ploidy. The additive effect of, and the intralocus interaction at, the bs_1 locus were established by one-way analysis of variance and regression analysis. Intralocus interaction at the bs_1 locus apparently regulates the appearance of symptoms on the leaf surface, whereas the additive effect and the intralocus interaction of the bs_1 locus affect disease development in the host plant. Therefore, the gene action(s) at the bs_1 locus may provide durable resistance by slowing down disease development.

Effect of black leaf streak disease on agronomic traits

Yield loss due to black leaf streak disease is 33 to 50% in plantain (Stover, 1983; Mobambo *et al.,* 1993) as a result of a reduced number of fruits per bunch and a lower fruit weight. Black leaf streak disease has no effect on plant height and suckering, but delays flowering and harvest by more than one month. The disease also causes premature fruit ripening (Stover, 1980; Mobambo *et al.,* 1993). In plantain landraces, normal fruit filling or ripening time, i.e. time from flowering to harvest, is 91 days. Black leaf streak disease significantly reduces this time. This premature fruit ripening is expected to have adverse effects on postharvest characteristics, such as a reduced shelf life. In addition, fruits are shorter and thinner, which generally results in lower quality fruit and lower market value. Black leaf streak disease thus has a negative impact on fruit bulking, probably as the result of a reduction in healthy leaf area (Mobambo *et al.,* 1993).

Fruits of susceptible plantain hybrids are unable to bulk fully. Less susceptible and partially resistant plantains are bigger, longer and heavier. Resistant plants have increased bunch weight due to complete fruit filling as they have more functional leaves for photosynthesis during the period between flowering and harvest (Craenen and Ortiz, 1998).

Genetic analysis of the plantain genome is difficult due to triploidy and high sterility. As shown earlier, ploidy manipulations (scaling up and down the number of chromosomes) and interspecific plantain-banana hybridization opened the path for the genetic amelioration of the crop and for the investigation of its genome. There are several associated effects of ploidy, parthenocarpy and resistance to black leaf streak disease on growth and yield characteristics of euploid hybrids. The number of copies of the resistance allele (bs_1) and of the parthenocarpy gene (P_1), as well as their intralocus interaction and ploidy level, have all been found to significantly affect bunch and fruit characteristics of euploid hybrids (Ortiz *et al.,* 1998a). Epistasis significantly affected fruit weight and size in one cross but not in another. Significant multiple regression models combining ploidy and genetic markers explained 15% to 85% of quantitative trait variation (QTV). The amount of QTV accounted by ploidy and genetic markers varied according to the characteristic and cross in which the markers were examined.

Linear and multiple regression models, coefficients of determination, and Durbin-Watson statistics were used by Craenen and Ortiz (1996) to determine the single and combined effects of the major locus for resistance to black leaf streak disease (bs_1) and of ploidy on bunch weight, fruit weight, fruit length and fruit girth in the progenies derived from crosses between a resistant diploid wild banana source and the susceptible French plantain landraces. Differences in yield were mainly due to changes in weight and circumference of the fruit, which are affected by the disease. The combined effect of ploidy and resistance to black leaf streak disease was partially responsible for QTV in yield. As a result of the gene interaction in the locus for resistance (bs_1), the partially resistant phenotypes showed higher yield than their more susceptible full sibs.

The performance of 20 euploid hybrids was compared with that of their parents to determine the influence of the disease on growth parameters and components of yield (Craenen and Ortiz, 1998). There were significant differences among the hybrids for all components of resistance, growth parameters, and yield components. For diploid hybrids, which often had a short growth cycle or early flowering, or both, the disease incubation time was significantly correlated with days to fruit filling ($P < 0.05$). However, for tetraploid hybrids that had a long growth cycle and delayed flowering, the correlation was not significant ($P \geq 0.05$). For diploid and tetraploid hybrids, disease evolution time and disease development time were both correlated ($P < 0.05$) with days to fruit filling. Bunch weight of tetraploid hybrids was correlated ($P < 0.05$) with disease development time as scored by the youngest leaf spotted at flowering ($r = 0.933$; $P < 0.001$). This result confirms that resistant hybrids with potentially high yield could be selected efficiently by recording the youngest leaf spotted at flowering.

Diploid or polyploid breeding

Most banana and all plantain cultivars grown by farmers in the tropics are triploids. Euploid hybrids derived from triploid-diploid crosses are either mostly diploids but some are tetraploids. They are seldom triploids. The most important goal in genetic population improvement programs for managing disease resistance in large populations is to enhance the frequency of favourable alleles controlling

the desired characteristics. Recurrent selection methods are the most common for increasing the frequency of favourable alleles in a cyclic fashion.

Theoretically, sq^2 recessive individuals will be selected out from a large diploid population where q is the allele frequency of a recessive gene and s is the intensity of selection against the recessive genotype. Similarly, in a large tetraploid population, sq^4 represents the proportion of recessive genotypes that are selected out. Hence, the change of allele frequency appears to be faster at the diploid than at the tetraploid level. This occurs because the recessive allele is only hidden in the heterozygous genotype at the diploid level while it will be included in the triplex, duplex and simplex at the tetraploid level. For example, if $q = 0.5$ and selection efficiency $s = 1$ at both ploidy levels, then the change in allele frequency after selection will be four times larger in the diploids than that for the tetraploids (25% vs 6.25%, respectively).

Population genetics theory also suggest that the smaller the s, the lower response to selection. When there are no escapes during screening, i.e. maximum selection efficiency, increases in the frequency of favourable allele will be maximum. With increased rates of escapes, breeding becomes inefficient and to the point that it may become worthless. As pointed out by Mendoza (1988) "*the degree of success in altering the genotypic structure of the population, by modifying its gene frequency, is a function of the precision in identifying and isolating the individuals carrying the attributes under selection. Any errors or 'escapes' during the process, depending on their magnitude, could alter the response to selection... A breeding effort can only be as efficient as the screening procedure permits.*"

Selection appears to be more effective in the early cycles when the frequency of the favourable allele is low, especially at the diploid level. However, if there are escapes owing to unreliable screening methods, particularly for a small population size, then a lowering of the response to selection will occur at a very low frequency of the favourable allele. With tetraploids, when the frequency of the favourable allele exceeds 0.4, the response to selection falls rapidly. When the frequency reaches 0.8 in the tetraploid population, the response to selection becomes practically nil for breeding purposes.

Outlook

The results of this research support the early views of *Musa* breeders (Ortiz, 1997; Vuylsteke 2000), who claimed that a broad-based, improved *Musa* germplasm with pest and disease resistance was necessary to achieve the sustainable production of this vegetatively propagated perennial crop. This germplasm was obtained by using conventional crossbreeding and may be further enhanced with the utilization of innovative methods for the introduction of additional genetic variation, e.g. ideotype breeding, polycross mating design or marker-aided introgression (Ortiz, 2001). In short, "*the prospects of banana and plantain breeding are unlimited and increased efforts will at once initiate a new phase of* Musa *evolution*" (Vuylsteke, 2001).

Manipulation of the *Musa* genome for its genetic betterment will be also facilitated by the available knowledge on the inheritance of most important characteristics in plantain and banana (Ortiz, 2000). Likewise, the information regarding fungal diseases, such as black leaf streak disease, and the interactions between the

pathogen and its host plant, provide the basis for a rational integrated management strategy to control the disease. For example, the partial resistance provided by the bs_1 gene (Craenen and Ortiz, 1997; Ortiz and Vuylsteke, 1994) can be easily incorporated into mixed cultivar systems common among the resource-poor farmers in the tropics (IITA, 1998). These farmers prefer cropping systems that provide intraspecific (cultivar mixtures) and interspecific (inter-cropping) diversity to maximise land, use labour efficiently and minimise the risk of crop failure. Deploying resistant hybrids in farmers' cropping systems in association with their own landraces is regarded as non-disruptive (IITA, 1999). In this suggested cultivar mixture, the resistant hybrids serve as inoculum traps that reduce the spread of the disease to the susceptible plantain landraces and may increase the bunch weight of the landraces that are preferred by farmers due to their culinary and rheological characteristics (IITA, 2000). On-farm participatory research undertaken by IITA will provide more insights into this proposal for deployment of resistance to black leaf streak disease using a cultivar mixture system (IITA, 2001). Data are still being recorded in a farm in south-eastern Nigeria.

A cultivar mixture system would preserve genetic diversity and provide new, high-yielding hybrids that may be incorporated in the local diet through novel processing methods. Introducing new cultivars may lead to losses of diversity in farmer's fields (Sharrock *et al.,* 2000), particularly when single-cultivar plantations are preferred over mixed farming. Furthermore, diseases, e.g. black leaf streak disease, may spread quickly into single-cultivar plantations of susceptible germplasm or when resistance breaks down in improved germplasm. Hence, cultivar mixtures may provide an "insurance" for a sustainable farming system.

Pyramiding genes from distinct germplasm sources may also enhance partial resistance in plantain and banana. For example, IITA hybrids, which show this kind of resistance, have alleles for resistance to black leaf streak disease from two sources: triploid plantains and diploid bananas. The resistance alleles are masked by intra and interlocus interactions in highly susceptible plantain parent landraces. The resistance alleles are mainly from the wild banana accession 'Calcutta 4' or the diploid banana cultivar 'Pisang lilin' (Hartman and Vuylsteke, 1999). The search for other sources of resistance against a wide range of strains (e.g. Fullerton and Olsen, 1991, 1993) appears mandatory to develop a strategy for durable resistance to black leaf streak disease. This requires urgent attention because resistance in at least two cultivars ('Paka' and 'T8') broke down after eight years of cultivation in the Cook Islands (Hartman and Vuylsteke, 1999).

The incorporation of different resistance types in the same genotype could potentially confer durable resistance to black leaf streak disease. Indeed, resistance alleles may be more stable depending on their mode of action and the particular resistance they control. For example, 'Calcutta 4' has a polygenic hypersensitive response that stops all development of the pathogen, but it also possesses recessive alleles controlling partial resistance in its plantain hybrids. This partial resistance simply slows disease development and may be more difficult to circumvent than the hypersensitive response, which already failed when screening young plants with Papua New Guinea strains. Hypersensitive responses are often associated with a gene-for-gene host-pathogen interaction (Flor, 1971), but this hypothesis has not been

tested in *Musa*. Resistance to black leaf streak disease provided by the recessive bs_1 gene may be stable in the host-plant because virulence requires a rare mutation to the dominant allele in the respective locus of the pathogen.

In conclusion, improved propagules with partial resistance to black leaf streak disease coming from distinct genetic sources, along with crop husbandry techniques are part of a holistic approach for long-term, sustainable productivity in *Musa* farming systems.

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Conventional breeding of bananas

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Abstract
Whereas ancestral bananas are fertile diploids, the main groups of bananas grown today are clones of plants, mostly triploids, which are reproduced entirely vegetatively and consequently difficult to breed. Conventional breeding techniques have yielded new varieties conventional breeding can accomplish only so much. Not all genetic combinations necessarily lead to useful hybrids. This communication presents the strategies which have been and are still being used in this field–namely the 3x/2x scheme and the creation of triploid hybrids from ancestral diploid varieties–and draws attention to their strengths and limitations.

Resumen - Mejoramiento convencional de los bananos
Mientras que los bananos ancestrales son diploides fértiles, los principales grupos de bananos que se cultivan actualmente son clones de las plantas, en su mayoría triploides, que se reproducen solo vegetativamente y, en consecuencia, son difíciles de mejorar. Las técnicas de mejoramiento convencional han producido nuevas variedades y el mejoramiento convencional tiene sur límites. Todas las combinaciones genéticas no llevan necesariamente a generar híbridos útiles. En este trabajo se presentan las estrategias que todavía están siendo utilizadas en este campo, a saber, el esquema 3x/2x y la creación de los híbridos triploides a partir de las variedades ancestrales diploides, y se destacan sus fortalezas y debilidades.

Résumé - Amélioration conventionnelle des bananiers
Alors que les bananiers ancestraux sont des diploïdes fertiles, les principaux groupes de bananiers cultivés aujourd'hui sont des clones de plantes, principalement triploïdes, qui se reproduisent selon un mode entièrement végétatif et sont donc difficiles à améliorer. Les techniques classiques d'amélioration ont produit de nouvelles variétés, et l'amélioration conventionnelle a ses limites. Toutes les combinaisons génétiques ne conduisent pas nécessairement à des hybrides utiles. Cette communication présente les stratégies qui ont été, et sont encore utilisées dans ce domaine, c'est-à-dire le schéma 3x/2x et la création d'hybrides triploïdes à partir de variétés ancestrales diploïdes, et insiste sur leurs avantages et leurs limites.

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[3]CIRAD, Montpellier, France
[4]INIBAP, Montpellier, France

Introduction

Bananas and plantains are difficult crops to breed because most of the important and popular varieties are highly sterile and therefore do not produce seeds. Furthermore, compared to many other important food crops, there is a relative lack of knowledge on *Musa* genetics and cytogenetics. Despite these constraints, important progress has been made in the genetic improvement of *Musa* in recent years and new varieties are now becoming available from breeding programmes. Major breeding programmes that use conventional breeding methodologies are located at the *Fundación Hondureña de Investigación Agrícola* (FHIA) in Honduras, the *Centre de Coopération Internationale en Recherche Agronomique pour le Développement* (CIRAD-FLHOR) in France and Guadeloupe, the International Institute of Tropical Agriculture (IITA) in Nigeria and Uganda, the *Centre Africain de Recherches sur Bananiers et Plantains* (CARBAP) in Cameroon and the *Empresa Brasiliera de Pesquisa Agropecuaria* (EMBRAPA) in Brazil.

In the last decades new banana varieties were mainly created by using conventional breeding techniques (Bakry and Horry, 1992; Jenny *et al.*, 1994; Menendez and Shepherd, 1975; Rowe and Rosales, 1992; Shepherd, 1968; Soares Filho *et al.*, 1992; Swennen and Vuylsteke, 1990; Tomekpé *et al.*, 1998). One of the peculiarities of bananas is the need to adapt these techniques to the genetics of polyploid plants.

This communication presents the strategies which have been and are still being used in this field, and draws attention to their strengths and limitations.

Constraints to the improvement of bananas

Whilst ancestral bananas are fertile diploids, the main groups of bananas grown today are clones of plants, mostly triploids, which are reproduced entirely vegetatively. For producers and consumers, this feature presents two advantages: triploidy gives the plant vigour, making it easier to grow than diploids; clonal propagation assures uniformity which facilitates management, both in the field and throughout the distribution and sale chain. Finally, triploidy ensures sterility of the fruit, enabling it to be eaten.

On the other hand, the nature of these plants also presents potential dangers for their cultivation, and obstacles to their improvement. First, the genetic uniformity of these plants facilitates the spread of diseases and increases the impact of the latter on banana plants. For example, the 'Cavendish' varieties throughout the world are all susceptible to leaf spot diseases. The same is true for plantains with regard to black leaf streak disease, whether in Africa, South America or Asia. Furthermore, the sterility of the clones currently grown is a considerable hindrance to their genetic improvement.

It is therefore clear that the primary needs in terms of breeding have to do with the various diseases which affect the crop. Among the most important is Fusarium wilt (caused by *Fusarium oxysporum* f.sp. *cubense*), and Sigatoka disease (caused by *Mycosphaerella musicola*) and black leaf streak (caused by *Mycosphaerella fijiensis*). However there are also other improvement criteria, especially for dessert

bananas for export, which have to do with fruit quality and post-harvest characteristics.

One of the first ideas was to draw on the natural existing genetic resources to find solutions for replacement varieties. Thus, during the 20th century, all of the 'Gros Michel', which was traditionally grown on commercial plantations, was gradually replaced by 'Cavendish' which is resistant to race 1 of Fusarium wilt. In certain areas, attempts were made to introduce 'Pisang awak' and 'Bluggoe' to replace or supplement the plantains susceptible to black leaf streak. These solutions are unfortunately of limited value because the natural varieties suitable for cultivation are very few, and are susceptible to other parasites, such as nematodes and weevils. Often the fruit produced is not the kind favoured locally and fails to gain acceptance. Finally, the problem of potential susceptibility ends up being passed on to the new clones through the creation of a new genetic uniformity which can easily be circumvented by the pathogens. The need for genuine genetic improvement is therefore real.

An improvement strategy: the 3x/2x scheme

The prerequisite to any genetic improvement strategy is to analyse the available tools. For conventional methods, it is necessary to identify the required qualities of fertility and useful characters of the germplasm which can be used. Since the 1920s, the questions tackled have been: which cultivars can be improved, and what characteristics can be introduced into them. Detailed analysis of the fertility of the main triploid cultivars resulted in the identification of a certain number of triploid clones which had retained residual female fertility, like 'Gros Michel' (*Musa* cv. AAA) and the French-type plantains (*Musa* cv. AAB). Conversely, certain clones turned out to be completely sterile, like 'Cavendish'[1] (*Musa* cv. AAA).

The search for sources of resistance to the main diseases was pursued by looking for wild varieties present in the area of origin of bananas, Southeast Asia. In this way, the variety *Musa acuminata* ssp. *burmannicoïdes* 'Calcutta' 4, among others, was identified, notably for its resistance to *M. musicola* and to *M. fijiensis*.

Armed with these tools, breeders crossed the various parents among themselves to improve the cultivated triploid clones, by combining the residual female fertility of the latter with the strong male fertility of the wild varieties (Figure 1). Detailed analysis of the mechanisms brought into play during these hybridizations has shown that the fertile triploid parent produced gametes during meiosis with a v ariable chromosome number, ranging from 11 to 66. Among these gametes, those with 33 chromosomes, i.e. with a true restitution nucleus, were particularly useful. Among the progeny the first priority was to look for tetraploid hybrids, the result of fusion between this restitution nucleus and a normal haploid gamete from the wild parent. The final result resembled more a fusion than a true hybridization, because recombination on the triploid side is low, and on the wild parent, being very homozygous, produces very homogeneous gametes. In this way hybrid

[1] It is now known that under certain stress conditions, it is possible to obtain pollen from 'Cavendish' clones.

tetraploids were produced with a genome similar to the complete genome of the triploid parent one is seeking to improve, supplemented by a wild haploid genome contributing sources of resistance. A certain degree of recombination would however explain the sometimes mediocre quality of the tetraploid hybrids obtained.

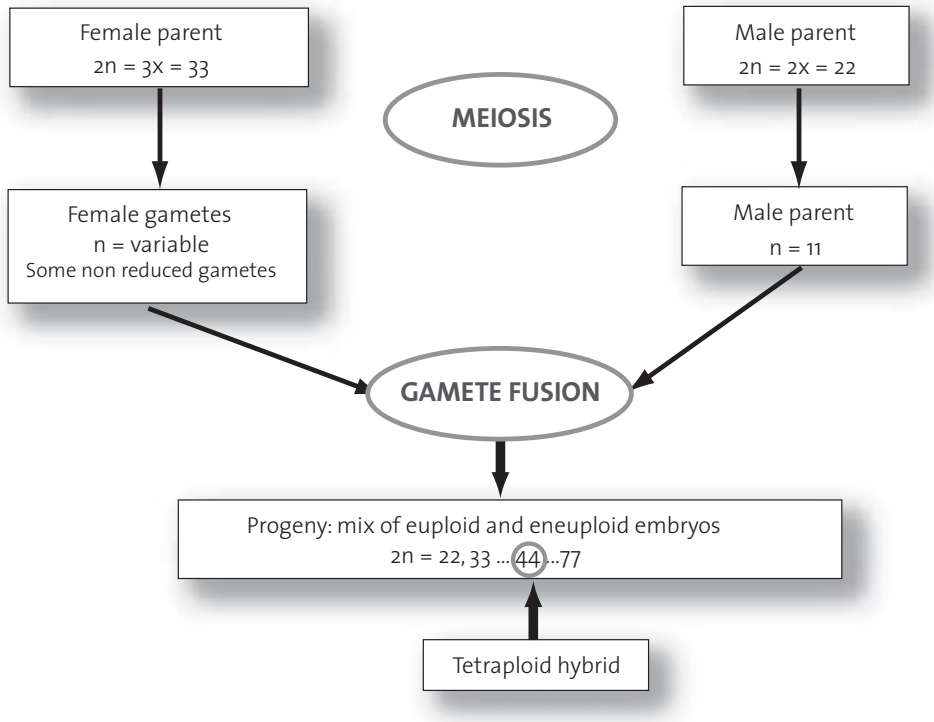


Figure 1. Scheme for creating tetraploid hybrids from triploid and diploid parents.

Two complementary methods exist to improve this strategy:

- It is possible to produce diploid hybrids which appear to be useful, and which can themselves be used in an improvement programme.
- The produced tetraploid hybrids, being more fertile than the triploid parent, can be reintroduced in the crossing schemes with a view to creating secondary triploid hybrids. One must not however forget that in this latter case the genetic gain obtained from nuclear restitution will be reduced by recombination which will occur during the meiosis of this tetraploid.

Nevertheless, many research organizations throughout the world produced hybrids using this scheme. As for cooking banana hybrids, the cultivars 'FHIA-21' (FHIA, Honduras), 'CRBP-39' (CARBAP, Cameroon) and 'Bita-3' (IITA, Nigeria) (Ortiz and Vuylsteke, 1998) should be mentioned. In the majority of cases, these hybrids were found to be resistant to both Sigatoka disease and black leaf streak disease, which was

the primary aim of their breeding. This immediate success can be attributed to the type of resistance used. Very often the wild male parent chosen was 'Calcutta 4' which is highly resistant (HR) to leaf spot diseases. It is generally accepted that this type of resistance depends on a smaller number of genes than partial resistance (PR), and consequently can be more easily transmitted during hybridization. The result however poses two questions: the exact transmission mechanism of these resistances is not known; and as a corollary, one cannot predict the durability of these resistances over time. Logically one would prefer to try to introduce into these hybrids partial resistance, known to be more durable but more complex, which has, for example, been tried by using parents such as M-53.

The 3x x 2x strategy described here also suffers from several limitations.

- From a purely technical point of view, this strategy depends on the existence of valuable triploid clones exhibiting exploitable female fertility. There are relatively few of these, limiting the possibilities of enlarging the genetic base of these crossings.
- The hybrid populations obtained by crossing are usually small.
- The percentage of tetraploid hybrids being small, the possibilities for selection are very limited. This technique cannot be used to work simultaneously on a large number of characters to be improved.

More recently, the realization of the existence of potentially active sequences of banana streak virus (BSV) contained within the *balbisiana* genome has further reduced the possibilities for using this strategy. Moreover, the most fertile triploid genomes are often of the AAB or ABB types. Such germplasm should not be used for breeding as long as the activation mechanisms of BSV are unknown. One might nevertheless use it to produce various diploid type AA hybrids, notably plantain diploids which constitute 50% of the 3x/2x descendants produced by certain plantain cultivars. Several dozen of these hybrids produced at CARBAP were found to be negative for IC-PCR and have never expressed any BSV symptoms in the field even under stress conditions in which nearly all tetraploid hybrids frequently present symptoms.

Lastly, the tetraploid nature of the hybrids formed often leads to problems of fruit quality. In dessert-type hybrids the firmness of the pulp is less in the tetraploids. This problem also exists in cooking hybrids, albeit less pronounced. In numerous polyploid plants, it has been found that the water content of the cells increases with ploidy level. If this proves true for bananas, it might explain the phenomenon. Moreover, at the tetraploid level, female fertility might be restored, often leading to the presence of seed in the fruit, which is unacceptable to present-day consumers.

Recent developments with biotechnological tools and a better understanding of the evolutionary processes of bananas have led to the introduction of another breeding strategy aimed at the production of triploid hybrids.

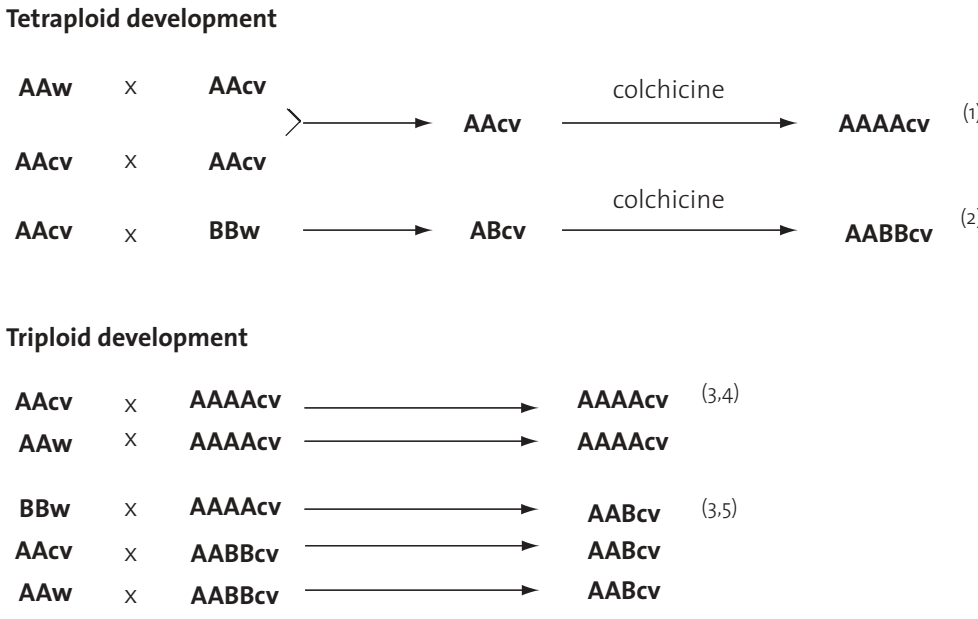
Creation of triploid hybrids from ancestral diploids

The natural emergence of triploid cultivars derived from ancestral diploid varieties is due to the accidental production of unreduced gametes in one of the diploid parents during hybridization (Simmonds, 1962). The 4x x 2x strategy is a copy of

this natural evolutionary process. The meiotic error leading to these unreduced gametes is replaced by a chromosome doubling in one of the parents using colchicine (Vakili, 1967; Stover and Buddenhagen, 1986; Bakry *et al*., 1997).

Unlike the scheme just described, this strategy does not attempt to improve existing varieties, but rather to create new improved varieties, close to the established targets, using ancestral varieties. These new hybrids should therefore combine all the classic characteristics of the banana requiring improvement, plus the improved characters for which the strategy was introduced.

The triploid hybrids are obtained by simple hybridisation between a diploid parent and a tetraploid parent (Figure 2). This hybridisation has to be the end of the procedure, since the product obtained is almost completely sterile, and can thus no longer be improved by conventional means. The tetraploid parent has previously been obtained by doubling with colchicine an ancestral diploid or an improved diploid. After treatment, the purely tetraploid nature of the parent is checked by flow cytometry. The success of the strategy rests on the judicious choice of the parents. These can either be natural diploid cultivars or improved diploid cultivars.



1. Nearly 20 clones developed at CARBAP and CIRAD.
2. Currently stopped because of BSV concern.
3. Annual hybrid population size of nearly 400 plants in the field.
4. 98% of the progeny is triploid.
5. Currently stopped because of SV concern.

Figure 2. Strategy for creating triploid hybrids from diploid material.

At CIRAD, where this method has been favoured for several years, progress has been made in characterizing available genetic resources and understanding the relationships between ancestral and cultivated varieties, thus facilitating the definition of pools of parent lines according to the desired results (Jenny *et al.*, 1999). Moreover, the development of molecular tools has made, and continues to make, this strategy more efficient. Among the most important results, it has been possible to demonstrate the uniparental inheritance of cytoplasmic organelles, which helps in identifying the phylogeny of bananas (Fauré *et al.*, 1994). *Musa* is one of the few species with biparental cytoplasmic inheritance: paternal inheritance of mitochondria and maternal inheritance of chloroplasts.

The extent of genetic variability within the *acuminata* genome has been related to the variability in fruit quality in the main cultivated groups. The most striking example is probably the strict relationship between the sub-species *M. acuminata banksii* and cooking bananas. It has thus been possible to produce triploid cooking banana hybrids of purely *acuminata* origin. The variability of the *acuminata* genome also permits variation in the type of fruit type and plant obtained, whether dessert or cooking, but also with regards to the fruit's sweetness or acidity, its length, the plant's number of suckers, and its yield, etc.

One of the main attractions of this strategy rests on making use of highly fertile parents, thus leading to a large progeny. In preliminary results, CARBAP identified in the 100 or so individuals of the progeny of a BB x AAA cross, about 20% of hybrids having useful resistance to black leaf streak disease (BLSD).

To sum up, the 4x x 2x strategy presents a certain number of undeniable advantages in genetics:

- The number of available parents is only limited by our knowledge of the germplasm, knowing that subsequently pools of parents that produce the targeted results will be formed.
- Using a tetraploid parent allows better control of the heritability of characters due to limited recombination within the polyploid parent.
- Highly fertile parents lead to the production of large populations in which it is possible to set up a true selection programme, possibly based on several improvement criteria.

In order to improve this strategy, it would be valuable to further enlarge the diploid crossing base either by crossing known diploids to obtain improved diploids or by more collection missions in the regions of interest. CARBAP is currently developing, from diploid plantain hybrids resistant to BLSD, second and third generation improved diploids (secondary and tertiary diploids obtained by crossing diploid plantain hybrids with different sources of resistance to BLSD) which are in the process of chromosome doubling so as to be integrated into this strategy. In the near future, the use of molecular markers should facilitate selection at the parent level (identification of the genes to be transferred) and hybrid level (identification of the genes effectively transferred). Nowadays for example, three QTL (Quantitative Trait Loci) have already been localized in relation to resistance to BLSD (Persley and George, 1999). Their use remains dependent on the completion of the genetic map being created by CIRAD. Among the future challenges,

a molecular characterization of fruit quality will be particularly important for the creation of dessert-type varieties which are competitive in the world market.

Once again however, BSV acts as a brake on the best use of this strategy. The B genome is excluded from breeding schemes because of possible activation of integrated viral DNA sequences within the genome. It is known that activation is linked to certain stresses including hybridisation and *in vitro* multiplication. These two stresses do not necessarily activate the same integrated sequences, and could therefore have additive effects (Lheureux *et al.*, 2003). It is therefore very important to study the B genome in more detail in order to be able, as soon as possible, to use it again for creating varieties.

- The range of banana species is not so rich that we can manage for their improvement without the one or two species from which cultivated bananas originated.
- In particular, the use of *M. balbisiana* in crossing schemes confers resistance, in particular to *Mycosphaerella musicola* and *M. fijiensis*, on the hybrids produced. This increased vigour renders the plants less susceptible to growth stresses.
- Finally, the high natural fertility of *M. balbisana* is an advantage for the production of large numbers of progeny.

Among the avenues to explore, one should mention collecting in the regions of origin of the species *M. balbisiana*, the analysis of the germplasm present in collections and genetic methods of improvement such as for example the extraction of the B genome from interspecific clones. In each case, it will be essential to gather international expertise and competence on the subject, and the PRO*MUSA* workshop on the diversity of the *M. balbisiana* genome held in Bangkok in 2002 was a good starting point.

Conclusion

At a more technical level, it emerges from this presentation that conventional breeding can accomplish only so much. Not all genetic combinations necessarily lead to useful hybrids. Crossing cooking bananas with dessert ones, for example, generally leads to intermediate hybrids of no real value. Not all combinations are possible, and many simply do not work, when using conventional hybridization techniques.

Conventional breeding methods should be viewed as just a part - admittedly a significant part - of a more general genetic improvement strategy. Unconventional techniques can usefully complete this arsenal (Novak, 1992; Sagi *et al.*, 1995; Sharrock *et al.*, 2000). For example, protoplast fusion is one of the ways which could increase the possibilities of overcoming certain fertility barriers in combining parental lines. This technique also allows transmission of intact genotypes by bypassing the recombination phenomena associated with meiosis, and also results in modification at the cytoplasmic level of the cells, thus potentially leading to novel results.

Mutagenesis and genetic transformation - aside from the arguments as to their appropriateness - might improve either the parents or, at the other end of the chain, the hybrids created by conventional hybridization and which lack certain characters. A complementary approach, recombinant DNA, has led to the production of the first

transgenic banana and plantain, but also to the generation of a large number of transgenic lines with agronomically useful genes. Transgenic plants transformed with genes encoding antifungal proteins are currently available for field-testing.

It is essential that *Musa* breeders and biotechnologists work together to accelerate improvement. In view of the limited resources being devoted to research into *Musa* improvement, and knowing the scale of the problems to be overcome, it is important to strengthen collaboration between the various institutions working on the problem, and to take advantage of all the resources already available to develop research on *Musa*. The Global Programme for *Musa* Improvement (PRO*MUSA*) aims to bring together all the scientists working on *Musa* genetic improvement, involving in the same programme geneticists, biotechnologists, but also pathologists and physiologists.

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Transgenic approaches for resistance to *Mycosphaerella* leaf spot diseases in *Musa* spp.

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Abstract

In smallholdings, average banana and plantain yields per unit have not increased significantly in the last 30 years. Increases in production are due almost exclusively to an increase in the area under cultivation. Increasing pest and disease pressure, especially from leaf spot diseases, and the deteriorating natural resource base have collectively been responsible for these low yields. Resistant high yielding bananas have been bred and supplied to smallholders in the 1990s after nearly 70 years of conventional breeding. This very slow progress was due to the high sterility, poor seed germination rate, need for interploidy crosses, the long generation cycle, which are inherent to bananas and plantains. A breeding program can only supply a few promising hybrids per year for further evaluation. The few selected hybrids are high yielding and resistant to some diseases but have usually lost other desired characteristics such as shelf life or pulp texture. Genetic transformation tools offer an opportunity for plant breeders to overcome the constraints imposed by the high level of sterility of the most popular cultivars. Good progress has been made in the development of a molecular toolbox for bananas and plantains in the areas of 1) cell suspension, 2) genetic transformation (particle bombardment and *Agrobacterium*-mediated transformation), 3) high expression of foreign genes, 4) insertion of multiple genes and 5) identification of genes for resistance to fungal disease.

Resumen – Enfoques transgénicos para la resistencia a las enfermedades de las manchas foliares en banano (*Musa* spp.)

Durante los últimos 30 años, los rendimientos promedio de los bananos y plátanos no han aumentado significativamente en las pequeñas fincas y los aumentos de producción se deben casi exclusivamente a un aumento del área bajo cultivo. El aumento de la presión de plagas y enfermedades, especialmente de las enfermedades de las manchas foliares, y el deterioro de la base de recursos naturales han sido responsables de manera colectiva de estos rendimientos tan bajos. En la década de los 90, se seleccionaron bananos resistentes de alto rendimiento los cuales

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fueron suministrados a los pequeños productores, después de casi 70 años de mejoramiento convencional. Este progreso tan lento se debió a una alta esterilidad, una tasa pobre de germinación de las semillas, una necesidad de cruzamientos interploídicos, un largo ciclo de regeneración, etc., inherentes a los bananos y plátanos. Básicamente, un programa de mejoramiento puede proporcionar solo unos pocos híbridos prometedores por año para realizar las evaluaciones consiguientes. Los pocos híbridos seleccionados son de alto rendimiento y resistentes a algunas enfermedades, pero usualmente pierden otras características deseadas como vida verde, textura de la pulpa, etc. Las herramientas de la transformación genética ofrecen una oportunidad a los fitomejoradores para vencer las limitaciones impuestas por el alto nivel de esterilidad en las variedades más populares. También se alcanzó un buen progreso en el desarrollo de una serie de herramientas moleculares para los bananos y plátanos en las áreas de (1) desarrollo de las suspensiones celulares; (2) tecnologías de transformación genética (bombardeo con partículas o transformación con *Agrobacterium*); (3) alta expresión de genes foráneos; (4) inserción de genes múltiples; (5) identificación de genes para la resistencia a enfermedades fungosas.

Résumé – Approches transgéniques de la résistance aux maladies foliaires causées par les *Mycosphaerella* chez les *Musa* spp.
Dans les exploitations de petite taille, les rendements en bananes et bananes plantain n'ont pas significativement augmenté au cours des 30 dernières années. L'augmentation de la production est due presque exclusivement à une augmentation de la surface cultivée. L'accroissement de la pression des maladies et ravageurs, et particulièrement des maladies foliaires, et la détérioration de la base de la ressource naturelle ont été collectivement responsables de ces faibles rendements. Des bananiers résistants et à rendement élevé ont été produits et distribués aux petits producteurs dans les années 90, après près de 70 ans d'amélioration conventionnelle. Ces progrès très lents sont dus à la stérilité élevée, au faible taux de germination des semences, au besoin de réaliser des croisements interploïdes et au long cycle de génération, qui sont propres aux bananiers et aux bananiers plantain. Un programme d'amélioration ne peut produire que quelques hybrides prometteurs par an pour leur évaluation ultérieure. Les quelques hybrides sélectionnés ont une production élevée et sont résistants à certaines maladies mais ont généralement perdu d'autres caractéristiques désirées, telles que la durée de conservation ou la texture de la pulpe. Les outils de transformation génétique offrent une opportunité aux sélectionneurs de surmonter les contraintes imposées par le niveau élevé de stérilité des cultivars les plus populaires. Des progrès importants ont été faits dans le développement d'une boîte à outils moléculaires pour les bananiers et les bananiers plantain dans les domaines : 1) des suspensions cellulaires ; 2) de la transformation génétique (bombardement de particules et transformation avec *Agrobacterium* ; 3) du niveau d'expression élevé de gènes étrangers ; 4) de l'insertion de gènes multiples et 5) de l'identification de gènes de résistance aux maladies fongiques.

Introduction

The predicted increase of the world's population to 8 billion people by 2025 (Harris, 1996) will require developing nations to dramatically increase crop yields. Technologies such as the application of fertilizers or pesticides will have to contribute, but the most environmentally safe and sustainable approach is the production and delivery of stress resistant high yielding cultivars. Until recently, new cultivars were produced by cross-breeding or the selection of induced or natural mutations. With the rapid advances in molecular biology, the genetic modification of tropical crops needs to be envisaged to accelerate and focus genetic improvement.

Bananas are one of the first domesticated crops (De Langhe and De Maret, 1999). Some 3000 years ago, between 3 to 8 plantain cultivars were introduced in Africa (De Langhe *et al.,* 1995) (Mbida *et al.,* 2001). Somatic mutations gave rise to about 120 plantain cultivars (Swennen, 1990) which are all susceptible to black leaf streak disease. Clearly, plantains have a very narrow genetic basis but this is also true for the entire *Musa* genus despite the existence of about 1200 accessions (Van den houwe *et al.,* 2000).

Average yields of bananas and plantains, hereafter called bananas, have not increased significantly in the last 30 years and increases in production are due almost exclusively to an increase in the area under cultivation. Average yields on smallholdings remain below 8 t/ha/yr but yields up to 80 t/ha/yr are possible. The gradual decrease in yields in the major banana growing regions has been attributed to increased pest and disease pressure and a deteriorating natural resource base. As a result, many rural communities in Africa are now unable to meet their basic needs for food and income.

Resistant high yielding bananas have been bred and supplied to smallholders in the 1990s after nearly 70 years of conventional breeding (Vuylsteke *et al.,* 1993a, 1993b, 1993c, 1994, 1995; Rowe, 1984; Rowe and Rosales, 1990). This extremely slow progress is due to high sterility, poor seed germination rate, the need for interploidy crosses (Swennen and Vuylsteke, 1993; Vuylsteke and Swennen, 1993; Ortiz and Vuylsteke, 1995) and the long generation cycle. Basically, a breeding program supplies only a few promising hybrids per year for further evaluation. Only 0.1% of the selected hybrids are high yielding and resistant to some diseases but they have lost other desired characteristics such as shelf life or pulp texture. Genetic transformation tools offer an opportunity for plant breeders to overcome the constraints imposed by the high level of sterility of the most popular cultivars (Swennen, 1994; Sági *et al.,* 1995a, 1995c, 1998a, 1998b).

In this article, we discuss the different molecular tools available for banana improvement, i.e. 1) embryogenic cell suspensions, 2) gene transfer technologies, 3) expression of foreign genes, 4) insertion of multiple genes and 5) gene identification. The current technology has the potential of producing several hundreds transgenic plants per day, in contrast to conventional breeding methods. Possible future scenarios for the production of bananas resistant to leaf spot diseases, such as strategies relying on meristem transformation, R-genes from banana and pathogen inducible promoters, are presented.

Transgenic bananas have been available at KULeuven since 1994. Yet, until now they could not be tested in the field because of a lack of national laws in African plantain-growing countries to regulate their release. This causes unnecessary delays in the further testing, fine-tuning and delivering of resistant bananas and plantains to smallholders.

Cell and tissue culture

In many plant species, genetic transformation is very simple and genes are transferred to callus obtained, for example, from wounded leaves as is the case with apples (De Bondt 1995). Even simpler is the flower dip method in *Arabidopsis,* which

does not necessitate an *in vitro* process for transformation (Bent, 2000; Clough and Bent, 1998). In bananas, embryogenic cell suspensions are still needed and the procedure is far from routine (Schoofs *et al.,* 1999). Unlike most dicots (De Vries *et al.,* 1988; Meijer *et al.,* 1999) and seedbearing monocots (Vasil and Vasil, 1986; Vasil, 1987), bananas are highly recalcitrant to embryogenesis. Four main procedures have been developed, each relying on different explants: zygotic embryos (Cronauer and Krikorian, 1988; Escalant and Teisson, 1989), rhizome slices and leaf sheaths (Novak *et al.,* 1989), immature (fe)male flowers (Escalant *et al.,* 1994; Grapin *et al.,* 1996; Grapin *et al.,* 1998) and proliferating meristem cultures (Dhed'a *et al.,* 1991; Schoofs, 1997).

Most embryogenic suspensions are produced from meristems or flowers, each method having advantages and disadvantages. For example, the former depends on extensive preparation of material before induction of embryogenesis, whereas the latter requires direct access to flowering banana plants.

At KULeuven the 'scalp' method (Schoofs, 1997) relies on rapidly proliferating cultures initiated from a shoot-tip meristem cultured on a medium containing high levels of cytokinin. Embryogenesis-competent scalps contain a high number of tiny white meristems with only a small amount of corm or leaf tissue. The shoot-tip is first screened for endophytes and if found positive either cleaned-up or replaced by an endophyte-free shoot-tip (Van den houwe *et al.,* 1998; Van den houwe and Swennen, 2000). The scalp method involves: 1) preparation of embryogenesis competent explants (scalps), which takes 5 to 14 months; 2) embryogenesis induction, which takes 4 to 7 months; and 3) suspension initiation and upgrading, which takes 3 to 6 months (Swennen *et al.,* 1998). Hence 12 to 27 months, depending on the cultivar (Schoofs, 1997), are needed before a suspension is ready for transformation.

The production of suspensions from East African Highland bananas is particularly cumbersome (Strosse *et al.* in press, Table 1). A broad range of cytokinins at varying concentrations was explored for scalp induction and it was found that TDZ (thidiazuron) was a good alternative to BAP (benzylaminopurine) (Table 2 and Figure 1). In fact, 10 mM TDZ could reduce by threefold the embryogenesis induction time (Strosse *et al.,* in press). The embryogenic response was found to depend on the genotype (Figures 2 and 3) and even on the selected line and the experiment, and varied between 0 and 22.2% (Strosse *et al.,* in press). Homogeneous complexes consisting of a high proportion of embryogenic callus and early-stage transparent embryos are preferred as inoculum but embryogenic cell suspensions remain more or less heterogeneous (Georget *et al.,* 2000).

Once cell suspensions are produced, they undergo quality control measurements at repeated intervals on regeneration potential, health status (Van den houwe *et al.,* 1998), DNA content (Roux *et al.,* in press a), true-to-typeness, etc. Between 10^4 to 10^5 somatic embryos per ml settled cell volume can be obtained. Hence, a 'Grande naine' cell suspension can produce 14 580 to 100 980 plants while 27 000 to 117 000 plants can be regenerated from an 'Orishele' suspension (Strosse *et al.,* in press) (assuming an inoculum of 1.5% settled cell volume in a 60-ml cell suspension maintenance medium and a twofold increase of cell volume after a two-week subculture). DNA content is assessed through flow cytometry and can show the loss

Table 1. Preliminary results using the scalp method (Jan 1998–Dec 2001).

Cultivar	Genome	Type	ITC code	Number of inoculated scalps	Responsive scalps*	Frequency (%)**	Highest frequency in a single experiment	EC	ECS	Ready for first applications
Calcutta 4[a]	AA	wild diploid		1872						
Grande naine	AAA	Cavendish	1256	2040	112	5.5	11.7	yes	yes	yes (4)[b]
GN FHIA	AAA	Cavendish		1296	11	0.8	2.9	yes	yes	
GN JD	AAA	Cavendish		1872	16	0.9	4.2	Yes	yes	
Williams BSJ	AAA	Cavendish	0570	456						
Williams JD	AAA	Cavendish		2808	99	3.5	22.2	yes	yes	yes (7)[b]
Ingarama	AAA-h	highland	0160	1104						
Mbwazirume	AAA-h	highland	0084	1128	240					
Nyamwihogora	AAA-h	highland	0086	864						
Agbagba	AAB-p	plantain	0111	576	3	0.5	0.5	yes	yes	yes[f] (1)[b]
Obino l'ewai	AAB-p	plantain	0109	336	3	0.9	2	yes	yes	yes (1)[b]
Orishele	AAB-p	plantain	0517	1200	29	2.4	5.8	yes	yes	yes (4)[b]
Burro cemsa	ABB	cooking	1259	504						
Total				16056	273					
Mean						1.1	3.8			

* Number of scalps forming embryogenic complexes

** % of scalps forming embryogenic complexes (RS/NS3)x100, with NS3 being the total number of scalps longer than 3.5 months in culture (long enough for first embryogenic complexes to form)

EC Embryogenic complex

ECS Embryogenic cell suspension

a Derived via zygotic embryo rescue. Seeds obtained from IITA, Nigeria

b Number of independently established cell suspension lines ready for first applications at the end of 2001

Table 2. Preliminary results using TDZ scalps (Jan 1998–Dec 2001).

Cultivar	Genome	Type	ITC code	Number of inoculated scalps	NS3*	Responsive scalps**	Frequency (%)***	Highest frequency in a single experiment	EC	ECS
Calcutta4[a]	AA	wild diploid		96	24					
Williams	AAA	Cavendish	0365	960	720	13	1.8	2.8	yes	yes
Igisahira gizanswe	AAA-h	highland	0083	264	24					
Agbagba	AAB-p	plantain	0111	144	24	1	4.2	4.2	yes	
Bluggoe	ABB	cooking	0010	144	144	4	2.8	5.6	yes	
Cachaco[b]	ABB	cooking	0643	144	144	4	2.8	5.6	yes	
Cachaco	ABB	cooking	0643	120						
Total				1872	1080	22				
Mean							1.7	2.3		

* Total number of scalps longer than 3.5 months in culture (long enough for first embryogenic complexes to form)
** Number of scalps forming embryogenic complexes
*** % of scalps forming embryogenic complexes (RS/NS3)x100
EC Embryogenic complex
ECS Embryogenic cell suspension
a Derived via zygotic embryo rescue. Seeds obtained from IITA, Nigeria
b Scalps derived from 1 µM TDZ meristem cultures instead of 10µM TDZ meristem cultures as in all other cases

of even 1 chromosome (Dolozel *et al.*, 1999). Rate of somaclonal variation can vary from very low (2%) (Côte *et al.*, 2000) to very high (99%) (data not published). Since high quality suspensions are rare, they are cryopreserved for backup purposes (Panis *et al.*, in press; Panis *et al.*, 1990).

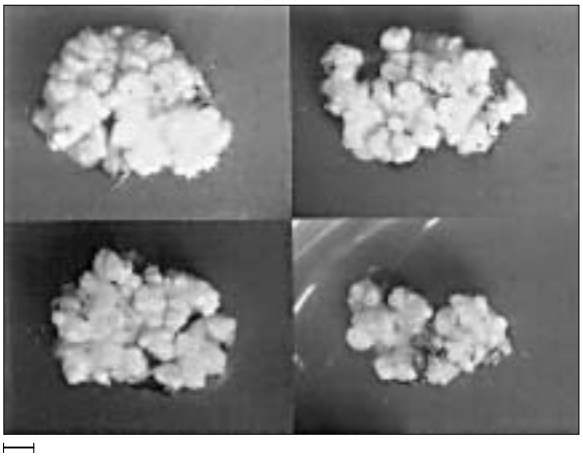

Figure 1.
Highly proliferating meristem cultures of 'Williams' (AAA) four months after inoculation of a 5-mm explant (apical dome fully covered by three to four leaf primordia and a few millimeters of corm beneath the apical dome) on MS based medium supplemented with 10 µM TDZ, bar = 769 µm.

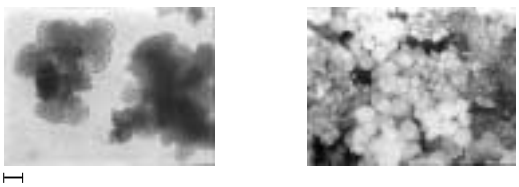

Figure 2.
Highly regenerable embryogenic cell suspensions of 'Grande naine' (AAA). Embryogenic cell clusters observed with (left) light microscope, bar = 95 µm and (right) germinating embryos one month after culturing on regeneration medium, bar = 370 µm.

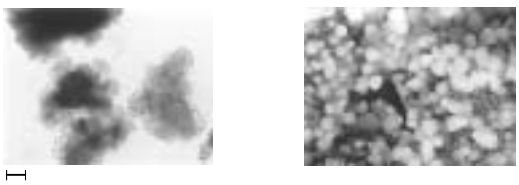

Figure 3.
Highly regenerable embryogenic cell suspensions of 'Orishele' (AAB). Embryogenic cell clusters observed with (left) light microscope, bar = 95 µm and (right) germinating embryos one month after culturing on regeneration medium, bar = 370 µm.

Gene transfer methods

Regenerable embryogenic cell suspension (ECS) cultures are the material of choice (Dhed'a *et al.*, 1991; Escalant *et al.*, 1994; Côte *et al.*, 1996, 1997) for the genetic engineering of bananas via particle bombardment-mediated transformation (PMT) and *Agrobacterium*-mediated transformation (AMT) (Ságі *et al.*, 2000). The former uses the biolistic gun device (Ságі *et al.* 1995a), whereas the latter uses cocultivation

with *Agrobacterium* (Pérez Hernández, 2000). Different methods and cultivars are being used (Sági *et al.,* 1995a; May *et al.*, 1995; Remy *et al.,* 1998a, 1998b; Becker *et al.,* 2000; Ganapathi *et al.,* 2001). Our comparative study involved ECS of four cultivars in the exponential growth phase (4+1 days after subculture). DNA plasmid, PMT and plant regeneration were according to Sági *et al.* (1995a, 1995b). For AMT transformation, evaluation of transient gene expression and selection and regeneration of transformants was according to Pérez Hernández (2000), Pérez Hernández *et al.* (1999) and Arinaitwe *et al.* (in press).

Results indicate that the efficiency of the two gene transfer methods is quite similar at the transient level with different promotors. In contrast, AMT was more efficient than PMT after selection (Arinaitwe *et al.*, in press) (Table 3). The higher number of plants regenerated using the AMT system in comparison with the PMT system confirmed AMT as the method of choice for transforming plant cells, as reported by Newell (2000).

Table 3. Shoot regeneration of four cultivars using the AMT and PMT methods.

Cultivar	AMT	PMT
Grande naine	117	76
Obino l'ewai	118	41
Orishele	93	05
Three hand planty	96	98

ECSs of four cultivars were co-cultivated with an *Agrobacterium tumefaciens* strain: AGL0 harbouring the binary plasmid pUbi-sgfpS65T; and EHA101 harbouring the binary plasmid pFAJ3000. Plasmid pFAJ3000 contains a *gusA* (ß-glucuronidase) gene driven by the CaMV 35S promoter and a *neo* gene under the control of the NOS promoter. Plasmid pUbi-sgfpS65T contains a *gfp* (green fluorescent protein) gene driven by the ubiquitin promoter (Arinaitwe *et al.*, in press). There was a difference in the expression of the two reporter genes used (Figure 4). This was, probably, due to differences in efficiency of the two *A. tumefaciens* strains used and the variable embryogenesis.

The effect of infection time on transformation frequency was investigated in 'Grande naine' and ''Three hand planty' (AAB) by using transient *gus* expression (TGE) and transient green fluorescent protein expression (TGFPE). In both cultivars and both marker genes, transient expression increased with increasing infection time (Table 4). With 'Grand naine', maximum transient expression was reached after 8 and 12 hours for TGE and TGFPE, respectively. More TGE was observed in Three hand planty' than 'Grande naine', possibly due to differences in the quality of the cell line.

Variable volumes (ml) of ECS were plated and uniformly spread over a 50-mm nylon mesh. Transient GFP expression indicates that T-DNA transfer was highest at 100±50 ml (Arinaitwe *et al.*, in press) but dropped sharply when the volume of ECS was increased to 300 and 600 ml. A decreased attachment and access to individual embryogenic cells or cell clusters by *Agrobacterium* is considered to be the cause. High TGFPE in small volumes is attributed to increased exposure of ECSs

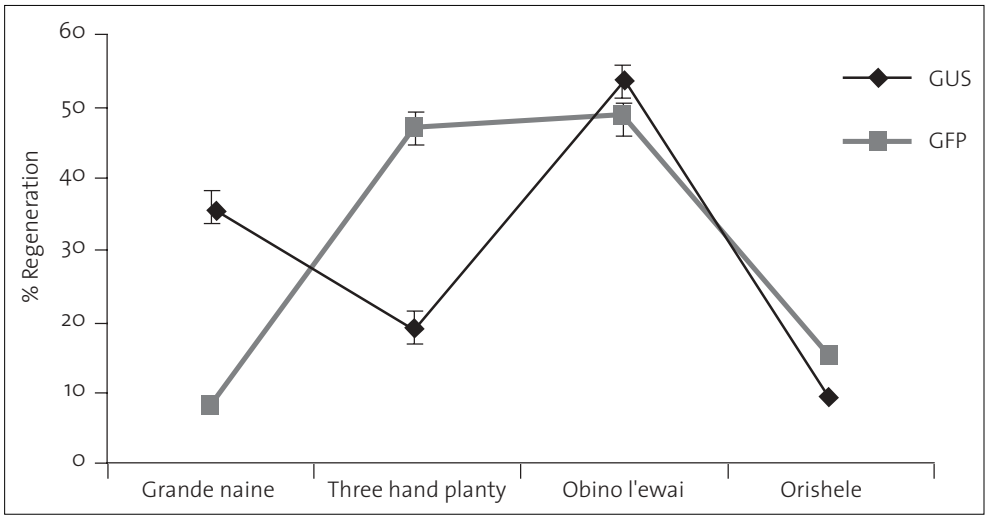

Figure 4. Percentage of regenerated shoots of four cultivars transformed via *Agrobacterium* (AGLO, pUbi-sgfpS65T with *gfp* gene; EHA 101, pFAJ 3000 with *gusA* gene).

to *Agrobacterium* since efficient spreading of thin layers of cells is achieved during the co-cultivation phase.

Upon subculture an ECS starts to multiply but its growth (Schoofs *et al.* 1999) and cell cycle (Roux *et al.* in press b) changes with age. Hence an effect of ECS age on transformation frequency is expected and could be confirmed (Figure 5). Cell competence for transformation increased from day 1 until day 7, beyond which it dropped. This period is thought to coincide with the exponential growth phase of the ECS (Ságí *et al.* 1995a, b). Efficient transformation of 7-day-old ECSs has been reported in cultivar 'Rasthali' (AAB) (Ganapathi *et al.* 2001).

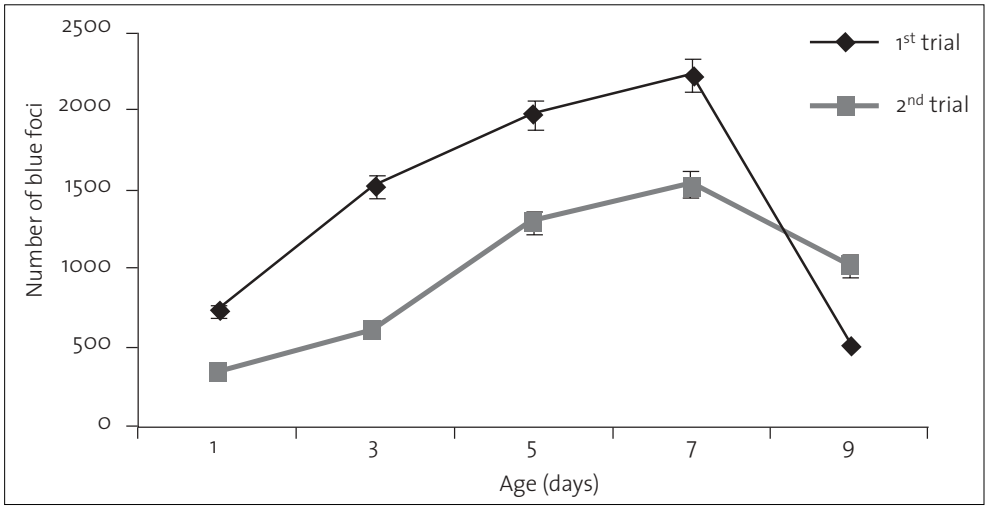

Figure 5. Effect of age of embryogenic cell suspension on transformation frequency: transient *gus* expression in cultivar 'Obino l'ewai' transformed via *Agrobacterium* (EHA 101; pFAJ 3000) (n=3).

Table 4. The effect of infection time on transient gene expression frequency (Mean±SE of the number of spots of gene expression per cell sample of 50 mg (fresh weight); at least 4-5 replications).

Marker gene	Cultivar	Infection time (hrs)					
		4	6	8	10	12	14
gus	Grande naine	794±87.9	881±91.8	>1500	>1500	>1500	>1500
gfp	Grande naine	209.7±24	272±41	365.7± 28	922.7±21	>1500	>1500
gfp	Three hand planty	1169.3±150	1311.7±95	>1500	>1500	>1500	>1500

The combined use of these and other factors resulted in a five-fold increase in transient expression compared to the original procedure. Representative results of these experiments are shown in Figure 6. One hundred mg (fresh weight) of control banana cells showed no background transient expression of the *gus* reporter gene (Figure 6A). In contrast, an average of 1500 blue foci was observed in the same amount of a cell line of the dessert banana 'Grande naine' (Figure 6B) using the improved method, in comparison with about 250 blue foci using the standard protocol. The uniform distribution of the transiently transformed cells also indicates the high efficiency of the improved procedure. Similarly increased transient *gus* expression rates have been observed in several bananas cultivars. Experiments are now in progress to determine if increased transient gene expression improves the yield of transgenic plants.

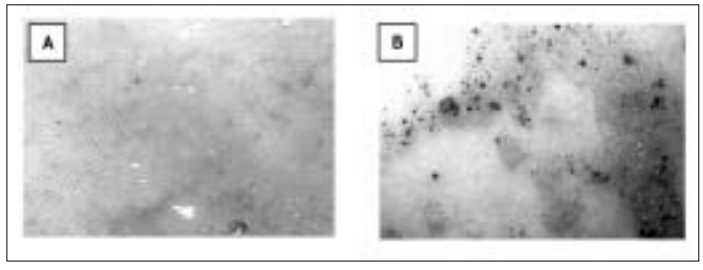

Figure 6. Transient GUS expression in a cell suspension culture of 'Grande naine' after co-cultivation with *Agrobacterium tumefaciens* EHA105 harbouring the GUS-intron containing binary vector pFAJ3000. A) 100 mg control cells showing no GUS expression, B) the same amount of transformed cells after six days of co-cultivation using the improved method.

Following confirmation that banana could be transformed (Ságí *et al.*, 1995a; May *et al.*, 1995), several groups looked for suitable promoters to improve the expression of heterologous genes. More than 25 heterologous and 1 homologous promoters have been tested (Table 5). In general strong transcription is obtained when genes are driven by the constitutive promoters such as the promoter from the maize ubiquitin, or the rice actin gene and promoters from the pregenomic RNA of banana streak badnavirus. Few tissue-specific promotors have been identified in banana but some promoter regions from the banana bunchy top nanavirus (BBTV) seem to have a potential for expression in vascular tissue.

Table 5. Promoters used in banana genetic transformation.

Promoter	Source of the promoter	Reference
CaMV35S	Cauliflower Mosaic Virus	Ságі *et al.*, 1992; Ságі *et al.*, 1994; Ságі *et al.*, 1995b; Ságі *et al.*, 1995c; CIRAD, 2001; Dugdale *et al.*, 1998; Pérez Hernández *et al.*, 1998; Ságі, 1998; Más *et al.*, 1999; Schenk *et al.*, 1999; Becker *et al.*, 2000; Dugdale *et al.*, 2001; Schenk *et al.*, 2001
CaMV35S [a]	Cauliflower Mosaic Virus	Más *et al.*, 2000
35S-AMV	Cauliflower Mosaic Virus Alfalfa Mosaic Virus	Ságі *et al.*, 1995c
35S-35S [b]	Cauliflower Mosaic Virus	Moy *et al.*, 1998; Moy *et al.*, 1999; Schenk *et al.*, 2001; Remy *et al.*, 1998b; Ságі *et al.*, 1995a; Ságі *et al.*, 1995b; Ságі *et al.*, 1995c
35S-35S-AMV	Cauliflower Mosaic Virus Alfalfa Mosaic Virus	Ságі *et al.*, 1994; Ságі *et al.*, 1995a; Ságі *et al.*, 1995b; Ságі *et al.*, 1995c; Ságі *et al.*, 1998a
Emu [c]	Recombinant ARE-*ocs-adh1*	Ságі *et al.*, 1995a; Ságі *et al.*, 1995b; Ságі *et al.*, 1998a; Remy, 2000
Act-1	Rice actin gene *act1D*	May *et al.* 1995; Ságі *et al.*, 1998a
Act-1	Banana actin gene	Hermann *et al.*, 2001b
Ubi	Maize ubiquitin	Grapin, 1995; Ságі *et al.*, 1995a; Grapin *et al.*, 1996; CIRAD, 2001; Ságі *et al.*, 1998a; Dugdale *et al.*, 1998; Moy *et al.*, 1998; Remy *et al.*, 1998b; Moy *et al.*, 1999; Schenk *et al.*, 1999; Becker *et al.*, 2000; Pérez Hernández, 2000; Ganapathi *et al.*, 2001; Schenk *et al.*, 2001
$(ocs)_3$mas [d]	Recombinant *ocs-mas*	Remy *et al.*, 1998b; Moy *et al.*, 1998; Moy *et al.*, 1999; Remy, 2000; Ganapathi *et al.*, 2001
Sc	Sugarcane bacilliform badnavirus (ScBV)	Schenk *et al.*, 1999
Badnavirus My Cv Go	Banana streak badnavirus My from Mysore cv. BSV Cv from Cavendich cv. BSV Go from Goldfinger cv. BSV	Ságі, 1998a; Schenk *et al.*, 2001; Remans *et al.* 2000
BBTV DNA 1 to 6 S1 S2	Banana Bunchy Top Virus	Dugdale *et al.*, 1998; Becker *et al.*, 2000; Hermann *et al.*, 2001a
BT1, BT2, BT3, BT4, BT5 [e]		Dugdale *et al.*, 2000
BT6.1 [f]		Dugdale *et al.*, 2001

[a] OCS enhancer plus Cauliflower Mosaic Virus promoter plus rice *act1* untranslated sequence
[b] Enhanced Cauliflower Mosaic Virus promoter
[c] Six copies of the 41-bp ARE (anaerobic responsive element) plus four copies of the 40-bp *ocs* (octopine synthase) enhancer plus the 5' end of a truncated *adh1* promoter linked to its first intron
[d] A tandem of three upstream activating sequences (UAS) of the octopine synthase gene (*ocs*) and a promoter/activator region of the mannopine synthase gene (*mas*)
[e] Banana Bunchy Top Virus DNA 1-5 intergenic regions plus maize ubiquitin (*ubi1*) intron
[f] Banana Bunchy Top Virus DNA-6 intergenic region plus intron mediated enhancement of maize *ubi1*, maize *adh1*, rice *act1* and sugarcane *rbcs* genes

Transformation for resistance to leaf spot diseases

Plants have developed a range of defense mechanisms against pathogens such as, the rapid death of the first infected cells (Colligne and Slusarenko, 1987) which prevents further pathogen spread. Other defense mechanisms include increased

lignification of the cell wall (Vance *et al.,* 1980), the synthesis of phytoalexines (Hahlbrock and Scheel, 1989) and the production of reactive oxygen species (Mehdy, 1994). The biochemical complexity of these mechanisms makes it difficult to develop molecular methods to breed for fungus resistance.

Of the large number of genes activated upon pathogen recognition by the plant is a group that encodes for pathogenesis-related (PR) proteins (Van Loon *et al.,* 1987; Linthorst, 1991). PR proteins have been defined as proteins encoded by the host plant but induced only under pathological or related stress conditions (Antoniw *et al.,* 1980). To date, more than 10 families of PR proteins have been classified, which include chitinases. In addition, plants express numerous other genes that encode for proteins with antimicrobial properties (Broekaert *et al.,* 1997; García-Olmedo *et al.,* 1998). These are small, stable and cysteine-rich peptides isolated from seeds of diverse plant species (Broekaert *et al.,* 1992; Cammue *et al.,* 1992; Terras *et al.,* 1992a, b; Cammue *et al.,* 1995; Osborn *et al.,* 1995). These plant defensins, or antifungal peptides, are highly active against a broad spectrum of phytopathogenic fungi. For example, two of these proteins (*Rs*-AFP1 and *Rs*-AFP2) have been isolated from radish (*Raphanus sativus*) seeds. The latter appears to be the most potent with IC_{50} values ranging from 0.4 to 25 µg/mL (Terras *et al.,* 1992a, b). Several of these antifungal peptide genes were shown to inhibit the growth of *Mycosphaerella* spp. under *in vitro* conditions (Cammue *et al.,* 1993) but were not toxic to human fibroblasts and erythrocytes (Terras *et al.,* 1992a, b; Cammue *et al.,* 1995) or to banana cells (Cammue *et al.,* 1993). We therefore focused our work on the insertion of these antifungal or antimicrobial proteins in banana with a different mode of action. Morphogenic defensins, like *Rs*-AFP2 from *Raphanus sativus,* cause a reduction in hyphal elongation and an increase in hyphal branching, whereas non-morphogenic ones, such as *Dm*-AMP1 from *Dahlia merckii* (Osborn *et al.* 1995), slow down hyphal elongation without a visible morphological effect.

Since it was demonstrated in tobacco that disease resistance can be increased by simultaneously integrating different antifungal proteins (Jach 1995), the frequency of co-transformations with particle bombardment was evaluated in three independent experiments (Remy *et al.* 1998a). In experiment 1, the selectable marker gene (gene A) and an antifungal peptide gene (gene B) were introduced into embryogenic cells of the plantain cultivar 'Three hand planty' in a linked position, *i.e.* the two genes were present on the same plasmid. In experiments 2 and 3, the plasmid with genes A and B were co-transformed with another plasmid that carried a different antifungal protein gene (gene C) which was thus not linked to genes A or B. Transgenic shoots were then regenerated from all three experiments and analysed by PCR for the presence of each foreign gene. Integration of these genes was also confirmed by Southern gel blot hybridisation in a number of selected plants from each experiment.

The number of plants carrying both gene A and gene B or C was used to calculate the co-transformation frequencies of linked genes and according to the following equation:

$\{\text{No. of (A+B or C)}^{+} / \text{No. of A}^{+}\} \times 100$

As one can expect, the linked genes co-existed in the transgenic plants at a high frequency that ranged between 90 to 100% in the different experiments. Similarly,

as expected, the unlinked gene showed a lower co-transformation frequency than the linked genes. However, this frequency was still remarkably high, in the range of 70 to 80%, probably due to efficient co-precipitation of the two plasmids on microparticles. Similarly to the results obtained by Hadi *et al*. (1996), this observation indicates that simultaneous bombardment of different plasmid molecules may be a convenient way for the introduction of multiple genes into crop plants.

Co-transformation frequency with *Agrobacterium* was also estimated with one of the several possible methods. Two *Agrobacterium* strains were used to introduce two reporter genes (*gfp* and *luc*) into bananas. The elegance of this experimental setup is the simultaneous and live observation of gene expression by imaging. This way, co-transformation frequencies were directly measured by co-expression frequency. As was expected, co-transformation frequency was relatively low because of the low probability that two bacterial cells will deliver their T-DNA molecules to the same plant cell. In our case, the average frequency of *gfp* and *luc* co-transformation in four cultivars was around 3% after three weeks and 4% two months after transformation (Ahmed, unpublished data).

The expression of antimicrobial peptides in transgenic bananas was analysed by ELISA using specific antibodies. Out of more than 150 single transformants, i.e. transgenic plantains expressing only one antimicrobial peptide, more than 10% had a relatively high concentration (between 0.05-0.12% of total soluble protein) in the leaf. In contrast, out of 16 double transformants, i.e. plants expressing a *Dm*-AMP1 and another antimicrobial peptide from onions (*Ac*-AMP1), 6 (37%) accumulated one or both peptides to at least four times the background level (Remy, 2000).

In order to assess tolerance to fungus in transgenic lines, a simple, sensitive and reproducible leaf disc bioassay has been developed (Remy, 2000). A 5-cm leaf disc was excised from transgenic plants grown in a greenhouse and inoculated *in situ* with fungi. Four days after infection, a differential disease response was observed between independent transformants, whereas a non-pathogenic fungus (e.g. *Fusarium* sp.) was unable to induce disease symptoms, indicating that the assay is specific for host-pathogen interactions. Transformations with different promoter-gene constructs resulted in a wide range of tolerance to fungus among the independent transgenic plants. Computer image capturing and software-based area calculation has been used to precisely measure the area of infected leaf and to classify independent transformants according to their tolerance. This procedure was used to screen 42 independent transgenic plantain lines expressing *Ac*-AMP1 (Pérez Hernández, 2000), among which 6 lines had 2 to 3 times less necrosis upon infection with *Colletotrichum musae* than the untransformed controls.

Towards an improved transformation technology

Efficient transformation techniques exist in banana but they rely on labour-intensive cell suspension technology. Moreover, controlled transgene expression needs to be developed for banana relying, among others, on native or heterologous developmental and tissue specific promoters and especially pathogen-inducible ones. This is needed because constitutive resistance leads to a decrease in fitness (Heil and

Baldwin, 2002). However, relatively few promoters are available for the regulated control of target gene expression.

Meristem transformation

Existing transformation technologies rely on cell suspensions, an elaborate, expensive and time-consuming process. A transformation procedure based instead on tissue culture of cell cultures would be more efficient and less dependent on the genotype. To this end, more than 12 000 explants of 'Grande naine' and 'Williams' were infected with *Agrobacterium* with or without sonication. Selection was performed either on a liquid or solid medium using different growth regulators. Independently of treatment, the frequency of putative transgenic cultures was about 0.5% in both cultivars after 2 to 3 months. This consistently low transformation frequency indicates that proliferating meristematic cultures may not be the ideal target for genetic transformation. This could be attributed to the heterogenous structure of the meristem containing few embryogenic cells. Meristem transformation may produce chimeras that are not easily identified or dissociated (Roux *et al.,* 2001). In addition, based on DNA behaviour in mouse embryonic stem cells (Lei *et al.,* 1996), embryogenic plant tissues in their early development may be more suitable for gene targeting than tissues that underwent several divisions and differentiation (Kumar and Fladung, 2001). However, given the little effort directed to the optimization of meristem transformation in banana, this avenue should be further explored.

Positive selectable marker genes

Once foreign genes are delivered to banana suspensions, transformed cells need to be harvested. With PMT, about 1 to 7 cells per 100 µl of a 33% settled cell volume suspension (± 25 mg fresh weight cells) are transformed, and up to 100 transgenic cells from twice the same volume with AMT. Because of this very low transformation frequency, selectable marker genes, e.g. the neomycin phosphotransferase gene (Fraley *et al.*, 1983) which confers resistance to aminoglycoside-type antibiotics such as kanamycin, neomycin and G-418 (geneticin), are used. Occasionally herbicide resistance genes are also used. These are negative selection systems. In banana, antibiotic resistance genes should pose no concern to the environment because there is no pollen in edible bananas, yet there is concern that such genes in genetically modified food organisms pose a hazard to human health (Fuchs *et al.,* 1992). Research is conducted to completely remove selectable marker genes (Puchta 2000).

In positive selection systems, transformed cells can convert a physiologically inert substance into a compound that stimulates growth. Hence, transgenic cells overgrow non-transformed cells that are starved rather than killed, e.g. the *gusA* (ß-glucuronidase) gene from *Escherichia coli* that hydrolyzes benzyladenine-glucuronide (Okkels *et al.* 1997) into active cytokinin and thus stimulates growth of transgenic cells. Other examples include the use of phosphomannose isomerase (PMI, or mannose-6-phosphate isomerase), an enzyme catalyzing the reversible isomerization of mannose-6-phosphate to fructose-6-phosphate, which serves as a precursor for the glycolytic pathway, and xylose isomerase (or D-xylose ketol-

isomerase) that interconverts D-xylose to D-xylulose, which after phosphorylation by xylulokinase enters the pentose phosphate pathway. The former enzyme is used to give transgenic cells a growth advantage over non-transgenic ones because most plant species cannot metabolize mannose (or mannose-6-phosphate) and in plant tissue cultures mannose has been known to be unable to support growth (Malca *et al.,* 1967). The *manA* gene of *E. coli* (Miles and Guest, 1984), which codes for PMI, has been successfully used in transformation experiments with cassava, maize, rice, sugar beet and wheat (see Suprasanna *et al.,* 2002 for references). No adverse nutritional effects were found (Reed *et al.,* 2001). Xylose, like mannose, cannot be metabolized by plant cells, whereas they can utilize D-xylulose because they express xylulokinase. So far, transformation with the xylose isomerase gene (*xylA*) (Wong *et al.,* 1991; Lee *et al.,* 1990) as a selectable marker was demonstrated in potato, tobacco and tomato (Haldrup *et al.,* 1998a, b). This enzyme is widely recognized as safe, since it is commercially used in the starch industry and for food processing.

The use of positive selectable markers genes has the additional advantage that it can increase the transformation frequency dramatically because no toxic substances are released from dying cells. Positive selection systems have proven their value in several crops (sugar beet, cassava, maize, wheat and rice). For an overview the reader is referred to Suprasanna *et al.* (2002). These novel selectable marker systems are being tested in banana in order to increase the transformation frequency but also to allow repeated transformation operations, and minimize the use of antibiotic and herbicide selectable marker genes.

Tagging, isolation and characterization of novel promoters and genes

The isolation of promoters of differentially expressed or inducible genes can be accomplished indirectly or directly. In the first approach, the promoter is isolated in parallel or subsequently to the characterization of a gene of interest by molecular techniques. However, when dealing with multigene families and pseudogenes or with genes that are developmentally regulated or exert a cell-specific pattern of expression this approach is likely to be extremely difficult.

Promoters can also be identified directly within the genome via tagging by transformation with a promoterless reporter gene and screening for individual transformants, in which reporter gene expression is activated. After plasmid rescue of the respective region from the genome or via direct genome walking (e.g. by inverse PCR or various anchored PCR techniques), the promoter region is isolated and sequenced. Via a combined screening of a population of transgenic plants for different parameters (e.g. abiotic and biotic stress factors, development and tissue-specific expression), several promoters for various genes can simultaneously be identified and thoroughly characterized without the *a priori* isolation and analysis of the corresponding coding sequence(s).

At KULeuven, several thousands of transgenic cultures can be produced in a relatively short time by using *Agrobacterium*-mediated transformation of embryogenic cell suspension cultures. Such a large number of transgenic cultures provides a reasonable chance of tagging interesting promoters. At present, the target

cultivars are 'Grande naine' and 'Three hand planty'. The tagging construct contains a promoterless luciferase gene linked to a T-DNA border and, so far, approximately 2500 and 4000 transgenic cultures respectively have been selected after transformation. The expression of the luciferase gene (*luc*) (De Wet *et al.,* 1985) is not destructive and therefore provides the opportunity for continuous detection of reporter gene activity in plants using low-light imaging techniques (Ow *et al.,* 1986; Millar *et al.,* 1992; Chia *et al.,* 1994). Screening for activated luciferase expression has been carried out under a liquid nitrogen-cooled CCD camera coupled to a sophisticated image capture and analysis system (Remy *et al.,* in press). A liquid nitrogen cooling system is used since it reduces the dark current to less than 1 electron per pixel per hour allowing long exposures of up to tens of minutes. LUC activity could be detected 80 minutes after bombardment and was clearly visible 40 minutes later with the codon-modified *luc+*gene (Sherf and Wood, 1994). The *luc+*gene showed a much higher and faster LUC activity than the wild type *luc* gene (Remy *et al.,* in press).

As a wide range of LUC activities was detected (from less than 5 to more than 300 relative grey levels/pixel), it is clear that this simple, fast and sensitive *in vivo* reporter gene assay can become a valuable tool in gene expression studies of bananas. In parallel with the multiplication of the tagged population, preliminary screenings have been performed for constitutive activation and for promoters inducible by temperature shock, salt stress and herbicide treatment. The frequency of cultures with detectable (constitutive) activation has been around 10% in different tagging experiments with 'Three hand planty' (Figures 7A and B). On the other hand, as expected, the frequency of inducible activation by specific conditions has so far been well below 1% (Figure 7C and D for salt-induced activation).

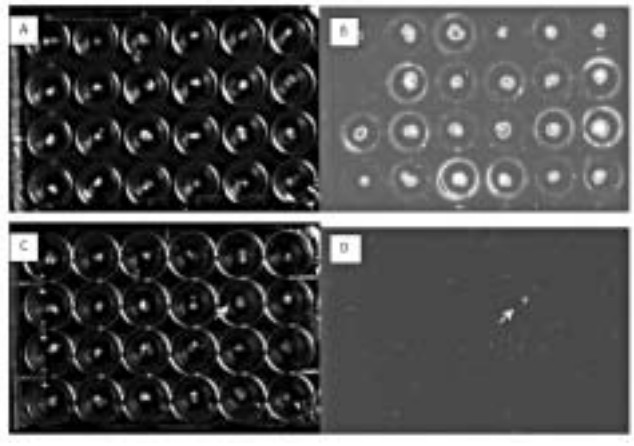

Figure 7.
Luciferase imaging of transgenic cultures of the plantain cultivar 'Three hand planty'.
A) Light image of a 24-well plate with cultures transformed with a 35S-luciferase construct for constitutive expression.
B) The same cultures screened for luciferase expression.
C) Light image of a 24-well plate with cultures transformed with a promoterless luciferase construct for tagging.
D) The same cultures screened for luciferase activation after salt stress (arrow indicates a positive culture).

In future, selected plants will be analysed by southern hybridization, as well as via a novel anchored PCR technique, to screen for individuals containing single insertions. Then, TAIL-PCR will be used to recover and clone the plant DNA flanking the luciferase gene. The cloned fragments will be sequenced, compared to sequence databases and analysed with standard bioinformatic tools for conserved regions and

putative transcription factor binding sites. Based on the sequence obtained, internal fragments of each promoter candidate will be hybridized to DNA purified from the original transformant as well as an untransformed control to confirm their genuine nature. Finally, for functional testing promoter-*gfp* fusions will be re-introduced to banana to confirm the constitutive or wound and pathogen pattern of expression as well the tissue specificity in an ectopic situation.

Confirmed promoters will then be utilized for disease control, for example with antifungal genes, and associated genes isolated and characterized to understand their role in plant-pathogen interactions. Except for promoter tagging of genes related to nematode feeding structures (Bartels *et al.*, 1997; Puzio *et al.*, 1999), this method has not been applied to studies on plant-pathogen interactions. Since salicylic acid (SA) and its analogs play an important role in the defense response of many plant species to pathogen attack, promotorless *luc* tagging in banana would be useful to unravel upregulated genes that are involved in widely different metabolic pathways including pathogen defense. SA treatment mimics osmotic and oxidative stress, mediates the oxidative burst that leads to cell death in the hypersensitive response, and acts as a signal for the development of systemic acquired resistance (Shirasu *et al.,* 1997).

Resistance genes are often proposed to control pathogen attack. Based on a "guard model" it is proposed that *R* genes, which are generated randomly, most likely through a birth-and-death process (Michelmore and Meyers, 1998), stand a better chance to induce resistance if identified from the plant family of which a certain cultivar needs to acquire additional resistance (Van der Hoorn *et al.,* 2002). Resistance genes from banana are currently unavailable for banana transformation although techniques are available and some sources do exist. A series of resistance gene analogs (RGAs) were isolated, using degenerate PCR primers targeting highly conserved regions in proven plant resistance genes (e.g. leucine-rich repeat sequences) (Wiame *et al.,* 2000). For an overview of the current situation and a proposed strategy to correct this situation, the reader is referred to Kahl (in press). In any case, there is a need not to focus only on a few resistance genes but on the simultaneous detection, identification and quantification of all transcripts at a given time and monitoring of gene expression patterns at various developmental stages or after specific treatments (Matsumara *et al.*, 1999) correlated to physiological or developmental processes. Much is expected from The Global *Musa* Genomics Consortium.

Increased expression

The search in badnaviruses for useful promoters to drive transgene expression in banana (Schenk *et al.,* 1999) resulted in two novel DNA fragments of 2105 bp (My) and 1322 bp (Cv) amplified from the upstream region of the coding sequence of two Australian banana streak badnavirus (BSV) isolates. Evaluation of the My and Cv promoters in transgenic banana demonstrated that these promoters could drive high-level expression of either the *gusA* or the *gfp* reporter gene in different tissues during vegetative development. For instance, *gus* activity in transgenic *in vitro* plants of the plantain 'Three hand planty' containing the My promoter were up to seven

times higher in leaf tissue and up to four times stronger in root and corm tissue compared to plants harbouring the maize ubiquitin promoter (Schenk *et al.,* 2001). The Cv promoter showed activities that were similar to the maize ubiquitin promoter in transgenic *in vitro* plants, but was significantly reduced in larger glasshouse-grown plants.

Enhancement of transgene expression levels by translational fusion of transgenes has been observed by different research groups. In an experiment, the sequence coding for a naturally occurring plant linker peptide was used to connect the sequence coding for two antimicrobial proteins (AMPs) in a polyprotein construct that was transformed to *Arabidopsis thaliana* (François *et al.,* 2002a, 2002b). The linker peptides used were based on the fourth linker peptide of the *Ib*AMP polyprotein precursor isolated from the seed of *Impatiens balsamina* (Tailor *et al.,* 1997). The heterologous polyprotein precursors were demonstrated to be cleaved post-translationally in *A. thaliana* thereby releasing the two AMPs (François *et al.,* 2002a, 2002b). Cleavage appeared to be complete as no immunoreactive polyprotein precursor could be detected in the transformed *A. thaliana* plants. A striking observation from the experiments was that the expression levels of the first protein were several times higher in plants transformed with the polyprotein constructs compared to plants transformed with the single protein construct. Expression levels as high as 3.1% of total protein content, as seen in some lines transformed with polyprotein constructs, have so far never been reported in literature for the nuclear expression of a transgene in leaves of transgenic plants.

In another experiment, enhanced expression of the gene coding for the antimicrobial peptide sarcotoxin IA was studied by fusing translationally the coding sequence of this gene to that of *E. coli* b-glucuronidase (GUS) (Okamoto *et al.*, 1998). Western blot analysis of transgenic tobacco plants demonstrated that the amounts of sarcotoxin IA present in the form of sarcotoxin IA-GUS fusion proteins were considerably higher than in tobacco plants transformed with the single sarcotoxin IA peptide construct.

It is assumed that a high transcription of genes coding for proteins that control fungi under *in vitro* conditions will increase resistance in plants. One of these strategies relies on strong promoters. However, much higher transgene expression levels can be achieved with chloroplast genetic engineering (Daniell *et al.,* 2002) because chloroplasts are polyploid. Thousands of copies of foreign genes per plant cell will generate extraordinarily high levels of foreign protein. Consequently, chloroplast transgenic plants can show a 25-fold increase in the accumulation of foreign gene products than nuclear transgenic plants (Lee *et al*., in press; Daniell *et al*. 2002). In tobacco this resulted in the accumulation of 45.3% foreign protein of total soluble protein (De Cosa *et al.,* 2001).

In related experiments, 21.5% of total soluble protein was demonstrated to be foreign and resulted in the protection against a fungal pathogen (DeGray *et al.,* 2001). Sidorov *et al*. (1999) and Ruf *et al*. (2001) achieved expression levels up to 5% and 50%, respectively. Moreover, in contrast to nuclear transformation where the integration of a transgene is random and in unpredictable numbers, chloroplast transformation facilitates the controlled integration in a pre-determined site, thereby influencing the expression of the transgene (Kumar and Fladung, 2001).

Field testing

The genetic modification of many tropical and subtropical crops offers the prospect of faster plant improvement (Ortiz, 1998; Sharma *et al.,* 2000). Banana may be next. Since 1994, putative fungal resistant transgenic plantains have been obtained at KULeuven and analysed in partnership with scientists from Cuba, Ecuador, India and Uganda. The non-toxicity of the expressed proteins in fruits, and in feeding tests with rats, suggests that these plants should be field-tested in the tropics for confirmation of resistance and biosafety evaluation. Contained fields and nurseries have been put in place, but the LMOs (living modified organism) have not been exported due to the absence of competent national authorities to approve the request for import and risk assessment studies (Sági *et al.,* 1998b).

The absence of a regulatory framework in most tropical developing countries is delaying the evaluation of LMO bananas and plantains and the cultivation of resistant plantains by smallholders. This is occurring despite the ratification of the Cartagena Protocol (Cartagena Protocol, 2000) and article 19(3) of the Convention on Biological Diversity (CBD) (Convention on Biological Diversity, 1994). The objective of the Cartagena Protocol (adopted in January 2000) is to ensure an adequate level of protection in the field for the safe transfer, handling and use of LMOs resulting from modern biotechnology that may have adverse effects on the conservation and sustainable use of biological diversity, taking also into account risks to human health, and specifically focusing on transboundary movements.

Currently, public opinion is influenced by feelings about "Frankenstein food" and by the "precautionary principle". The former calls for more scientific data whereas the latter should allow for approvals for field-testing. Indeed, the "precautionary principle" should not be used to stop field-testing but to guide scientists under what circumstances field-testing should be conducted. Besides, scientific data from the field can be used to further improve current requirements. Edible bananas are particularly suitable since they are both seed and pollen sterile. Thus, the introduced gene(s) remain confined to the transformed plant. Banana LMO plants should have been among the first plants to be tested in developing countries.

The development and field release of transgenic plants have been much debated. It is clear that the deployment of transgenic plants should be safe (Custers, 2001). Therefore, ecological risk assessment studies need to be conducted on matters dealing with the invasiveness of the transgenic crop (can it become a weed in the natural habitat?), on the invasiveness of the transgene itself (gene flow into wild relatives) and the environmental side effects of the transgenic products (on non-target organisms, for example) (Amman, 2001). To avoid any type of invasiveness, research is conducted to introduce "reproductive isolation barriers" into crop plants, the biosafety of transgenic crops being one of the driving forces. Examples are male sterility (there are no viable pollen, hence no outcrossing) and the terminator technology (seeds cannot germinate without chemical application). Complementary strategies rely on the cultivation of sexually incompatible crops and respecting isolation barriers, i.e. crops that can intercross are separated by a crop that cannot intercross (Obrycki *et al.,* 2001). The industry and environmentalists favour reproductive isolation barriers, but the strategy would seriously handicap future

breeding as it would require that an interesting gene be inserted in each cultivar separately, which is very cumbersome and costly. However, a transgenic plant of interest should become part of a conventional breeding programme and used for further crossing (Dodds *et al.,* 2001). In the case of banana, this means that diploids should also be transformed for use in the current breeding programmes.

Conclusion

The predicted increase of the world's population in the coming years poses many challenges to developing countries to feed their population as more than 90% of the population increase will occur there. But since agricultural productivity is currently low, there are many opportunities to improve that situation. Technical solutions, such as biotechnology, are not the only solution but form part of a package to be used in synergy with an agro-ecological approach. The technology being in the plant material, biotechnology ensures benefits to smallholders without changing local cultural practices, as long as the appropriate features are considered. The 2001 United Nations Human Development Report unequivocally states that biotechnology offers "the hope of crops with higher yields, pest- and drought-resistant properties and superior nutritional characteristics - especially for farmers in ecological zones left behind by the green revolution" (UNDP, 2001).

Many opponents raise ethical questions but blocking the development and application of biotechnology can also be construed as unethical. Zero risk does not exist. The important point is that biotechnology poses risks that are equal to the risks encountered in conventional breeding (NRC, 2000). "A process that is safer shouldn't be given up because it cannot be elevated to an impossible standard of absolute safety" (Trewavas, 2000). In the end, what counts is zero harm.

Breeding has long been used to suppress plant diseases and pathogen-resistant cultivars quickly became popular and grown as homogeneous crops. Pathogens can eventually overcome resistance and become epidemic, forcing breeders to introduce a cultivar with a new resistance trait. The battle never ends as pathogens always try to circumvent recognition by resistant plants.

To protect crops better, plant cultivars that differ in their resistance mechanisms should be mixed, as in natural plant populations (Dangl and Jones, 2001). For example, the deployment of different rice cultivars resulted in a 94% reduction in the occurrence of rice blast (Zhu *et al*., 2000). Smallholders would be best served by interplanting into their existing banana plot banana plants resistant to leaf spot diseases consisting of cultivar(s) in which a single or a combination of foreign genes have been integrated. Given that it should be possible target proteins both intra- and intercellularly, a broad-spectrum resistance to banana leaf spot should be achievable.

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Mutagenesis and somaclonal variation to develop new resistance to *Mycosphaerella* leaf spot diseases

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Abstract

Mycosphaerella leaf spot diseases can reduce fruit yield by up to 50%. Chemical strategies exist to combat these diseases, but they are environmentally unsound, hazardous and very expensive for many farmers. The only sustainable means to reduce the use of pesticides is breeding for tolerant cultivars. Whereas breeders are looking for genetic variability to develop new varieties, edible *Musa* cultivars are multiplied vegetatively, polyploid and sterile. Spontaneous somatic mutation has already contributed largely to obtaining new cultivars of *Musa*. Nevertheless, the rate of occurrence is too low to satisfy practical breeding needs. Mutations can be induced by physical or chemical mutagens. With the development of tissue culture techniques, *in vitro* mutagenesis and somaclonal variation raised hopes in the 1980-1990s. In spite of this, very few useful and stable mutants/somaclones were obtained. The multicellular structure of meristems which leads to chimerism is certainly an impeding factor. Additionally, the random process in mutation induction calls for the screening of several thousand plants after treatment. Recently, following a five-year FAO/IAEA/DGIC coordinated research project, it has been possible to overcome these two barriers by mutagenic treatment of embryogenic cell suspensions and by establishing an early mass screening method resting on infiltration of juglone, a toxic metabolite of *Mycosphaerella fijiensis*. After screening approximately 4000 plants, 15 putative mutants showed tolerance to this metabolite. These plants must be evaluated for their resistance to *M. fijiensis* infection under controlled conditions and field experiment.

Resumen - Mutagénesis y variación somaclonal para desarrollar nueva resistencia a las enfermedades de la mancha foliar por *Mycosphaerella*

Las enfermedades de las manchas foliares causadas por *Mycosphaerella* spp. afectan significativamente el cultivo bananero y puede reducir el rendimiento de la fruta en hasta un

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50%. Existen estrategias de control químico para combatir estas enfermedades, pero estas causan daños al ambiente, son peligrosas y muy costosas para muchos agricultores. El único medio sostenible para reducir el uso de plaguicidas es el mejoramiento de los cultivares tolerantes. Los mejoradores que están investigando la variabilidad genética para desarrollar nuevas variedades, toman en cuenta que las variedades comestibles de *Musa* se multiplican vegetativamente, son poliploides y estériles. La mutación somática espontánea es una fuente de variación que ya ha contribuido en gran medida en la obtención de las nuevas variedades en *Musa* spp. Sin embargo, la tasa de ocurrencia es muy baja para satisfacer las necesidades prácticas de mejoramiento. Las mutaciones pueden ser inducidas por mutágenos físicos o químicos. Con el desarrollo de las técnicas del cultivo de tejidos, la mutagénesis y variación somaclonal *in vitro* elevaron las esperanzas en las décadas de los 80 y 90. A pesar de esto, se obtuvieron pocos mutantes o somaclones útiles y estables. La estructura multicelular de los meristemas que conduce al quimerismo es ciertamente un factor de impedimento. Adicionalmente, el proceso aleatorio para inducir la mutación requiere el cribado de varios miles de plantas después del tratamiento. Recientemente, después de realizar un proyecto de investigación de cinco años coordinado por FAO/IAEA/DGCI, fue posible vencer estas dos barreras mediante un tratamiento mutagénico de las suspensiones de células embriogénicas y el establecimiento de un método de cribado masivo temprano que se basa en la infiltración de juglone, un metabolito tóxico de *Mycosphaerella fijiensis*.. Después de realizar el cribado de aproximadamente 4000 plantas, 15 mutantes putativos mostraron tolerancia a este metabolito.

Résumé – Mutagénèse et variation somaclonale pour développer de nouvelles résistances aux maladies foliaires causées par *Mycosphaerella* spp.
Les maladies foliaires causées par *Mycosphaerella* spp. peuvent réduire le rendement de jusqu'à 50%. Des moyens chimiques existent pour lutter contre ces maladies, mais ils sont nocifs pour l'environnement, dangereux et très coûteux pour nombre de fermiers. La seule manière durable de réduire le recours aux insecticides est de créer des cultivars tolérants. Sauf que pour y arriver, les sélectionneurs ont besoin de variabilité génétique et que les cultivars de bananiers sont polyploïdes, stériles et multipliés végétativement. Les mutations somatiques spontanées ont déjà passablement contribuées à l'obtention de nouveaux cultivars, mais leur fréquence est trop faible pour satisfaire les besoins des sélectionneurs. Des agents mutagènes chimiques et physiques peuvent provoquer des mutations. Dans les années 1980-1990, le développement des techniques de culture de tissus, mutagénèse *in vitro* et variation somaclonale ont soulevé bien des espoirs. Malgré cela, peu de mutants/somaclones ont été obtenus. La structure multicellulaire des méristèmes, qui produit des chimères, est sans doute un facteur limitant. De plus, l'induction de mutations est un processus aléatoire qui nécessite le criblage de plusieurs milliers de plants. Récemment, suite à un projet de recherche de cinq ans coordonné par FAO/IAEA/DGIC, il a été possible de contourner ces deux obstacles en faisant subir un traitement mutagène à des suspensions de cellules embryogéniques et en mettant au point une méthode de criblage précoce basée sur l'infiltration de juglone, un métabolite toxique de *Mycosphaerella fijiensis*. Après avoir criblé 4000 plants, 15 mutants présomptifs ont montré une tolérance à ce métabolite. Ces plants doivent être évalués pour leur résistance à *M. fijiensis* sous des conditions contrôlées et en champ.

Introduction

Bananas and plantains (*Musa* spp.) are a staple food of millions of people and rank among the top five food commodities. However, *Mycosphaerella* leaf spot diseases can reduce fruit yield by 50% (Mourichon *et al.*, 1997). Chemical control stategies exist to combat these diseases, but they are environmentally unsound, hazardous and very expensive for many farmers (Persley and George, 1999). Breeding of resistant

cultivars is the only sustainable mean to reduce the use of pesticides. But edible *Musa* are difficult to breed since they are polyploid and sterile.

Spontaneous mutations are at the origin of almost all of the edible banana and plantain cultivars (Buddenhagen, 1987). The best example is the spontaneous banana mutant 'Cavendish' from Vietnam, which is resistant to Fusarium wilt (race 1) and which replaced 'Gros Michel' in the 1950s and 1960s (Ploetz, 1994). The discovery of this banana mutant saved the banana industry from collapsing but as a consequence, the export trade relies on a very narrow genetic base since only one or two triploid cultivars of the subgroup Cavendish dominate the export market (Risède and Tézenas du Montcel, 1997).

Banana is probably the best example in the history of agriculture of the pathological perils of monoclone culture. Indeed, without clonal diversification, the trade can hardly be expected to survive indefinitely and the generation and use of genetic variability may be the only remaining option. Spontaneous somatic mutation has already contributed largely in obtaining new cultivars in *Musa* spp. Nevertheless, the rate of occurrence is too low to satisfy practical breeding needs. Mutations can be induced by tissue culture (somaclonal variation) and/or by physical or chemical mutagens (induced mutants). With the development of tissue culture techniques, *in vitro* mutagenesis and somaclonal variation raised hopes in the 1980 and 1990s. So far, however, very few useful and stable mutants/somaclones have been obtained. The multicellular structure of meristems, which leads to chimerism is certainly an impeding factor. Additionally, the random process of mutation induction calls for the screening of several thousand plants after treatment. Recently, following a five-year FAO/IAEA/DGIC coordinated research project, it has been possible to overcome these two barriers. This paper presents the improved methodology and the potential use of mutants in genetic improvement programmes.

Advantages and limitations of induced mutation and somaclonal variation

Somaclonal variants

In vitro propagated plants are not necessarily true to type. Off-type plants might differ permanently (i.e. somaclonal variation) or temporarily from the source plant as a result of an epigenetic or physiological effect. The term 'somaclonal variation' was introduced to describe the genetic variation in plants regenerated from any form of cell culture. Larkin and Scowcroft (1981) advocate the view that somaclonal variation represents a new source of variability and therefore constitutes a powerful tool to the breeder. Nevertheless somaclonal variation from micropropagated banana and plantain should not be overestimated as a source of novel variability for use in genetic improvement (Vuylsteke *et al.*, 1991). A narrow spectrum of variants has been obtained through somaclonal variation. It is becoming increasingly clear that somaclonal variation is usually undesirable (Vuylsteke *et al.,* 1996).

Some off-types have improved agronomical traits, such as the higher yield of the 'French reversion' variant plantain and the short stature of dwarfs (Vuylsteke *et al.*, 1996). Regarding disease resistance, a 'Cavendish' banana was recovered in Taiwan

for its tolerance to Fusarium wilt (Hwang *et al.*, 1993). More recently Trujillo *et al.* (1996) reported the selection of a somaclonal variant CIEN BTA-03 tolerant to Sigatoka disease. But cytogenetic and molecular characterization revealed that CIEN BTA-03 was in fact a tetraploid clone and was not part of the Cavendish subgroup to which the parental 'Williams' (AAA) belongs (Gimenez *et al.*, 2001).

Induced mutants

The *Musa* mutation induction system based on *in vitro* techniques to recover mutant plants and micropropagate desirable mutants was developed by Novák *et al.* (1990) in the FAO/IAEA Laboratories. It is now applied in a few *Musa* breeding programmes. Gamma irradiation is the main physical mutagen used to induce genetic variation. More recently, Roux (1997) standardized the methodology to provide guidelines to mutation induction programmes in *Musa* spp. Shoot-tips excised from clones representing the different genomic constitutions of the genus *Musa* were treated with 10 doses from 10 to 100 Gy of a ^{60}Co gamma irradiation source at a dose rate of 44 Gy/min. For each *Musa* accession, 200 explants were treated for sensitivity testing and 20 non-irradiated explants were used as control. Radiation sensitivity and post-irradiation recovery were assessed by measuring the survival rate, the propagation rate, the shoot height and the fresh weight.

The different *Musa* accessions showed different responses according to their ploidy level and genomic constitution. The following ranges of doses are recommended:

- 10 to 20 Gy of gamma irradiation for diploid clones 'Calcutta 4' (AA) and 'Tani' (BB);
- 30 to 40 Gy of gamma irradiation for the triploids 'Three hand planty' (AAB), 'Grande naine' (AAA), 'Williams' and 'Kamaramasenge' (triploid, formerly classified as AB);
- 40 to 50 Gy of gamma irradiation for the triploid 'Cachaco' (ABB).

From the FAO/IAEA mutant varieties database, two banana accessions were registered as improved mutant varieties: 'Novaria' for early flowering and 'Klue hom thong KU1' for its bunch size and cylindrical shape from which larger bananas can be selected.

Other desirable variants/putative mutants have been identified for release or further confirmation trials. Examples are shown in Table 1.

Most of the improved characteristics are agronomic features. Disease resistance seems to be difficult to obtain through mutation induction techniques. Consequently, Smith *et al.* (1995) used an original strategy. Instead of irradiating an agronomically superior but susceptible genotype, they irradiated 'Dwarf parfitt', an extra dwarf Cavendish banana that has shown a high level of resistance to race 4 of Fusarium wilt. Following radiation, 35 M_1V_3 (M: Mutagenic treatment; V: vegetative generation) out of 500 explants irradiated at 20 Gy were recovered that possessed improved agronomic characteristics (taller plant size, increased yield and no choking). Most importantly these selections appeared to retain the resistance to race 4 derived from the mother plant 'Dwarf parfitt'.

Bhagwat and Duncan (1998) used gamma irradiation (8 to 20 Gy) and chemical mutagens to make 'Highgate' (AAA) tolerant to *Fusarium oxysporum* f.sp. *cubense*. Twelve weeks after inoculation in the greenhouse, 0.3 to 0.9% of the regenerated plants derived from irradiated explants and 1.9 to 6.1% of chemically treated explants had less than 10% vascular invasion in their corms with no external symptoms. These plants were considered tolerant to the fungus and were multiplied, *ex vitro*, for field screening.

Table 1. List of putative mutants obtained.

Country	Parent clone	Selected clone	Selected traits	Technique	Place of induction
Cuba	SH-3436	SH-3436-L9	Reduced height	Gamma rays	Cuba
	Parecido al Rey	6.44	Reduced height	Gamma rays	IAEA
Malaysia	Pisang rastali	Mutiara	FOC* tolerance	Somaclonal variation	Malaysia
	Novaria		FOC tolerance	Somaclonal variation	Malaysia
Philippines	Lakatan	LK-40	Reduced height	Gamma rays	IAEA
	Latundan	LT-3	Larger fruit size	Gamma rays	IAEA
Sri Lanka	Embul	Embul-35 Gy	Earliness	Gamma rays	Sri Lanka
Austria (IAEA**)	Grande naine	GN35-I to GN35-VIII	Tolerance to toxin from *Mycosphaerella fijiensis*	Gamma rays	IAEA

* FOC: *Fusarium oxysporum* f.sp. *cubense*
**IAEA: International Atomic Energy Agency.

Even though the traditional shoot tip mutation induction technique has permitted to obtain useful mutants, the following limitations are impeding its wider use:

- The treatment of shoot-tips with mutagenic agents (physical or chemical) results in a high degree of chimerism. This is a serious obstacle to mutation techniques since it is not yet possible to distinguish mutated cells from none mutated cells.
- Since mutation induction is a random process, efficiency requires the need to treat and screen as many plants as possible. However, a bottleneck occurs due to the time spent on field screening. The current methods of field screening are also site-specific and involve considerable resources: large numbers of technicians, hours of work, fertilizer, logistic support and high cost.

Recent technical achievements

Origin of embryogenic cell suspensions

In order to screen efficiently for characters such as disease resistance, an efficient method to overcome chimerism after mutagenic treatment is needed (Roux *et al.*, 2001). Considering this, somatic embryogenesis is the most promising method since somatic embryos are assumed to be of single cell origin (Halperin, 1966). In some species, histological studies confirmed the single cell origin of somatic embryos. They develop either directly from an explant or as secondary embryos at the surface of older somatic embryos (Litz and Gray, 1992). Grapin *et al.* (1998) stipulated from cytological studies on somatic embryo ontogenesis in *Musa* that a unicellular origin was more than likely. However such studies can only be performed on few somatic

embryos and thus the extrapolation of these findings to a large number of somatic embryos is risky.

Colchicine treatment and ploidy analysis with flow cytometry, used by Roux *et al.* (2001) to monitor the efficiency of 3 different micropropagation techniques in dissociating chimeras, was applied to verify the unicellular origin of somatic embryos from ECS. After treating cell suspensions with colchicine (Figure 1), the embryos were subsequently transferred on a regeneration medium in test tubes. As soon as green plantlets with shoot and roots were obtained, leaves pieces of 0.5 cm^2 were excised and their ploidy measured through flow cytometry before acclimatization (Table 2). The majority of the regenerated plants were triploid. Among the treated cells, the proportion of regenerated hexaploid plants (5.3%) remained very low compared to triploid plants. We think that triploid cells have a comparative advantage over induced hexaploid cells during culture. In contrast to shoot-tip cultures that were treated with colchicine (Roux *et al.*, 2001), no mixoploids among the regenerated plants were observed, which confirms the single cell origin of embryos. Thus embryogenic cell suspensions seem to be the material of choice for mutagenic treatments.

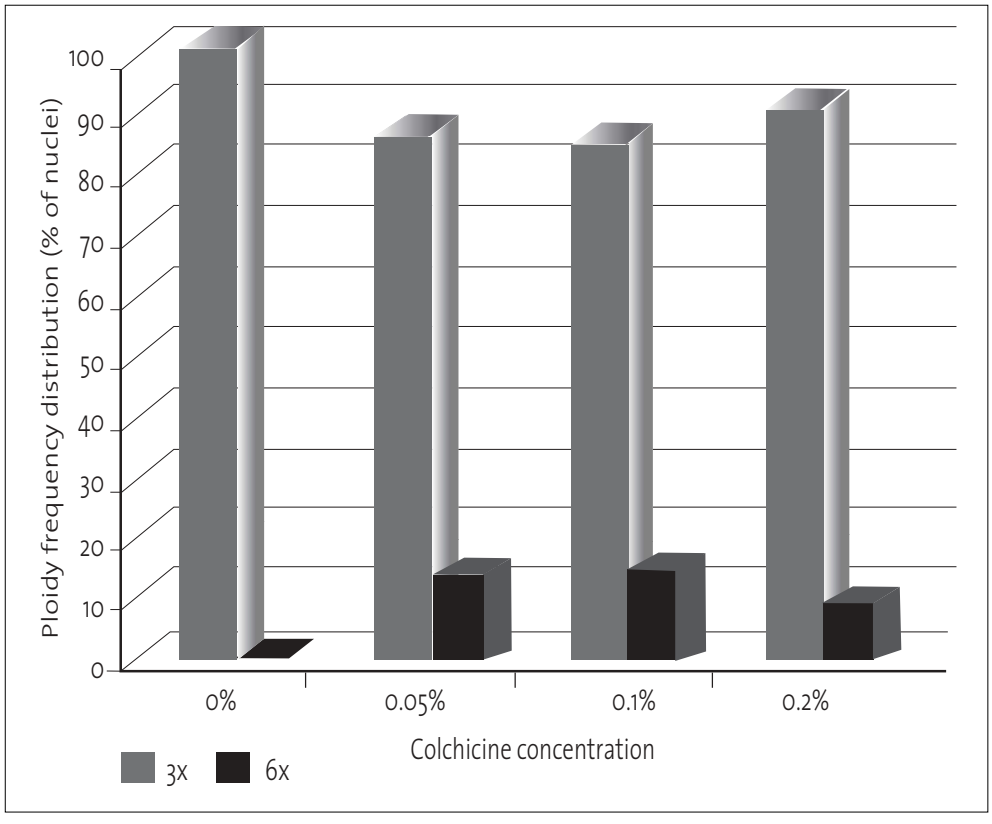

Figure 1. Ploidy frequency distribution of 'Williams' suspension cells (cell-line 124T) with triploid (3x) and hexaploid (6x) nuclear DNA content observed 15 days after colchicine treatment (w/v).

Table 2. Ploidy distribution of regenerated plants from colchicines-treated ECS of the triploid cell line 124T.

Colchicine concentration (%)	Number of regenerated plants	Ploidy		
		3n	6n	Chimeric mixoploidy (3n+6n)
0	108	108	0	0
0.05	63	58	5	0
0.1	37	36	1	0
0.2	88	84	4	0
	296	**286**	**10**	**0**

Mutagenic treatment of embryogenic cell suspensions

Two cell lines of the cultivars 'Williams' and 'Three hand planty' were sieved using a 1000-mm pore size mesh to obtain a fine suspension. The ECS were then subcultured in the maintenance medium (ZZ) in 100-ml flasks at a concentration of about 3% of settled cell volume (SCV). Three days after subculture 0.5 ml of cells were transferred to a sterile Petri dish and the medium was removed. The cells aggregates were then irradiated at doses ranging from 0 to 250 Gy with 25 Gy intervals using a ^{60}Co gamma source at a dose rate of 30 Gy/min. After irradiation, the cells were resuspended in maintenance ZZ medium in centrifuge tubes and transferred to 100-ml Erlenmeyer flasks at different quantities according to the parameter to be analyzed. To study the effect of gamma radiation on the growth of ECS, fresh weight gain and regeneration capacity were determined. The results were expressed in percentage of the control (non-irradiated cells) at all doses.

The two radiosensitivity curves for 'Williams' and 'Three hand planty' are quite similar. After 28 days, at 75 Gy, the cells' weight gain (CWG_{75}=0,84g) was 50% of the control (CWG_0= 1,68g) (Figure 2A).

To measure the regeneration capacity, green plantlets were counted and transferred to Magenta GA7 boxes containing semi-solid R_3 regeneration medium for further growth before acclimatization. The radiosensitivity curve was obtained by comparing the number of regenerated plantlets for each dose with the control plants (from non-irradiated ECS) (Figure 2B). Radiation at a low level seems to stimulate the regeneration capacity especially in 'Williams'. We must, however, take into account that in control plants, the density of embryos in the temporary immersion system vessels was too high and hence, a considerable number of small plantlets could not develop. In both genotypes no plants regenerated above 200Gy. The number of regenerated plants drops drastically above 50 Gy for 'Williams' whereas for 'Three Hand Planty' the number of regenerated plantlets decreases less dramatically. The regenerated plants were transferred to the greenhouse for early mass screening for tolerance to black leaf streak disease.

Establishment of an early mass screening method

Genetic variability is a prerequisite before selecting for disease resistance. A technique that can reliably identify resistant plants is then adopted to screen the populations (Lepoivre *et al.*, 1993). The Plant Pathology Unit at the *Faculté Universitaire des Sciences*

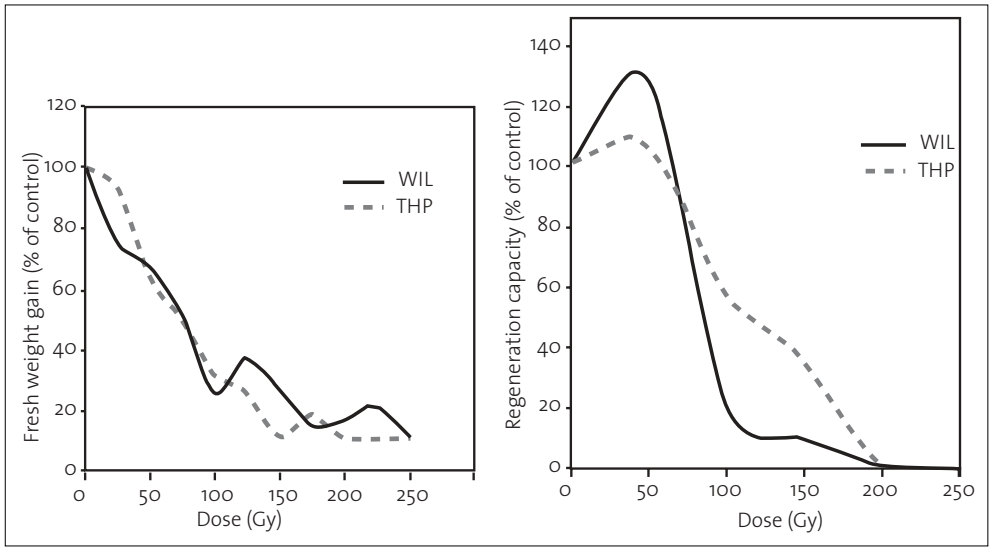

Figure 2. Effect of gamma radiation on : (A) fresh weight gain 28 days after irradiation and (B) regeneration capacity at 60 to 90 days after irradiation. All parameters are expressed as a percentage of the non-irradiated ECS (control). Cell suspension cultures of the cultivars 'Willliams' (WIL) and 'Three hand planty' (THP) were used. For each treatment the equivalent of four Petri dishes were measured.

Agronomiques de Gembloux (FUSAGx) developed a rapid and early screening protocol for tolerance to black leaf streak disease in banana and plantain. The method is based on the infiltration of juglone (5-hydroxy-1,4-naphthoquinone), a toxic metabolite of *Mycosphaerella fijiensis*. After developing different bioassays, it was concluded that slow lesion development in cultivars exhibiting a partial resistance to black leaf streak disease was correlated with lower sensitivity to *M. fijiensis* toxins (Lepoivre, 1995). The toxin is probably not involved in the initiation of the infection but could serve as a secondary determinant of the pathogenecity, contributing to the lesion expansion in cultivars exhibiting partial resistance to black leaf streak disease (Harelimana *et al*., 1997). The prospect of utilizing plant tissue cultures to generate and identify novel genetic variants has sparked the interest of researchers for many years (Dix, 1996). Nevertheless in *Musa*, *in vitro* heterotrophic tissues are not suitable targets to perform the screening with such toxin (Harelimana *et al*., 1997). Our first goal was thus, under greenhouse conditions, to determine the lowest concentration of juglone which enables the differentiation between the susceptible cultivar 'Grande naine' and the partially resistant cultivar 'Fougamou'. After performing 10 assays with 4 to 8 replicates we concluded that 25 ppm was the most suitable concentration of juglone to distinguish between a partially resistant and a susceptible plant. This dose was thus further used to screen the plants regenerated from irradiated shoot tips.

Four batches (100 meristems/batch) of 'Grande naine' were irradiated at 35 Gy and propagated over four subcultures. The plants were then acclimatized in the greenhouse. The early mass screening method can be divided in three steps:

1. Plant preparation: The acclimatized plants which reached the six leaf stage are maintained at 90 to 100% relative humidity for 48 hours to open the stomata.

2. Infiltration: The second open leaf of each plant was infiltrated on its under surface with juglone (25 ppm), crude extract (500 ppm) and 10% methanol (control). The amount of each infiltration was 20µl.
3. Necrose observation: 48 hours after infiltration, the plants were observed for necrosis and compared with control plants (non-irradiated 'Grande naine' = positive control; non-irradiated 'Fougamou' = negative control).

To date, from a total of 3 728 'Grande naine' plants screened, 15 putative mutants (0.4%) were selected for their tolerance to 25 ppm of juglone (Table 3).

Table 3. Inventory of irradiated meristems from the cultivar 'Grande naine', multiplied, regenerated into plants, screened and retained for their partial resistance to 25ppm of juglone.

Batch	Irradiated meristems	Number. of shoots in M_1V_1	Number of plants screened in M_1V_4	Number of plants retained
A (ST)	100	142	780	8
B (ST)	100	105	512	0
C (MA)	100	110	1351	4
D (MA)	100	140	1085	3
TOTAL	400	497	3728	15

ST: propagated by shoot tip culture; MA: propagated by the multi-apexing culture technique
M_x: Mutagenic treatment; V_x: Vegetative generation.

The two first batches were propagated by shoot-tip culture and the two second batches were propagated by the multi-apexing technique to dissociate more efficiently chimeras (Table 3). Among the young banana plants selected for their tolerance to juglone, some were showing an increased content of anthocyanin (Figure 3).

Even though we may not have directly obtained mutant genes controlling for resistance, genes responsible for anthocyanin biosynthesis may have been activated and indirectly provided tolerance to black leaf streak disease. Atanassova *et al.* (2001) studied the effect of mutations affecting anthocyanin biosynthesis during tomato and pepper development under stress conditions. They found four genes, which had a kind of universal effect on tomato and pepper germination as they increased the germination potential of the individual accession under a relatively large range of stresses.

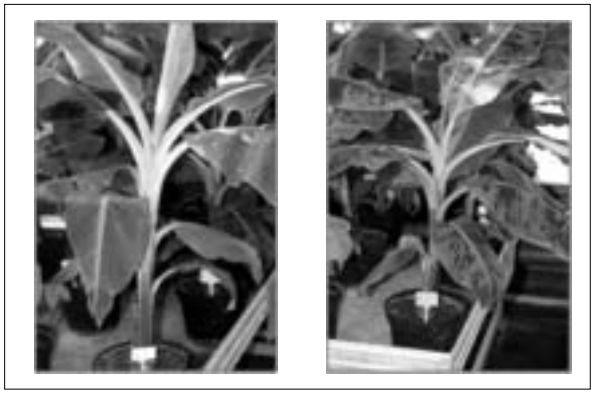

Figure 3.
Regenerated plants from irradiated shoot tips of the cultivar 'Grande naine':
A) susceptible to 25 ppm of juglone;
B) putative mutant, tolerant to 25 ppm of juglone.

Thus the establishment of morphological marker correlated with tolerance to stress even at one plant growth stage, could be useful. The pre-selected plants and their suckers were transferred to the FUSAGx for further screening by inoculation under control conditions.

Conclusion and future directions

Over the last five years, considerable efforts have been assembled to speed up the *in vitro* mutagenesis process and make it more efficient. Mutation induction techniques as well as other genetic improvement strategies will benefit from the use of embryogenic cell suspensions and the establishment of early mass screening techniques. Tolerance to *Mycosphaerella fijiensis* obtained after mutagenic treatment still needs to be confirmed under field conditions. Such mutants may not have the required agronomic characters but they would be very useful in genetic studies and may help in discovering genes responsible for resistance or susceptibility.

Mutation induction should no longer be seen as an independent genetic improvement strategy but more as a tool which can contribute to functional genomics and genetic improvement programmes based on cross-breeding or genetic transformation. For example, a disease resistant mutant from a diploid *Musa*, could be used as a parent line in cross-breeding programmes and also help in understanding the mechanism of resistance and permit the isolation of genes to be used in genetic transformation.

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Reaction of banana genotypes to black leaf streak disease in the State of Acre in Brazil

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Abstract
Black leaf streak disease (caused by *Mycosphaerella fijiensis* Morelet) is the most severe disease affecting commercial varieties of banana of economic importance in the world. Its occurrence was verified in Brazil in 1998, in the State of Amazonas, and it has spread in the plantations through out the State of the Acre, severely attacking the varieties of the Terra subgroup. The objective of this study was to evaluate seven banana genotypes from the *Embrapa Mandioca e Fruticultura*, under two cultivation systems (with and without cultural practices) with the goal of obtaining low environmental impact alternatives to control the disease. The evaluations regarding severity were accomplished on a monthly basis, in ten plants of each genotype, using a descriptive scale. 'FHIA-01', 'FHIA-02', 'Caipira', 'FHIA-21', 'PV 42-85' and 'Thap maeo' presented resistance to black leaf streak disease whereas 'SH 36-40' proved to be susceptible. There was no significant effect of the cultivation system on the severity of black leaf streak disease.

Resumen – Reacción de los genotipos de banano a la Sigatoka negra en el estado de Acre, Brasil
La Sigatoka negra (causada por *Mycosphaerella fijiensis* Morelet) es la enfermedad más severa que afecta las variedades comerciales de banano de importancia económica en todo el mundo. La enfermedad se confirmó en Brasil en 1998, en el Estado de Amazonas, y luego se propagó a las plantaciones a través del Estado de Acre, atacando severamente las variedades del subgrupo Terra. El objetivo de este estudio consistió en evaluar siete genotipos de banano de *Embrapa Mandioca e Fruticultura*, en dos sistemas de cultivo (con y sin empleo de prácticas culturales), con el fin de obtener alternativas de control para la enfermedad con un bajo impacto ambiental. Las evaluaciones con respecto a la severidad se realizaron mensualmente en diez plantas de cada genotipo, utilizando una escala descriptiva. Los resultados mostraron que los genotipos 'FHIA-01', 'FHIA-02', 'Caipira', 'FHIA-21', 'PV 42-85' y 'Thap maeo' resultaron resistentes mientras que el genotipo 'SH36-40' fue susceptible a la Sigatoka negra en los dos sistemas de cultivo.

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Résumé – Réaction de génotypes de bananiers à la maladie des raies noires dans l'Etat de Acre, Brésil
La maladie des raies noires (causée par *Mycosphaerella fijiensis* Morelet) est la plus sévère des maladies qui affectent les cultivars commerciaux économiquement importants. Sa présence au Brésil a été confirmée en 1998, dans l'Etat d'Amazonas, d'où elle s'est propagée à l'Etat de Acre, affectant sévèrement les variétés du sous-groupe Terra. L'objectif de cette étude a été d'évaluer sept génotypes de bananiers de l'*Embrapa Mandioca e Fruticultura* sous deux systèmes de culture (avec ou sans pratiques culturales) dans le but de développer des méthodes de lutte contre la maladie dont l'impact sur l'environnement serait faible. Les criblages furent réalisés sur une base mensuelle, 10 plants par génotype, et utilisant une échelle descriptive. 'FHIA-01', 'FHIA-02', 'Caipira', 'FHIA-21', 'PV 42-85' et 'Thap maeo' ont présenté une résistance à la maladie des raies noires, tandis que 'SH 36-40' s'est avéré susceptible. Il n'y a pas eu d'effet significatif du système de culture sur la sévérité de la maladie des raies noires.

Introduction

Black leaf streak disease is the most devastating disease of banana worldwide. It can cause losses up to 100% if no control measure is taken (Cordeiro *et al.*, 1998).

In the State of Acre, banana is the most consumed fruit and is considered a staple food among the poor populations. It is also exported to other States. The disease was observed for the first time in Brazil in early 1998 (Cordeiro *et al.*,1998), in the municipalities of Tabatinga and Benjamim Constant, and in Rio Branco and Acrelândia at the end of 1998 (Ritzinger *et al.*, 1999; Cavalcante *et al.*, 1999).

Resistant varieties are not only less expensive to control pathogens than fungicides, they are also preferable from an environmental point of view (Pereira *et al.*, 1999).

The present work aims to evaluate the resistance to black leaf streak disease of banana cultivars under the weather and soil conditions found in Acre and using two cultivation systems.

Material and methods

The research was conducted in Embrapa's experimental station in Acre, Rio Branco. Seven genotypes ('PV-4285', 'FHIA-21', 'Caipira', 'FHIA-01', 'FHIA-02', 'SH-3640' and 'Thap maeo') were evaluated for their resistance to black leaf streak disease under two systems of cultivation: a traditional system (weeding) and a more intensive system (weeding, trimming, shedding and fertilization).

A randomized complete block design (7 genotypes x 2 cultivation systems) with 5 replications was used and data were recorded on 10 leaves/plant.

Disease severity was assessed on a monthly basis, starting from the sixth month after planting. The disease was observed in individual leaves, using the following scale (Stover, 1971 modified by Gauhl, 1994):

0 = absence of symptoms
1 = less than 1% of lamina with symptom (presence of streaks and/or $\geq$ 10 spots)
2 = 1 to 5% of lamina with symptoms
3 = 6 to 15% of lamina with symptoms
4 = 16 to 33% of lamina with symptoms

5 = 34 to 50% of lamina with symptoms and
6 = 51 to 100% of lamina with symptoms

The variables were submitted to an analysis of variance (F test) and the averages were compared using Scott & Knott's test (1974) at 1% of significance.

Results

According to the analysis of variance (Table 1) the genotype had the most significant effect on disease severity of black leaf streak disease. There was no interaction between the cultivation system and the genotypes nor isolated effects of the cultivation system on the severity of the disease.

Table 1. Analysis of variance of disease severity of black leaf streak disease in seven genotypes grown under two cultivation systems.

Source of Variation	DF	Average Square
Block	4	51.2714
Genotypes (G)	6	1356.9333*
Cultivation system (CS)	1	66.0571ns
Interaction (G X CS)	6	10.3238ns
Error	52	24.8868
VC (%)	15.30	

*Statistically significant at probability 0.01.

As presented in Figure 1, disease severity was highest on 'SH-3640' (55,10%), followed by 'Thap maeo' (39%) and 'FHIA-21' (33,3%). The genotypes 'Caipira', 'FHIA-01' and 'FHIA-02' were similar whereas the hybrid 'PV-4285' presented the lowest severity (19,70%) (Figure 2).

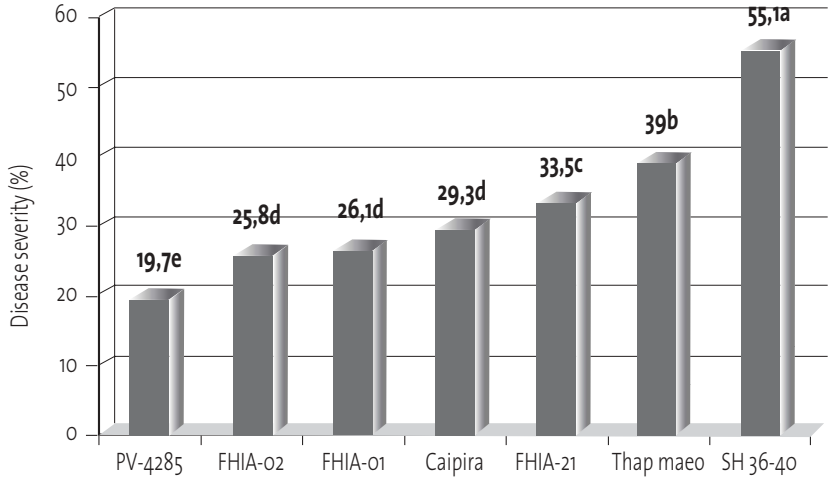

Figure 1. Average disease severity of black leaf streak disease in seven genotypes.

Figure 2.
'PV 42-85' free of black leaf streak disease.

Figure 3.
'SH 36-40' showing signs of black leaf streak disease.

The cultivation system (weeding, shedding, trimming and fertilization) did not influence the severity of black leaf streak disease in 'PV-4285', 'Caipira', 'FHIA-01', 'FHIA-02', 'Thap maeo' and 'SH-3640'.

For the data recording shooting, it was observed that the genotypes 'FHIA-01', 'FHIA-02', 'Caipira', 'FHIA-21', 'PV 42-85' and 'Thap maeo' were more resistant to black leaf streak disease than 'SH 36-40', which was the most susceptible to the disease (Figure 3).

Conclusion

The genotypes 'FHIA-01', 'FHIA-02', 'Caipira', 'FHIA-21', 'PV 42-85' and 'Thap maeo' presented resistance to black leaf streak disease. The genotype 'SH 36-40' is susceptible to black leaf streak disease. There was no significant effect of the cultivation system on the severity of black leaf streak disease.

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The International *Musa* testing programme (IMTP): a worldwide programme to evaluate elite *Musa* cultivars

J.V. Escalant

Abstract
The International *Musa* Testing Programme (IMTP) is a collaborative effort coordinated by INIBAP to evaluate, in suitable sites worldwide, elite *Musa* cultivars produced by breeding programmes and promising accessions from the INIBAP collection. Established in 1989, IMTP trials are designed to be replicated anywhere in the world and aim to evaluate elite clones for resistance and/or tolerance to black leaf streak disease, Sigatoka disease and Fusarium wilt. Phase II of IMTP started in 1996 when 15 countries initiated their field plots using 9 elite clones from Honduras, Taiwan, Brazil and Cuba. This presentation summarizes the results. The analysis of the results indicates that disease development time is not a reliable parameter for evaluating resistance levels and that the infection index is a more reliable parameter. Among the cultivars tested, 'FHIA-23' and 'SH-3436-9' displayed a good level of resistance using 'Pisang Ceylan' as a resistant reference. This conclusion is consistent with the youngest leaf spotted score obtained in most countries. A correlation was found between the infection index at bunch emergence and the average finger weight across sites and genotypes. So far, 23 research institutes in 21 countries are participating in IMTP III.

Resumen - Programa Internacional de Evaluación de *Musa* (IMTP): un programa mundial para evaluar las variedades elite de *Musa*
El Programa Internacional de Evaluación de *Musa* (IMTP) es un esfuerzo colaborativo coordinado por INIBAP cuyo fin es evaluar las variedades elite de *Musa* en sitios apropiados alrededor de todo el mundo. Los ensayos del IMTP son diseñados para poder replicarlos en cualquier lugar del mundo. El IMTP primero se desarrolló para realizar evaluaciones detalladas de material nuevo con el fin de obtener información sobre su resistencia o tolerancia a las Sigatokas negra y amarilla y al marchitamiento por Fusarium. La fase II del IMTP empezó en 1996 cuando 15 países diferentes establecieron sus parcelas en el campo utilizando

INIBAP, Montpellier, France

9 diferentes cultivares elite de Honduras, Taiwán, Brasil y Cuba. En esta presentación se brinda una apreciación global de los resultados. Un análisis de los resultados obtenidos indicó que el tiempo de desarrollo de esta enfermedad no es un parámetro confiable para evaluar los niveles de resistencia. Inversamente, los índices de infección parecen ser un parámetro más confiable. Dentro del material evaluado, 'FHIA-23' y 'SH-3436-9' mostraron un buen nivel de resistencia en comparación con la referencia resistente 'Pisang Ceylan'. Esta conclusión es consistente con el conteo de la hoja más joven manchada obtenido en la mayoría de los países. Se observó una buena correlación entre el índice de infección durante la emergencia de racimos y el peso promedio del dedo por el sitio y por el genotipo. Actualmente, 23 institutos de investigaciones en 21 países están participando en la fase III del IMTP.

Résumé – Le programme international d'évaluation des *Musa* (IMTP) : un programme international pour évaluer les cultivars d'élite *Musa*

Le programme international d'évaluation des *Musa* (IMTP) est un effort de collaboration coordonné par l'INIBAP pour évaluer, dans des sites appropriés du monde entier, des cultivars de bananiers produits par les programmes d'amélioration et des accessions de la collection de l'INIBAP. Etablis en 1989, les essais IMTP sont conçus pour être répliqués n'importe où à travers le monde et vise à évaluer les clones d'élites pour leur résistance et/ou tolérance à la maladie des raies noires, la maladie de Sigatoka et la fusariose. La phase II de l'IMTP a débuté en 1996 lorsque 15 pays ont mis en place leurs parcelles expérimentales pour 9 clones d'élites provenant du Honduras, Taïwan, Brésil et Cuba. Cette présentation résume les résultats. L'analyse des résultats indique que le temps de développement de la maladie n'est pas un paramètre fiable pour évaluer les niveaux de résistance et que l'indice d'infection est plus fiable. Parmi les cultivars testés, 'FHIA-23' et 'SH-3436-9' ont démontré un bon niveau de résistance par comparaison au témoin 'Pisang Ceylan'. Cette conclusion est consistante avec le score obtenu pour la plus jeune feuille nécrosé obtenu dans la plupart des pays. Une corrélation a été observée entre l'indice d'infection à l'émergence du régime et le poids moyen des doigts pour tous les sites et génotypes. Jusqu'à maintenant, 23 instituts de recherche dans 21 pays participent à la phase III de l'IMTP.

Introduction

The International *Musa* Testing Programme (IMTP) is a collaborative effort coordinated by INIBAP to evaluate, in suitable sites worldwide, elite *Musa* cultivars produced by breeding programmes and promising accessions from the INIBAP collection. Taking into account local conditions, IMTP trials are designed to be replicated anywhere in the world. The programme was developed to evaluate new germplasm in order to obtain information on their resistance/tolerance to black leaf streak disease and Sigatoka disease, caused respectively by *Mycosphaerella fijiensis* and *M. musicola,* and to Fusarium wilt, caused by *Fusarium oxysporum* fsp. *cubense*. IMTP trials can also be used to conduct basic research on the pathogen and its host, such as epidemiological studies, host-pathogen relationships of the different strains of a pathogen, and adaptability and productivity studies.

Two protocols have been developed in response to the demand from national programmes to evaluate germplasm under local conditions while recognizing the need for more detailed research at a limited number of sites. The two types of evaluation are:

1) performance evaluations which use a simplified protocol to obtain data on cultivar or hybrid performance under local conditions and basic data on disease resistance or tolerance;

2) and in-depth evaluations which are more complete disease resistance evaluations carried out at a smaller number of sites. These sites are used to screen new improved hybrids and, if requested by breeding programmes, parental breeding lines. They can also provide opportunities for basic research on host-pathogen interactions. A standard procedure for data management and statistical analysis has been developed and the guidelines, which have been revised in the wake of phase II of the programme, are made available in English, French or Spanish to the participating programmes.

Phase I

The programme was established in 1989 as a partnership between National Agricultural Research Systems (NARS), INIBAP breeders and pathologists from several institutes. The objective was to use multilocation trials to identify banana and plantain hybrids meeting local requirements which small-scale farmers could use to replace susceptible cultivars. A second objective of IMTP was to stimulate *Musa* breeding programmes by providing information on the response of their improved cultivars to pathogens. A indirect effect of IMTP has been to increase the capacity of national organisations to carry out research on banana and plantain.

The programme began by evaluating germplasm from the *Fundación Hondureña de Investigación Agrícola* (FHIA) for resistance to black leaf streak disease. Seven tetraploid hybrids from a wide variety of genetic backgrounds were tested along with several reference diploid clones (wild and edible) that represented the whole range of reactions to black leaf streak disease, i.e. from highly resistant to highly susceptible. The trials were conducted in six countries. Site managers were trained, and provided with technical guidelines and funding to carry out the trials. Four years later, the detailed results were published and three hybrids were recommended for distribution: 'FHIA-01' and 'FHIA-02', two dessert banana cultivars with outstanding performance and high resistance to black leaf streak disease, and 'FHIA-03', a cooking banana also with excellent performance and resistance to black leaf streak disease. Over the last ten years these clones have been distributed to more than 50 countries. In view of the success of the programme, INIBAP was asked to develop the programme further.

Phase II

In IMTP II, germplasm was assessed for resistance to *M. fijiensis* and *M. musicola*. Four breeding programmes contributed germplasm (Table 1) and the number of test sites was increased to 37, even though the trials were financed by the participating institutes. The majority of IMTP II trials were set up in 1996-1997. The complete report includes results on resistance to black leaf streak disease from sites in Cameroon, Costa Rica, Honduras, Nigeria, the Philippines,Tonga and Uganda and to Sigatoka disease from one site in Colombia. A complete analysis was not possible because of missing data, for reasons that include natural catastrophes, in places like Cameroon, Costa Rica, Tonga, Thailand, Cuba and India. This presentation summarises the IMTP II results.

Table 1. Improved cultivars included in the resistance trials of IMTP II.

Clone	Origin[1]	Genome
PV-03.44	EMBRAPA	AAAB
PA-03.22	EMBRAPA	AAAB
SH-3436-9	INIVIT	AAAA
FHIA-01	FHIA	AAAB
FHIA-03	FHIA	AABB
FHIA-17	FHIA	AAAA
FHIA-23	FHIA	AAAA
GCTCV-119	TBRI	AAA
GCTCV-215	TBRI	AAA

[1]Breeding programme

EMBRAPA: *Empresa Brasileira de Pesquisa Agropecuaria*, Brazil; FHIA: *Fundación Hondureña de Investigación Agrícola*, Honduras; INIVIT: *Instituto Nacional de Investigación en Viandas Tropicales*, Cuba; TBRI: Taiwan Banana Research Institute, Taiwan.

Resistance to black leaf streak disease

The data presented come from the trials in Costa Rica, Cameroon (Figure 1) and Tonga which are considered as representative of Latin America, Africa and Pacific Asia respectively. The response of the clones to black leaf streak disease varied according to the biotic and abiotic factors present in each country.

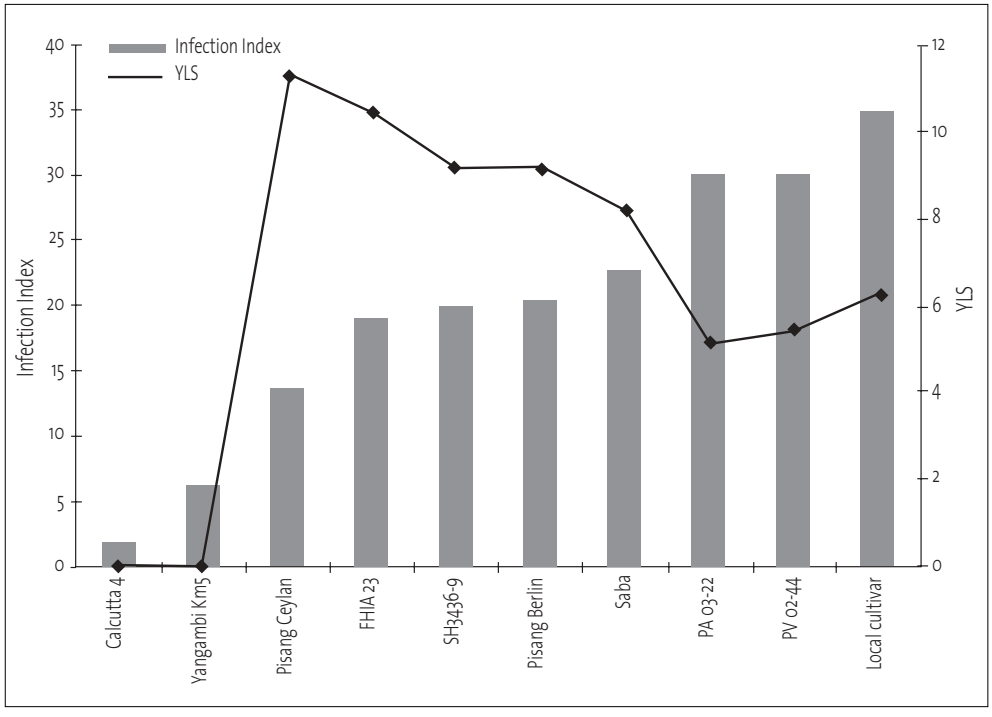


Figure 1. Infection index of black leaf streak disease and youngest leaf spotted (YLS) of different genotypes in Cameroon.

'FHIA-23' and 'SH-3436-9' from Honduras, had an average infection index similar to that of the resistant reference cultivar 'Pisang Ceylan' suggesting that 'FHIA-23' and 'SH-3436-9' are resistant to black leaf streak disease (Table 2). The conclusion is consistent with the youngest leaf spotted (YLS) score obtained in most countries.

Table 2. Infection index of black leaf streak disease and youngest leaf spotted (YLS) of various genotypes in Costa Rica and Tonga.

Costa Rica			Tonga		
Clone	**Infection Index**	**YLS**	**Clone**	**Infection Index**	**YLS**
PV-03.44	36.1	8.3	PV-03.44	29.9	7
PA-03.22	34.4	8.2	PA-03.22	34.8	7
SH-3436-9	24.6	7.4	SH-3436-9	13.5	11
FHIA-23	18.4	7.8	FHIA-23	61	11
References			References		
Calcutta 4	10.6	7.9	Calcutta 4	1.8	
Yangambi Km5	19.1	7.9	Yangambi Km5	20	11
Pisang Ceylan	18.7	8.5	Pisang Ceylan	19	10
Saba	32.3	8	Saba	20.3	9
Local cultivar	45.6	4.9	Local cultivar	30	5

Agronomic performance

Bunches of 'FHIA-23' and 'SH 3436-9' weighed on average 30.6 kg and 22.3 kg, with a maximum of 39.4 kg in Cameroon and 28.8 kg in Tonga. Although, the local cultivars differed between countries, their average bunch weight was 16.5 kg, with a maximum bunch weight of 22.8 kg in Tonga (Figure 2). This substantiates the improved performance of FHIA hybrids. However, FHIA and INIVIT hybrids had longer growth cycles than local reference cultivars with an average of 474 and 420 days for 'FHIA-23' and 'SH-3436-9', respectively.

Discussion

Disease development time (DDT) was not a reliable measure of resistance possibly because of difficulties in interpreting leaf symptoms under certain conditions. For example, when disease pressure is high, stage 1 lesions may coalesce due to their high number and appear similar to a stage 6 necrotic lesion. The infection index seems to be a more reliable parameter. Besides being comparable between countries, the results can be used to classify the new hybrids.

The same clone in different country can display a different tolerance to black leaf streak disease. Tolerance being influenced by many factors, e.g. management, soil fertility, pathogen pressure, presence of other pathogens and climatic conditions, it is not possible to generalize the results. The effect of these factors on yield is not easy to demonstrate or quantify. However, work at the *Centre africain de recherches sur bananiers et plantains* (CARBAP) in Cameroon has demonstrated an effect of

black leaf streak disease on bunch weight. A correlation between the infection index at bunch emergence and fruit weight averaged across sites and genotypes (r=-0.71) was found using IMTP data (Figure 3). The correlation suggests that the numbers of characteristics to record could be reduced, thus simplifying data collection and management. It would also reduce the need for visual interpretation of symptoms in the field.

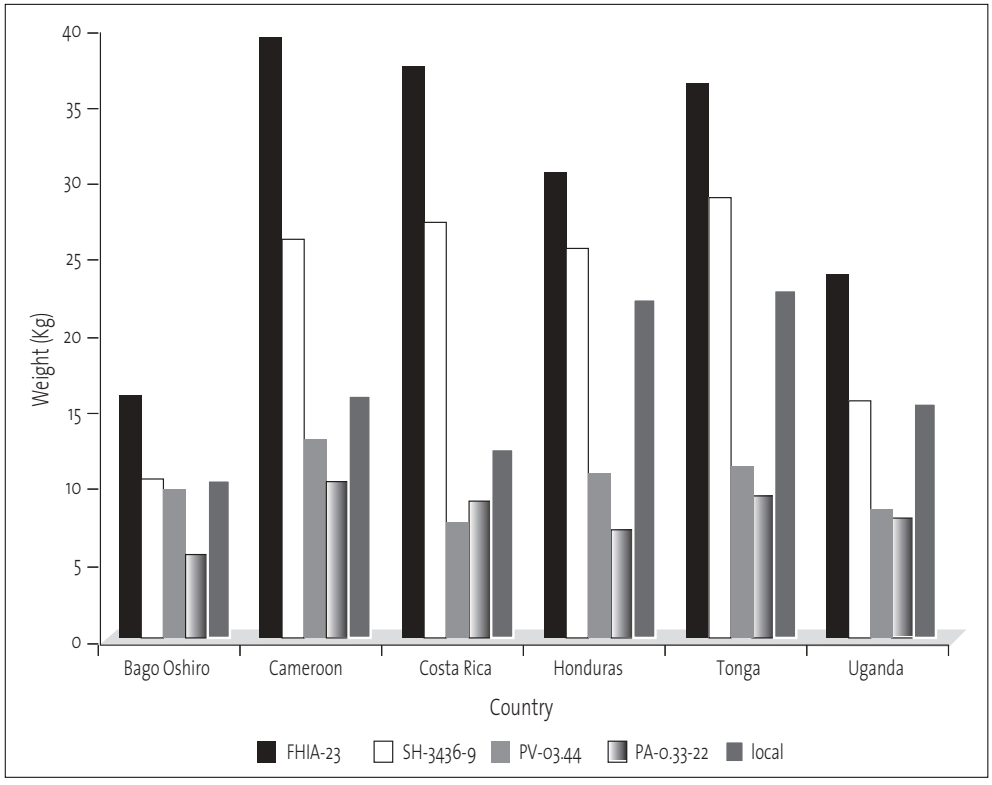


Figure 2. Average bunch weight of various hybrids in different locations.

Unexpectedly, in some sites, the highly resistant clones 'Calcutta 4' and 'Yangambi km5' had stage 6 necrotic lesions. Further investigations are needed to determine whether the effect was due to aggressive strains of *M. fijiensis* or to a new pathogen.

Conclusion

FHIA hybrids had consistently the best yields in trials. With few exceptions, their bunches were heavier than those from other improved and local cultivars. FHIA hybrids also responded well to careful management and to fertilizer. In summary, FHIA hybrids performed well under a range of conditions; the better the conditions the better their performance. The improved hybrid 'FHIA-23' had the best performance in all the trials to evaluate resistance to Sigatoka diseases.

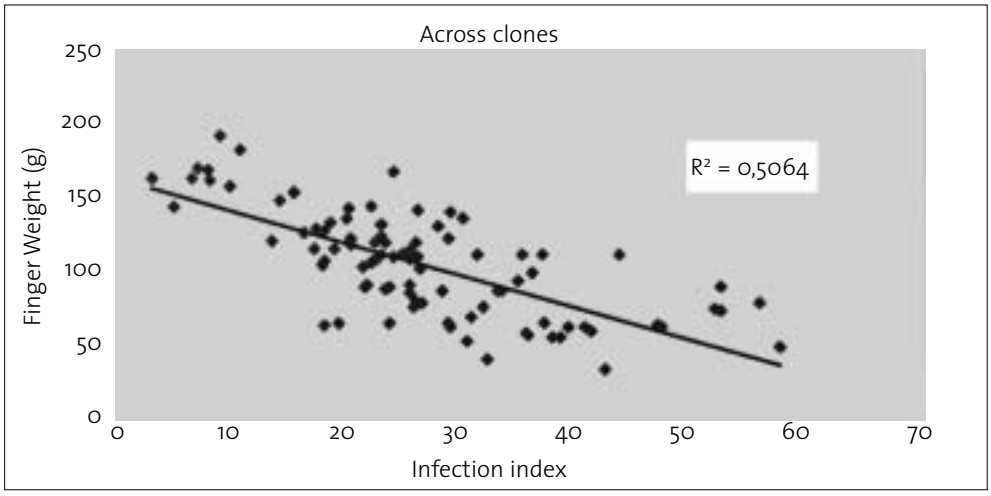


Figure 3. Correlation between the infection index at bunch emergence and fruit weight averaged across sites and genotypes.

Cultivar 'GCT CV 119' deserves special attention as it had the lowest discoloration scores for two races of *F. oxysporum* f.sp. *cubense* and high bunch weight under conditions of good crop husbandry. It is important to stress that resistance alone is not useful. It needs to be combined with good production, and acceptable post-harvest and organoleptic traits. Improved banana varieties contribute not only to reducing disease incidence but also to improving food production. The complete data and statistical analysis will be published shortly[1].

IMTP II database

All the information from IMTP II, including agronomic trains and host plant response, for all genotypes and all sites, is compiled in a database to facilitate access to the data on new *Musa* germplasm compiled throughout the world. The database is also included in the CD-ROM, 'Evaluation of *Musa* germplasm for resistance to Sigatoka diseases and Fusarium wilt'. The CD-ROM also contains the technical guidelines, a comprehensive analysis of phase II results entitled 'Evaluating bananas: a global partnership', a catalogue of candidate and reference clones, including those for IMTP III trials, and the transfer agreement to obtain genetic material. The CD-ROM contains all the information needed to participate in IMTP III.

IMTP III

In 2001, 450 consignments of germplasm accessions were sent from the INIBAP Transit Centre (ITC) to 23 institutes in 21 countries participating in IMTP III. Thirty-five cultivars including plantains, cooking bananas and dessert bananas are available

[1] To receive the final report please contact Jean-Vincent Escalant, the IMTP coordinator at INIBAP.

for evaluation. Eleven institutes are carrying out "in-depth" studies to evaluate improved varieties, which will involve epidemiological and ecological research. Thirteen institutes will be evaluating the performance of varieties against local diseases and conditions following a more simplified format of data gathering (Table 3). For the first time two private companies will be carrying out evaluations. The first results are expected in 2003.

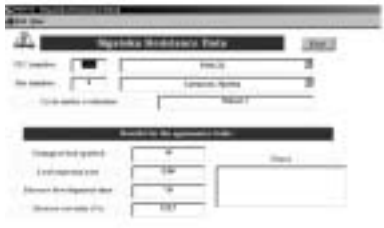
Table 3. Institutes and countries involved in IMTP III

Country	Institute	Phase III
Australia	Queensland Horticultural Institute (QHI)	Not confirmed
Bangladesh	Bangladesh Agricultural Research Institute (BARI)	Performance
Burundi	*Institut de recherches agronomique et zootechnique de la communauté économique des pays des grands lacs* (IRAZ)	Performance
Cameroon	*Centre africain de recherches sur bananiers et plantains* (CARBAP)	In-depth
China	South China Agricultural University (SCAU)	Performance
Colombia	*Corporación Colombiana de Investigación Agropecuaria* (CORPOICA)	In-depth
Costa Rica	*Corporación Bananera Nacional* (CORBANA)	In-depth
Dominican Republic	*Centro para el Desarrollo Agropecuario y Forestal* (CEDAF)	Performance
Haiti	*Institut Interaméricain de Coopération pour l'agriculture* (IICA)	Performance
Honduras	*Fundación Hondureña de Investigación Agrícola* (FHIA)	Performance
India	National Research Center on Banana (NRCB)	In-depth
Indonesia	Central Research Institute for Horticulture (CRIH)	Performance
Malaysia	Malaysian Agricultural Research and Development Institute (MARDI)	In-depth
Mexico	*Instituto Nacional de Investigaciones Forestales y Agropecuarias* (INIFAP)	In-depth
Nicaragua	*Universidad de León* (UNAN León)	Performance
Peru	*Servicio Nacional de Sanidad Agraria* (SENASA)	Performance
Philippines	Bureau of Plant Industry – Davao National Crop Research and Development Center (BPI – DNCRDC)	In-depth
Philippines	Dole Asia Research ; Stanfilco	In-depth
Philippines	Lapanday Fruit Company	In-depth
Rwanda	*Institut des sciences agronomiques du Rwanda* (ISAR)	Performance
Sri Lanka	Agricultural Research Station (ARS)	Performance
Uganda	National Agricultural Research Organization (NARO)	In-depth/ Performance
Vietnam	Vietnam Agricultural Science Institute (VASI)	Performance

All except two institutes asked to evaluate leaf spot diseases demonstrating the worldwide impact of leaf spot diseases on banana production. Black leaf streak disease is the main leaf spot of banana in the world. Leaf spots are also caused by *M. musicola* and *M. eumusae*, the latter recently discovered in Southeast Asia and easily confused with *M. fijiensis*. In the framework of IMTP, a training course for IMTP III participants was organized by INIBAP and CIRAD, and hosted by the Malaysian Agricultural Research and Development Institute (MARDI). The aims were to standardize methods of data collection and evaluation of leaf spot diseases and to

train researchers to identify leaf spot diseases by morphological differences in the anamorph stages of the fungi.

Data entry forms



Recommendations of session 4

Progress has been made towards the creation of new varieties resistant to black leaf streak disease, either through conventional and/or modern technologies. New tetraploid hybrids resistant to black leaf streak disease are already available and some of these are widely grown around the world. Good progress has also been made in the development of a molecular toolbox for bananas and plantains in the area of the genetic transformation.

Musa balbisiana genome

The presence of the activable form of the banana streak virus (BSV) in interspecific hybrids (AxB), hinders the production of a new generation of triploid hybrids. Access to all the *balbisiana* diversity is important because of the BSV related problem but also to get a better knowledge of the existing diversity in the B genome and its contribution in the interspecific hybrids.

It is recommended to study the diversity of the Musa balbisiana *genome with both morphological and molecular traits. It is also recommended to promote and facilitate new collecting missions.*

Breeding for resistance

All possible sources of resistance to pests and diseases are needed to genetically improve *Musa* cultivars. Previous studies report on resistance to *Mycosphaerella* spp. of *Musa schizocarpa* (genome S) *and Musa textilis* (genome T). Cultivars containing T and S genomes have also been reported in Papua New Guinea as being highly resistant. Prospecting for new sources of resistance to *Mycosphaerella* spp., as well as other diseases, should be easier since all *Musa* species, except *Musa textilis,* are covered by the facilitated access provision in the recently signed International Treaty on Plant Genetic Resources for Food and Agriculture Treaty.

It is recommended to anticipate the needs of genetic improvement programmes by screening the T and S genome species as well as subspecies of Musa acuminata *and other* Musa *spp. to detect new possible sources of resistance to pests and diseases.*

Durability of resistance

The erosion of resistance is a problem which should be addressed to ensure the durability of resistant improved cultivars.

The pathogen populations should be characterized in areas where the resistance of hybrids appears to be decreasing.

Computer modelling of black leaf streak disease epidemics is a way to predict the durability of resistance and to evaluate different disease management strategies. However, the development of such a model requires more quantitative parameters to describe disease epidemics and the evolution of the pathogen population in response to the selection pressure exerted by resistant hosts.

It is recommended to study the structure of the population at field and regional level in mixed-cultivars plots combining vertical and horizontal resistances.

Mutation induction

Mutation induction techniques should no longer be seen as an independent genetic improvement strategy but more as a tool that can contribute to cross-breeding programmes by increasing genetic diversity in parental lines. For example, the barley *MLO* gene that confers complete resistance to powdery mildew was obtained by mutagenesis. Mutants could also help in understanding the mechanism of resistance. Induced deletion mutants and aneuploids in particular will be useful to map or locate genes of interest and molecular markers.

Genetic transformation

Triploid cultivars of banana are often pollen and seed sterile and as such they should benefit from simpler risk assessment protocols regarding geneflow.

It is recommended to encourage the development of national legislation to allow field testing of transgenic Musa *plants, to collect data, to guide further research and regulation.*

It is also recommended to continue the development of transgenic banana plants to allow in-depth studies on plant development and on plant-pathogen interactions and to increase resistance to Mycosphaerella.

Genetic transformation is also recommended to identify and isolate genes of resistance using the 'knockout' strategy on resistant cultivars. This will also be very useful to study host-pathogen interaction.

Session 5

Integrated disease management

Management of *Mycosphaerella* leaf spot diseases in Australia

R. Peterson[1], **K. Grice**[1] and S. De La Rue[1]

Abstract

Mycosphaerella musicola is the major leaf spot pathogen affecting banana in the tropics, whereas *Mycosphaerella musae* and *M. musicola* dominate in the sub-tropical areas of Australia. Control strategies include chemical and cultural practices. In tropical areas, sprays are applied at intervals of 10-14 days during the wet season and 21-28 days during the dry season. Annually, 18-24 protectant and systemic fungicide sprays are applied with petroleum oil (5L). Since the early 1980s, the banana industry has fought to exclude this disease from the production areas by monitoring and creating a barrier of resistant plant material. Black leaf streak disease has been detected in the Cape York area eight times in the past 20 years and was limited to a few plants and in all cases was successfully eradicated.

In April 2001, black leaf streak disease was identified in the main production area of North Queensland. An intensive survey (April-June) indicated the disease was restricted to the Tully area. Black leaf streak disease was found on 14 commercial properties and on 11 clumps of unmanaged plants. An eradication programme commenced in September 2001 across approximately 4500 ha of banana plants and involved deleafing commercial plantings to zero disease, weekly fungicide applications for six months and the destruction of all non-managed plants. Black leaf streak disease has not been detected on commercial plantations for eight months and on non-managed plants for four months. It was not detected in 1550 samples assessed during January to April 2002.

Manejo de las *Mycosphaerella* en Australia

La Sigatoka amarilla (causada por *Mycosphaerella musicola*) es la principal enfermedad foliar que afecta a los bananos en los trópicos, mientras que la mancha foliar (*Mycosphaerella musae*) y la Sigatoka amarilla predominan en las áreas subtropicales de Australia. La estrategias de control incluyen la aplicación de los químicos y las prácticas culturales. En las áreas tropicales, los rociados se aplican a intervalos de 10-14 días durante la estación húmeda y de 21-28 días durante la estación seca. Anualmente, se aplican 18-24 rociados de fungicidas protectores y sistémicos mezclados con el aceite mineral (5L). Desde inicios de la década de los 80, la industria bananera ha luchado para erradicar esta enfermedad en las áreas de producción monitoreando y creando un barrera de material vegetal resistente. La Sigatoka negra fue detectada en el área de Cabo York ocho veces durante los últimos 20 años y fue limitada a unas pocas plantas y todos los casos fueron erradicados exitosamente.

[1] Queensland Department of Primary Industries, Mareeba, Australia.

En abril de 2001, la Sigatoka negra fue identificada en la principal área de producción de Queensland del Norte. Una encuesta intensiva (abril-junio) indicó que la enfermedad estaba restringida al área de Tully. La Sigatoka negra fue descubierta en 14 propiedades comerciales y en 11 grupos de plantas sin manejar. En septiembre de 2001 empezó un programa de erradicación en aproximadamente 4500ha de bananos e incluyó el deshoje total de las siembras comerciales, aplicaciones semanales de funguicidas durante seis meses y la destrucción de todas las plantas sin manejo. La Sigatoka negra no fue detectada en las plantaciones comerciales durante ocho meses y en las plantas sin manejo durante cuatro meses. Tampoco se detectó la Sigatoka negra en 1550 muestras evaluadas durante enero-abril de 2002.

Résumé - Gestion des *Mycosphaerella* en Australie

Mycosphaerella musicola est le principal agent pathogène des maladies foliaires dans les tropiques, alors que *Mycosphaerella musae* et *Mycosphaerella musicola* dominent dans les régions sub-tropicales d'Australie. Les stratégies de contrôle incluent des pratiques chimiques et culturales. Dans les zones tropicales, les pulvérisations sont appliquées à des intervalles de 10-14 jours pendant la saison humide et de 21-28 jours pendant la saison sèche. Sur une année, 18-24 pulvérisations de fongicides protecteurs et systémiques sont appliquées avec de l'huile de pétrole (5L). Depuis le début des années 1980, l'industrie bananière a lutté pour exclure cette maladie des zones de production par une surveillance et la création d'une zone tampon de matériel résistant. La maladie des raies noires a été détectée huit fois au cours des 20 dernières années dans la zone de Cape York ; elle était limitée à quelques plantes et, chaque fois, elle a été éradiquée avec succès.
En avril 2001, la maladie des raies noires a été identifiée dans la principale zone de production du North Queensland. Un inventaire intensif (avril-juin) a indiqué que la maladie était restreinte à la région de Tully. La maladie des raies noires a été trouvée dans 14 plantations commerciales et dans 11 groupes de plantes n'ayant subi aucun traitement. Un programme d'éradication a commencé en septembre 2001 sur environ 4500 ha de bananeraies ; il comprenait la suppression des feuilles dans les plantations commerciales jusqu'au niveau zéro de la maladie, des applications hebdomadaires de fongicides pendant 6 mois et la destruction de toutes les plantes non traitées. La maladie des raies noires n'a pas été détectée dans les plantations commerciales pendant huit mois et pendant quatre mois sur les plantes non traitées. Elle n'a pas été détectée dans les 1550 échantillons analysés entre janvier et avril 2002.

Introduction

Mycosphaerella musicola, the cause of Sigatoka disease, was first recorded in Australia in 1924 (Benson, 1925) and is still the predominant leaf disease of banana in tropical Australia. In the sub-tropical areas of south Queensland and New South Wales, the major leaf disease is Mycosphaerella speckle (caused by *Mycosphaerella musae*) followed by Sigatoka disease. In the Pacific region Sigatoka disease was reported from the Sigatoka Valley in Fiji in 1912 (Philpott and Knowles, 1913). Black leaf streak disease, caused by *Mycosphaerella fijiensis*, was recorded later, in 1963 (Rhodes, 1964), but has since become the dominant leaf disease in the region.

The integrated approach used to control *Mycosphaerella* pathogens involves the use of cultural practices and fungicides. In wet tropical areas, fungicide sprays are applied at 10-14 day intervals during the wet season (December–April) and 14-28 days during the remainder of the year, for a total of 18-24 applications per year. Mancozeb is the main protectant fungicide used, and the application rates and length of withholding periods vary depending on the formulation used. Systemic fungicides are used predominately during the wet season when conditions are more conducive to

disease development. Current registrations include propiconazole (Tilt®, Bumper® and Aurora®), tebuconazole (Folicur®) and benomyl (Benlate®). Cultural practices used to minimize the risk of disease development include good drainage and air circulation, location (not adjacent to a permanent body of water, buildings or rainforest) and most importantly inoculum reduction (deleafing).

Loss of sensitivity to fungicides

Sensitivity of *M. musicola* populations to systemic fungicides was assessed using a technique based on the Fungicide Resistance Action Committee (FRAC) guidelines, but using conidia instead of ascospores. Conidia are produced at an earlier stage of disease development than ascospores and are readily available during the winter and spring months. Ascospores are produced in summer and autumn and are not readily available during the remainder of the year due to phytosanitary regulations and deleafing practices. For the triazole fungicides (propiconazole and tebuconazole) germtube growth after 72 hours was expressed on a sensitivity graph (germtube growth at 4 fungicide concentrations in relation to growth in the absence of the fungicide). For benomyl, percent germination was recorded after 48 hours. Sensitivity graphs were used to establish the effective concentration required to give a 50% reduction in germtube growth (EC50).

Baseline data was established for each fungicide by averaging the EC50 of more than 20 populations of *M. musicola*. These were selected from unsprayed plants at least 25 km away from the nearest commercial/sprayed block of bananas. The sensitivity of populations from fungicide-treated plots was assessed by comparing the graph and the EC50 with the baseline average. An 8 to 16-fold increase in the EC50 is considered a moderate shift while a more than 16-fold increase is a severe shift in sensitivity.

A shift in sensitivity was first detected with propiconazole in 1995. In limited surveys over the last 2 to 3 years, moderate to severe shifts in sensitivity to triazole fungicides were detected:

- Shifts in sensitivity to tebuconazole were detected in populations that had not been sprayed with tebuconazole, but with propiconazole;
- Cross resistance from propiconazole to tebuconazole is 100%, but the reverse is variable;
- Shifts in sensitivity to triazoles occurred in isolated plantations and was linked to excessive use (8-12 applications), the number of consecutive applications (3-5) and applications to heavily diseased plants (no deleafing);
- Shifts in sensitivity were reversed when the product or similar products were withheld for 6-12 months;
- Resistance to benomyl was high across most populations.

Strategies to minimise the risk of resistance to fungicides based on FRAC guidelines were developed and implemented in 1996 in consultation with growers and government representatives. Some of the cultural and chemical related strategies included:

- Regular deleafing to remove heavily infested leaf material prior to the application of a systemic fungicide;

- A maximum of 6 triazole fungicides to be used in any one season;
- A maximum of 2 consecutive applications of triazoles;
- A 4-month triazole-free period from July to October.

Black leaf streak disease

Black leaf streak disease was first identified in Australia in 1981 at Bamaga on the tip of the Cape York Peninsula and throughout the Torres Strait area. It has since been found at 7 other locations in the sparsely populated Cape York area. Areas affected have ranged from only a few plants to an entire residential area (Weipa, population 3000), and to a 36-ha commercial plantation of organically grown bananas. In all cases the outbreaks were eradicated by destroying all plant tissue at the site of the infection and within a surrounding buffer area. Plants were destroyed by burying, ploughing or burning. In most cases the destroyed plants were replaced with resistant/tolerant banana cultivars. Regular intense surveys have failed to detect black leaf streak disease at any of these sites except at Bamaga. Bamaga was the first attempt at eradication and the program has been refined considerably over the past 15-20 years.

In April 2001, *M. fijiensis* was detected in the Tully area where approximately 55% of northern Queensland bananas are produced. An intensive survey indicated the disease was restricted to the Tully Valley area and was only a recent introduction (6-12 months). The identification of the organism was initially complicated by the lack of conidia and sporodochia due to heavy rainfall in the area. Drying and wetting of leaf material failed to produce the sporulating structures required for microscopic identification. All samples were therefore visually assessed and suspicious samples were analysed by using the PCR (Polymerase Chain Reaction) method. Dr Juliane Henderson from the Cooperative Research Centre for Tropical Plant Protection in Brisbane modified and refined the PCR protocol based on methods published by the Natural Resource Institute (Johanson, 1997). In the 12 months to 31st March 2002, more than 8600 banana leaf samples have been assessed and 2979 samples have been PCR tested. Black leaf streak disease has been positively identified on only 13 commercial properties and on 12 clumps of unmanaged banana plants.

An eradication programme was started in September 2001 and was based on:

- *M. fijiensis* having no dormant structures;
- *M. fijiensis* only affects *Musa* species. There are no alternative hosts;
- *M. fijiensis* survives in leaf tissue:
  - >20 weeks in leaf in canopy (Gauhl, 1994);
  - 4-8 weeks in leaf tissue in contact with the ground (Peterson *et al.,* 1998);
- Deleafing and placing leaves in piles reduces by about 80% the potential of inoculum production.

Programme consisted of three components:

- Maintaining all commercial banana plantations (4500 ha) at zero visible disease levels for 6-8 months to ensure all inoculum is destroyed;

- Weekly spray programme (systemic and protectant) for at least 6 months to prevent any ascospore release from plant remains establishing new infections;
- Eradication/destruction of all non-managed plants (not sprayed or deleafed) in the entire area.

Results

In commercial plantations:

- All blocks were monitored (every 2nd row) at 4 to 6-week intervals;
- >95% of the area have had zero visible disease since December 2001;
- Over 8600 samples have been collected and examined:
  – No black leaf streak disease has been recorded since August 2001.

In un-managed plants:

- > 28 000 stems and > 22 000 suckers have been destroyed;
- No black leaf streak disease has been recorded since November 2001;
- All land plots (8500) have been visited and revisited between January to March 2002, and 6382 stems and 6610 suckers have been destroyed;
- No black leaf streak disease has been found in the 6382 stems.

Spray programme:

- 13 systemic and 14 protectant fungicide sprays have been applied from August 2001 to February 2002;
- Oil caused damage when applied in hot dry conditions to plants that were water-stressed;
- Trifloxystrobin (temporary registration) caused considerable damage when applied in hot conditions. Damage was reduced when applied early morning or late afternoon.

The success of this programme can be attributed to the full participation of growers in combination with the unseasonable dry weather conditions experienced between August 2001 and April 2002. Conditions during this period were not conducive to the development of Sigatoka diseases.

Future plan – establish disease-free areas

Sentinel plant network:

- >130 sites of 8-10 plants have been established throughout the area on a 1-km grid near sites where black leaf streak disease has been confirmed. Spacing was increased to a 10-km grid when >15 km from a known black leaf streak disease site. All plants are inspected and sampled for disease identification at 4-week intervals.

Commercial plantations:

- Monitoring of commercial plantations is to be conducted at 6 to 8-week intervals and all diseased leaves are to be sampled;
- Sites where un-managed plants have been destroyed are to be inspected for regrowth. All diseased tissue is to be collected for identification;
- A period of 12 months, including an average wet season, without an outbreak should demonstrate an area free of black leaf streak disease.

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Spread and management of black leaf streak disease in the Dominican Republic

P.E. Jorge and T. Polanco

Abstract
In the Dominican Republic, black leaf streak disease, caused by *Mycosphaerella fijiensis*, was first identified in 1996 in the province of Montecristi, on the Northwest side of the country, a region with prevalent dry conditions. It was then identified in Hato Mayor on the southeast side on July, 1998. The disease appeared in 1999 in the Provinces of Sánchez Ramírez, Samaná, Dajabón, Santiago Rodríguez and Monte Plata, located on the Northwest and East sides, suggesting spread to these areas from the first two identified regions. In the year 2000, it was first identified in the Southwest, in the Provinces of Azua and San Juan de la Maguana. Today, black leaf streak disease continues to spread, moving to the central portion of the country, the largest plantain growing area, where favorable environmental conditions are common.
Previous to the appearance of black leaf streak disease, measures regarding management of Sigatoka disease were not very intensive. After the appearance of black leaf streak disease in 1996 growers have changed their practices to compensate for the presence of the disease. Currently, the management methods adopted by growers include deleafing, application of fungicides, fertilization and, to a lesser extent, planting tolerant-resistant materials, mostly FHIA hybrids. Deleafing and application of fungicides are done on a routine basis, determined by climatic conditions. Deleafing is usually done weekly during the rainy seasons and monthly otherwise. A similar pattern is observed for the application of fungicides. Fertilization is implemented to assure a prompt recovery from the stress induced by the disease. Few growers rely on climatic/biological disease forecasting strategies.

Resumen – Propagación y manejo de la Sigatoka negra en República Dominicana
En República Dominicana, la Sigatoka negra, causada por *Mycosphaerella fijiensis*, fue identificada por primera vez en 1996 en la provincia de Montecristi, en el noroeste del país, una región con condiciones secas prevalecientes. Se identificó en Hato Mayor en la parte sudeste en julio de 1998. En 1999, la enfermedad apareció en las provincias de Sánchez

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Ramírez, Samaná, Dajabón, Santiago Rodríguez y Monte Plata, localizadas en el noroeste y oriente, sugiriendo su propagación a estas áreas de las dos regiones identificadas inicialmente. En el año 2000, la enfermedad fue identificada por primera vez en el sudoeste, en las provincias de Azua y San Juan de la Maguana. Actualmente, la Sigatoka negra continua propagándose, moviéndose a la parte central del país, la principal área bajo el cultivo de plátano, donde existen condiciones ambientales favorables.
Previas a la aparición de la Sigatoka negra, las medidas con respecto al manejo de la Sigatoka amarilla no fueron muy intensivas. Después de la aparición de la Sigatoka negra en 1996 los productores han cambiado sus prácticas de cultivo para compensar la presencia de la enfermedad. Actualmente, los paquetes de manejo de la enfermedad adoptados por los productores consisten en el deshoje, aplicación de fungicidas, fertilización y, en un menor grado, siembra de materiales tolerantes o resistentes, principalmente los híbridos de la FHIA. El deshoje y la aplicación de funguicidas son actividades que se realizan sobre una base habitual determinada por las condiciones climatológicas. Los deshojes se realizan aproximadamente cada semana durante las estaciones lluviosas, y mensualmente durante las estaciones secas. Un comportamiento similar se observa para la aplicación de los fungicidas. La fertilización se implementa para asegurar una rápida respuesta y recuperación del estrés inducido por la enfermedad. Pocos productores confían en estrategias de preaviso biológico y climatológico de la enfermedad.

Résumé – Propagation et gestion de la maladie des raies noires en République Dominicaine
En République Dominicaine, la maladie des raies noires, causée par *Mycosphaerella fijiensis*, a été identifiée pour la première fois en 1996 dans la province de Montecristi, dans la partie nord-ouest du pays, une région où les conditions de sécheresse prévalent. Elle a été ensuite identifiée à Hato Mayor sur la côte sud-est en juillet 1998. La maladie est apparue en 1999 dans les provinces de Sánchez Ramírez, Samaná, Dajabón, Santiago Rodríguez et Monte Plata, situées dans les parties nord-ouest et est, ce qui suggère une propagation vers ces zones depuis les deux premières régions identifiées. En 2000, la maladie a d'abord été identifiée dans le sud-ouest, dans les provinces d'Azua et San Juan de la Maguana. Aujourd'hui, la maladie des raies noires continue de s'étendre, en se dirigeant vers la partie centrale du pays, la plus grande zone de culture des bananes plantain, dans laquelle des conditions d'environnement favorables sont fréquemment rencontrées.
Avant l'apparition de la maladie des raies noires, les mesures visant à contrôler la maladie de Sigatoka n'étaient pas très intensives. Après l'apparition de la maladie des raies noires en 1996, les planteurs ont modifié leurs pratiques pour compenser la présence de la maladie. Aujourd'hui, les méthodes de gestion adoptées par les planteurs incluent l'effeuillage, l'application de fongicides l'application d'engrais et, dans une moindre mesure, l'utilisation de plants résistants/tolérants, principalement des hybrides de la FHIA. L'effeuillage et l'application de fongicides sont réalisés de manière routinière, en fonction des conditions climatiques. L'effeuillage est effectué de façon hebdomadaire pendant la saison des pluies, et mensuellement le reste du temps. La même périodicité est utilisée pour l'application des fongicides. La fourniture d'engrais est réalisée afin d'assurer une récupération rapide du stress induit par la maladie. Seul un petit nombre de planteurs ont recours à des stratégies de prévision du développement de la maladie basées sur des facteurs climatiques/biologiques.

Introduction

The Dominican Republic occupies the eastern two thirds of the Caribbean island of Hispaniola and has an area of 48 422 km^2. The Republic of Haiti occupies the remainder of the island. Both countries are divided by the Cordillera Mountains which

reach 3175 meters and separate the southern and northern parts of the island. The mountains are important barriers for the natural movement of inoculum of black leaf streak disease (teleomorph: *Mycosphaerella fijiensis* Morelet; anamorph: *Paracercospora fijiensis* (Morelet) Deighton), and the movement of plant material between the two regions. Banana and plantain production is mainly in the southwestern and northern parts of the country. Thus, production of *Musa* species is isolated and growers from either side of the mountains do not exchange planting material and there is little commercial exchange of fruits.

The country is divided into eight regions by the Ministry of Agriculture and *Musa* production occurs in all regions. Banana production is mainly in the Southwest and the Northwest and plantain production is mainly in the Cibao Central, including the Northeast, North and North Central regions, where a large proportion of the population lives. Plantain is the third most important vegetable crop, after rice and beans. Plantain is consumed within the country, whereas a large part of the bananas, whether grown by using conventional or organic methods, are exported.

The average rainfall from 1961 to 1990 (Secretaría de Estado de Agricultura, 1998) from the different regions are shown on Table 1. Full expression of black leaf streak disease in the Dominican Republic is dependent on rainfall and humidity, as temperatures in banana and plantain areas do not limit the development of the disease. Figure 1 shows symptoms of the disease in dry and humid regions. Generally, the disease is limited to the first stages in dry regions and fully expressed in humid regions.

Humid regions Dry regions

Figure 1. Symptoms of black leaf streak disease in the dry and humid regions of the Dominican Republic.

Table 1. Average rainfall in the Dominican Republic by region for the period between 1961 and 1990.

Region	Rainfall (mm/year)
North central	1200-1350
Northeast	1200-1350
North	1200-1350
East	1342-1583
Central	1400-1800
Northwest	< 600
South	< 600
Southwest	665

Source: Secretaría de Estado de Agricultura, 1998. Anuario estadistico de planificación sectorial agropecuaria.

The distribution of plantain and banana crops is shown in Table 2 (Secretaría de Estado de Agricultura, 1998). More information on farm size and number of farmers can be found in Perez (2000), Departamento de Sanidad Vegetal (2000) and Secretaría de Estado de Agricultura (2000).

Table 2. Area planted with plantain and banana in the Dominican Republic.

Region	Plantain (ha)	Banana (ha)
South	12 500*	3105
Southwest	5764	5722
Central	3750	-
East	625b	-
North Central	14 333	2062
Northeast	3139	4280
North	9000	3585
Northwest	2876	6201
Total	51 987	24 955

Source: Secretaría de Estado de Agricultura, 1998

* Personal communication, Secretaría de Estado de Agricultura.

Pathogen identification

The spread of black leaf streak disease in the Americas, and the appearance of the disease in Cuba in 1990 (Vidal, 1992) and in Jamaica in 1994-1995 alerted the Dominican authorities. In 1995, the Department of Plant Protection, *Secretaría de Estado de Agricultura de la República Dominicana*, started a project to monitor the disease, in collaboration with Haiti's phytosanitary authorities and the Animal and Plant Health Inspection Service of the United States Department of Agriculture (APHIS/USDA).

Black leaf streak disease was first recorded in 1996 in Guayubín, province of Montecristi, at a farm of 125 ha planted with banana and plantain. Distribution within the farm was limited mainly to plantain. At the time, *M. fijiensis* was identified by the Plant Pathology Laboratory at CIRAD/AMIS, Montpellier, France. In May 1997, the pathogen was also identified, with the assistance of Mary E. Palm from the United States Department of Agriculture, as *P. fijiensis* based on the morphology of the conidia and conidiogenous cells in her report to the *Secretaría de Estado de Agricultura*. This was the first official diagnosis of the causal agent of black leaf streak disease in the Dominican Republic. Also in 1997, Fouré surveyed the different production areas to determine the distribution and incidence of black leaf streak disease in the country (Fouré, 1997).

In 1999, T. Polanco, in collaboration with Jean Carlier and Marie Zapater from CIRAD, isolated *M. fijiensis* from the northwestern, northeastern and central eastern regions of the Dominican Republic (Polanco, 1999). Rivas *et al.* (2001) compared the isolates with those from South America, Central America and the Caribbean. The isolate from the Dominican Republic was found to be closely related to the Cuban isolate.

Spread of black leaf streak disease

The spread of black leaf streak disease in the Dominican Republic up to May 2002 is shown in Figure 2.

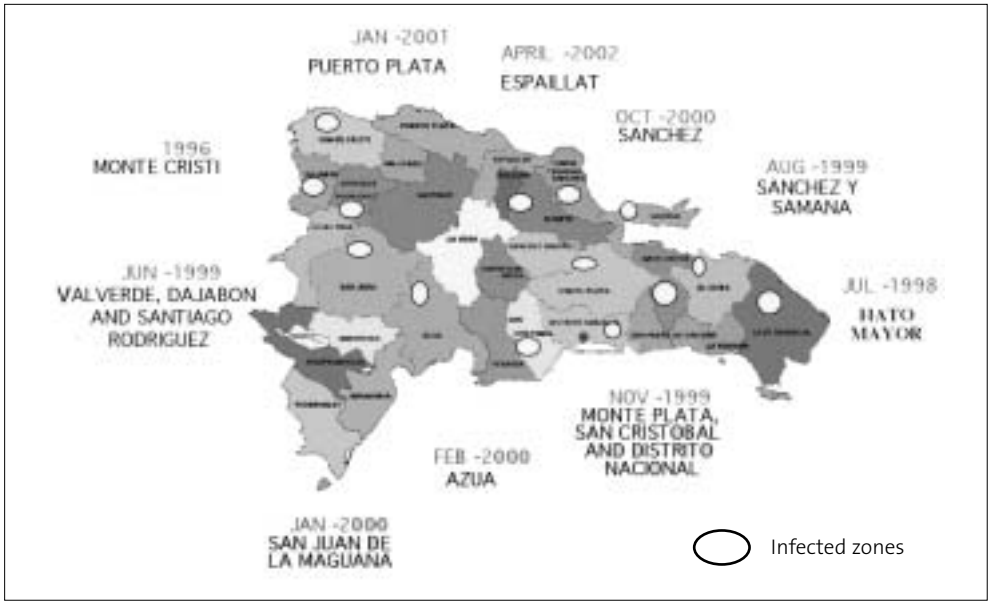

Figure 2. Spread of black leaf streak disease in the Dominican Republic up to May 2002.

Northwestern region

The northwest region has approximately 6250 ha of banana and 2876 ha of plantain (Table 2). Bananas are mostly grown for export to Europe, where there is a market for traditional and organic fruits.

The northwestern region of the Dominican Republic where black leaf streak disease was first identified in 1996, is mostly characterized by environmental conditions that do not favour the development of the disease. The rainy-humid conditions that favour disease development prevail for only short periods, usually in May and November-December. There were less than 600 mm of rain/year for the period 1961-1990, usually distributed over 70 days per year (Secretaría de Estado de Agricultura, 1998). Thus, most of the region is considered to be low risk for black leaf streak disease because of the climate.

In June 1999, black leaf streak disease was identified in the Provinces of Valverde, Dajabón, Santiago Rodriguez and other communities of Montecristi (Polanco 1999).

Eastern region

The eastern region includes the provinces of Hato Mayor, La Altagracia, El Seibo, La Romana and Higuey. Rainfall is an average of 1342-1583 mm per year distributed

over 122-137 days (Secretaría de Estado de Agricultura, 1998). Because of the favourable conditions, black leaf streak disease has caused considerable damage in the region. Many growers have changed from plantain to another crop and the plantains harvested are very small.

Black leaf streak disease was identified in the eastern region in the Provinces of Hato Mayor and El Seibo in the communities of Hato Mayor, Sabana de la Mar and El Valle, respectively (Figure 2). The disease was identified in 1998, i.e. two years after the original identification in the northwest and it appears that the disease had been introduced through planting material.

Before the occurrence of black leaf streak disease in 1998, approximately 1875 ha of plantain were grown in Hato Mayor and El Seibo but today, only 625 ha are planted with the crop because of the disease. Even though the area and the production are small, plantain is of economic and social importance for the region. Previously the crop supplied the regional market and provided food and economic support to several thousand small-scale farmers. Today, demand for plantain is satisfied by supply from other producing areas.

The hybrid 'FHIA-21' is being introduced to this region as an alternative, but acceptance has been limited.

Central region

The central region includes the provinces of Monte Plata, San Cristóbal and Santo Domingo-Distrito Nacional and production of plantain is approximately 3750 ha (Table 2). Rainfall is an average of 1400-1800 mm/year (Table 1).

The disease appeared in 1999 in the three provinces, and affected the communities of Bayaguana, Monte Plata, San Cristobal, Yamasá and Villa Mella. The occurrence of the disease appeared to have been caused by hurricane George in September 1998. Hurricane George moved from the southeast to the southwest and winds blew strongly into the middle of the island, suggesting that introduction was from the south region, which was already affected.

Southwestern region

The southwestern region includes the provinces of Azua and San Juan de la Maguana. Approximately 5722 ha of banana, mostly organic (Azua) and 5764 ha of plantain are grown in the region (Secretaría de Estado de Agricultura, 1998; Departamento de Sanidad Vegetal, 2000).

Annual rainfall for the region was an average of 665 mm in 1961-1990 (Table 1) and was mostly distributed over 62 days, mostly in November-December. Thus, the region is at low risk from outbreaks of black leaf streak disease (Secretaría de Estado de Agricultura, 1998).

Black leaf streak disease was reported on plantain from a small locality in the province of San Juan de la Maguana in January 2000; however, production in the province is not important. The disease was identified on organic bananas in Azua, in February 2000. Spread of the disease in Azua has been limited by the weather conditions; in contrast to Sigatoka disease which is moderately prevalent.

North, north-central and northeastern regions (Cibao Central)

The Cibao Central includes the north-central, northeastern and north regions, and includes the provinces of La Vega, Espaillat, Sánchez, Samaná, Puerto Plata and others. Production of plantain is 26 472 ha (Table 2) and of banana 9927 ha. Both crops are mostly produced without irrigation, and are dependent on rainfall for the water supply.

These regions have an average rainfall of 1200-1350 mm/year, with 122-173 days of rain per year (Secretaría de Estado de Agricultura, 1998). These conditions suggest that black leaf streak disease has the potential to cause considerable damage to plantain.

In August 1998, black leaf streak disease was found on plantain in the northeastern region in the provinces of Sánchez and Samaná but not in the north-central and northern regions. In 2001, symptoms were recognized on plantain in the community of La Isabela, province of Puerto Plata in the northern region. The disease has caused considerable damage to the point where crops have been abandoned, or deleafing and fungicide treatments implemented to ensure production, as was the case in La Isabela.

In March 2002, black leaf streak disease was identified in the north-central region in the community Hoya del Camú, province of La Vega, and in April 2002 in the north in the community of Moca, province of Espaillat. In both instances the disease affected plantain. In Moca, symptoms were diagnosed at a farm of 1.5 ha. Information from the growers suggests that the disease was introduced through plant material from infected areas.

Southern region

In the provinces of Barahona and Neiba, most of the area planted with plantains is irrigated. In this region, 5807 hectares of plantain are irrigated. Rainfall in 1961-1990 was on average less than 600 mm/year and was distributed over 48-69 days (Secretaría de Estado de Agricultura, 1998). As a dry region, there is little likelihood of serious damage from black leaf streak disease, unless favourable conditions were to occur for prolonged periods.

Management of black leaf streak disease

In general, management of black leaf streak disease in the Dominican Republic depends on the pressure exerted by the disease. The type of crop (banana or plantain), the size of the production unit, whether production is conventional or organic and the prevailing environmental conditions determine the techniques used to control black leaf streak disease. A technical package was only adopted in response to the spread of the disease within the country. Limited preventive measures had been taken when the disease was not present.

Cultural practices

Banana growers and, to a lesser extent, plantain growers have adopted the practice of deleafing. This is done every 7-10 days during the rainy season when the disease pressure is high, and every 30 days in the dry season when disease pressure is low.

In the eastern region, where environmental conditions favour the disease and the pathogen is present, only growers who have adopted deleafing and biological monitoring of the diseased have survived. Production of plantain in the region declined from 1875 ha before the appearance of black leaf streak disease in 1998 to 625 ha today.

Chemical control

Conventional banana production is dependent on the use of fungicides, e.g. triazoles, dithiocarbamates, benzimidazoles, mineral oils and to a lesser extent, strobilurins (Polanco, 1998). Organic banana growers use mineral and vegetable oils and organic products, e.g. citric acid and waxes. Preliminary data obtained by Polanco (personal communication) showed that there was no difference between mineral oil alone and mixtures of oil with the organic products. This work is to be repeated.

Conventional and organic banana growers spray between 7 to 10 times a year, and sometimes up to 15 times, depending on rainfall. Sometimes treatments are not effective or may have been unnecessary because of faulty forecast or monitoring.

Large-scale banana plantations, conventional or organic, are usually sprayed by plane in the northwestern and south-western regions. Small-scale growers use motorized high-pressure pumps. In general, plantain growers do not use fungicides.

Resistant hybrids

FHIA hybrids, especially 'FHIA-21' and to a lesser extent 'FHIA-20', were introduced before the identification of the disease in the country. At present, there are 600 000 to 800 000 'FHIA-21' plants in affected and not affected areas, representing less than one percent of the total planted area. Production is for the fresh and industrial markets.

Research plots have been established to compare native plantains, 'FHIA-21' and 'Rulo' for their response to the technical package needed for a successful crop of 'FHIA-21' and to different management practices including deleafing and fertilizer treatment. When black leaf streak disease is absent, cultural practices and chemical control are not implemented, unless if there is a high incidence of Sigatoka disease.

As a preventive measure, the replacement or planting of new areas with the resistant clone 'FHIA-21' has recently been recommended and plants are available from the Ministry of Agriculture. Acceptance of 'FHIA-21' by growers has been limited mainly because the most important plantain production areas do not have the disease or the spread is very limited, and consumers prefer the fruit of local cultivars.

Little has been done to diversify the gene pool of banana, and production is entirely with 'Cavendish' cultivars. This is mainly because of the requirements of the banana market and the prevalence of dry conditions in the banana production areas.

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Microbiological control of black leaf streak disease

A. S. Riveros, C. I. Giraldo and A. Gamboa

Abstract
Isolates of bacteria from the phyllosphere of tomato and banana were obtained from CATIE: *Bacillus cereus, Bacillus* sp., *Serratia marcescens, Serratia entomophila*, unidentified strains with glucanolytic and chitinolytic capacity (GS2-GS3-GC1-GBC2, SE/PO_2, White) and one isolate of *Mycosphaerella fijiensis* collected recently in Turrialba, Costa Rica. Crude culture filtrates of some microorganisms inhibited ascospore germination and the growth *in vitro* of *M. fijiensis* colonies. The two filtrates with the greatest effect resulted in changes to the ultrastructure of *M. fijiensis* hyphae, when examined under a scanning electronic microscope, in comparison with untreated tissue.

Resumen – Control microbiológico de la Sigatoka negra
Los aislados de la colección del CATIE obtenidos de la filosfera de tomate y hojas de banano: *Bacillus cereus, Bacillus* sp., *Serratia marcescens, Serratia entomophila* y cepas no identificadas con capacidad glucanolítica y quitinolítica (GS2-GS3-GC1-GBC2, SE/PO_2, White) y un aislado de *Mycosphaerella fijiensis* recolectado recientemente en Turrialba, Costa Rica, fueron utilizados para preparar filtrados de los cultivos líquidos. Los filtrados crudos de estos microorganismos se evaluaron bajo condiciones *in vitro* con el fin de determinar la germinación de las ascosporas y el crecimiento de las colonias de *M. fijiensis* (agente causal de la Sigatoka negra en banano y plátano). Los resultados muestran un efecto inhibitorio importante de algunos de estos filtrados en diferentes etapas de desarrollo de *Mycosphaerella*. La observación, bajo un microscopio electrónico de barrido, de las estructuras del hongo tratado con dos filtrados prometedores, muestra claras alteraciones de ultraestructura en el tejido tratado en comparación con el testigo sin tratamiento.

Résumé – Lutte microbiologique contre la maladie des raies noires
Des isolats de bactéries de la phyllosphère de tomates et de bananiers ont été obtenus du CATIE : *Bacillus cereus, Bacillus* sp., *Serratia marcescens, Serratia entomophila*, des souches non identifiées ayant une capacité glucanolytique et chitinolytique (GS2-GS3-GC1-GBC2, SE/PO_2, White) et un isolat de *Mycosphaerella fijiensis* collecté récemment à Turrialba, au Costa Rica. Les filtrats bruts de certains micro-organismes ont inhibé la germination des ascospores et la germination *in vitro* de colonies de *M. fijiensis*. Les deux filtrats qui ont eu le plus d'effet ont induit des changements de l'ultrastructure des hyphes de *M. fijiensis*, en microscopie électronique à balayage, par rapport aux tissus non traités.

CATIE, Turrialba, Costa Rica.

Introduction

During the seventies, agriculture was characterized by an undiscriminated use of agrochemicals. The situation has changed little since then, but international and national regulations have imposed changes aimed at reducing pollution and making agriculture sustainable. There are two types of agriculture: 1) high input agriculture characterized by high productivity that is limited by environmental factors and 2) low input agriculture with production that, in addition to environmental factors, is limited by pests, diseases and weeds.

Changes in the market and the influence of globalization are forcing a reconsideration of research in agriculture. The preferences of consumers play an increasingly important role that affects the work of multidisciplinary teams made up of researchers, ecologists, producers and industry.

The Tropical Agricultural Research and Higher Education Center (CATIE) has defined one of its research objectives as the implementation of methodologies focused on the biological control of the most common diseases and pests affecting economically important tropical crops, such as those belonging to the Musaceae family.

CATIE started studies on *Musa* and *M. fijiensis* with a project financed by AID/ ROCA (USA) the first phase of which started in July 1984. Other projects which followed were financed by RENARM (USA), CIRAD (France), INIBAP, INCO-*Musa*, Natural Resource Institute (NRI; UK), CINVESTAV (Mexico) and FONTAGRO.

These collaborative projects not only spurred research on biological control but also on the control of black leaf streak disease, a disease that was already threatening banana and plantain production. Other outcomes were the development of systems for somatic embryogenesis, cell suspensions, plant pathology, cryopreservation, genetic transformation and the genetics of *M. fijiensis* populations.

Since then, the Plant Protection Unit at CATIE has developed integrated pest management (IPM) practices for black leaf streak disease based on the preservation of the environment, reduced risks to farmers, the rural population and consumers, and the sustainability of traditional agriculture. Countries included within the CATIE mandate have a rich biodiversity which may contain materials or products, e.g. genes of wild plants or biopesticides, that might be useful in IPM programmes.

Research into the biological control of black leaf streak disease at CATIE encouraged researchers involved in the AID/ROCAP-USA project, e.g. Dr Elkin Bustamante and his team who were the first to work on the project. After a careful study of the different aspects of the parasitic relationship between *Musa* and *M. fijiensis*: the biology and morphology of the pathogen, the phenology and physiology of the plant, the phyllosphere, soil (importance of rhizobacteria, endophytic fungi and organic amendments), and methods of internal and external inoculation, a research programme was constructed to better study these aspects (Figure 1).

Step 1. Identification of antagonistic microorganisms

The purpose was to isolate microorganisms antagonistic to *M. fijiensis* and to evaluate their effectiveness under greenhouse and field conditions. One hundred and twenty isolates with chitinolytic activity were obtained from plants of cv. 'Grande naine' coming

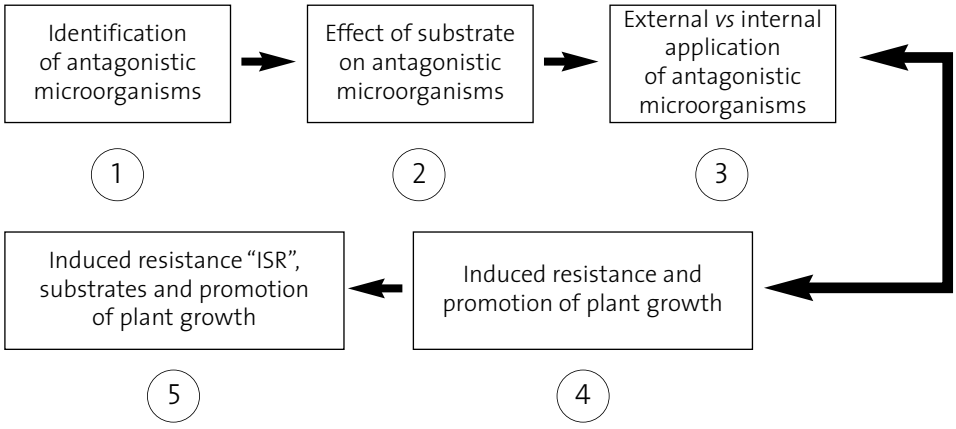

Figure 1. Steps involved in research on biological control at CATIE (1986-2001).

from two different locations: an area of high incidence of black leaf streak disease and an area of low incidence of the disease. The 'low incidence site' provided the highest population of microorganisms, which was evaluated on chitin agar. Thirteen bacterial strains were selected on the basis of their chitinolytic activity (*Serratia marcescens*, *Serratia entomophyla* and *Bacillus* spp.). Under greenhouse and field conditions the level of control of *M. fijiensis* was 40% in comparison with a level of 60% using fungicides (González, 1994; González *et al.* 1996).

Regarding glucanolytic activity, 196 strains of bacteria were collected from plants of cv. 'Grande naine' of which 37 belonged to the genus *Bacillus*. The microorganisms were purified and evaluated on glucan-agar and glucan nutrient-agar media. Seven strains with glucanolytic activity were selected. GS2, GBC2, BS3 and BC1 showed antagonistic effects, inhibiting germination of *M. fijiensis* ascospores in 25% of the cases and reducing germination tube length in 47%. Four strains were tested in the presence and absence of glucan. Commercial glucan being expensive, a common source of glucan from agricultural waste was used (Talavera-Sevilla, 1996; Talavera *et al.*, 1998a, b).

Step 2. Effect of substrate on antagonistic bacteria

The effect of different substrates on the growth and survival of antagonistic bacteria were investigated. The aim was to modify the physical and nutritional conditions in order to inhibit germination and establishment of the pathogen and favour antagonistic organisms.

The bacterial strains used were: *Serratia marcescens* R1, *Serratia entomophila* A100 and *Bacillus cereus* A30. The substrates tested, singly or in combination, were leaf extract, milk, foliar fertilizers, molasses, cassava starch, glucan and chitin. The highest recovery level of bacteria was observed in molasses which had positive effects on antagonistic microorganisms. A combination of milk and molasses increased multiplication and survival of R1 and A30 (Ruiz-Silvera *et al.*, 1997a, b).

Plants treated with a combination of chitin, yeast and calcium nitrate alternated with commonly used fungicides, reduced the number of fungicide treatments by 40% in comparison with fungicides alone (Arango-Ospina, 2000).

Step 3. Internal vs external application of antagonistic microorganisms

The objectives of the study were to evaluate the effects of R1 and A30 applied externally in combination with Silwet L-77, Nu-Film 17 and mineral oil, and to evaluate an endophytic inoculation method via the roots. R1 was compatible with stickers, mainly mineral oil. Mineral oil in combination with antagonistic microorganisms reduced disease severity in comparison with the controls. The best colonization of internal tissues was when A30 was applied directly inside the plant (Miranda, 1996).

Step 4. The phenomenom of induced resistance and promotion of plant growth

Stimulation by pathogens, non-pathogenic microorganisms, and by substances of biological or non-biological origin can induce resistance in susceptible plants Induced resistance to disease and growth promotion have potential for controlling disease (Figure 2).

Four bacterial and one fungal suspensions were applied to the rhizosphere; KH_2PO_4 and K_2HPO_4 solutions were applied to the leaves as abiotic exogenous inducers. In a second experiment, microorganisms were evaluated with the addition of sugarcane pulp, sugarcane filter press and coffee husks to the rhizosphere. *Pseudomonas fluorescens* (PRA25), *P. cepacia* (AMMD) and *Trichoderma harzianum* (Th) plus substrates increased growth the most and reduced disease in comparison with the controls (water and substrates). However, the lowest percentage was obtained with propiconazole (Tilt®). There was a significant and negative correlation between them (Gutiérrez, 1996).

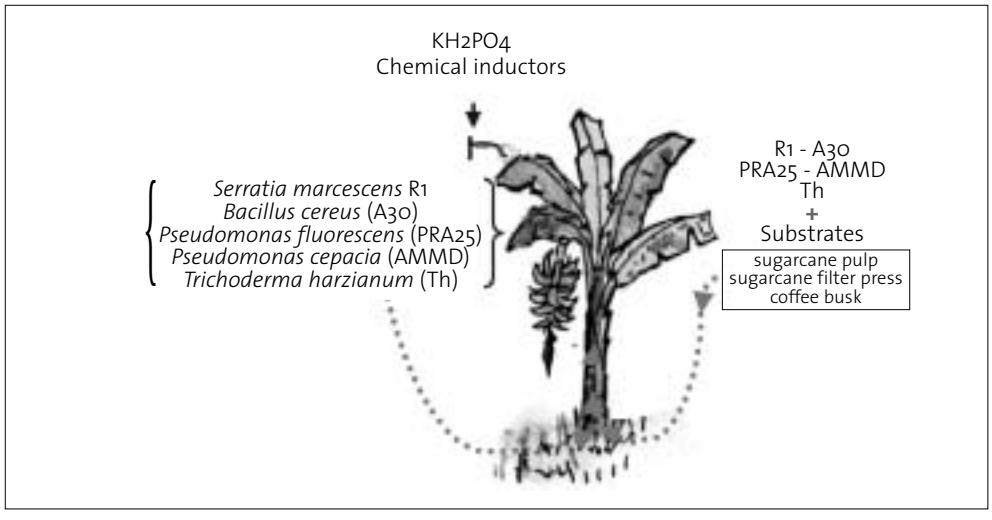

Figure 2. Illustration of the concept of induced resistance to control black leaf streak disease (© S. Belalcázar, 2002).

Step 5. Induced systemic resistance (ISR)

Investigations improved the understanding of the use of organic amendments, antagonistic microorganisms, substrates as energy sources in combination with mycorrhizal fungi and organic extracts known as efficient microorganisms, EMs (Okumoto *et al*., 2001; Ayuso, 2000; Sanchez Garita *et al*., 1998; Okumoto, 1992).

Induced systemic resistance (ISR) to disease results from the inoculation of lower leaves, or roots, with restrictive pathogens, non-pathogenic races of pathogens, non-pathogens, products of pathogen or non-pathogens, and organic or inorganic chemicals. ISR is also referred to in the scientific literature as SIR or SAR (Ku,? 2001).

The objective of the research was to evaluate, under greenhouse conditions, the response of cv. 'Grande naine', as an example of a banana cultivar susceptible to black leaf streak disease, and of 'FHIA-23' as an example of a clone resistance to the disease, in the presence of 3 resistance inducers and one foliar substrate as an energy source. The inducers were PRA25 bacteria and culture filtrate from germinating spores of *M. fijiensis* strains according to Riveros and Lepoivre (1998), and Acilbenzolar-S-metil (BION®,), a synthetic inducer provided by Syngenta. ISR was higher in 'FHIA-23' than in cv. 'Grande naine'.

Vermicompost increased ISR. BION® resulted in high ISR in both cultivars. Rhizobacteria and *M. fijiensis* filtrate induced resistance only in 'FHIA-23' when in the presence of an energy source. In the field, BION® reduced disease incidence in cv. 'Grande naine' in comparison with conventional control measures (Patiño, 2001).

Massive applications of antagonistic bacteria or fungi on crops could have unforeseen effects on the environment. The objectives of the study were to evaluate the *in vitro* biological activity of microbiological filtrates on a *M. fijiensis* ascospore preparation, the growth of *M. fijiensis* colonies, and the effects of two filtrates on the cell structure of *M. fijiensis*. Emphasis was put on the isolation of strains with glucanolytic and chitinolytic activity.

Materials and methods

Bacterial strains used in this study were obtained from CATIE's Plant Protection Unit Collection:

- *Bacillus cereus* (A30), isolated from tomato leaves (*Lycopersicon pimpinelli*), Turrialba, Costa Rica (Okumoto, 1992).
- *Serratia marcescens* (R1), isolated from banana leaves (*Musa* sp.), Limon province, Costa Rica (González, 1994).
- *Serratia entomophila* (SE), isolated from Canterbury Valley (New Zealand) from the digestive tract of the scarabid *Costelytra zealandica* (donated by Trevor Jackson from the AgResearch Lincoln Laboratory in 1994).
- GS2, GS3, GC1, GBC2, bacteria with glucanolytic activity isolated from banana leaves, Indiana farm, Siquirres, Costa Rica (Talavera-Sevilla, 1996).
- SE/PO2, isolated from deep well water, Carmen de Siquirres, Costa Rica (Gamboa, personal communication).

• White, chitinolytic bacteria, isolated from plantain leaves, La Montaña farm, CATIE, Costa Rica (Arango-Ospina, 2000).

• Extracts of the fluid obtained from suspensions of the conidia of *M. fijiensis* isolated from La Montaña farm, Turrialba, Costa Rica.

Microbiological extracts were prepared on Petri dishes containing nutrient agar medium (DIFCO). Two boxes per bacterial strain were inoculated with 20 ml bacterial solution previously maintained at 4°C. The bacterial suspension was uniformly distributed over the medium using a glass handle and the boxes were incubated at 30°C for 2 days. Once the bacteria started growing, sterile distilled water was added to the medium and the bacterial suspension removed with a scalpel; approximately 20 ml of each bacterial suspension was transfered to sterile vials. The absorbency at 600 nm of a 3-ml solution was measured with a spectrophotometer. One ml of each suspension with an optical density of 1.2 was transferred to 200 ml of sterile nutrient medium (DIFCO) and incubated for 12 hours at 30°C and 150 rpm. Absorbency was measured again at 600 nm and gave values of 1.2 ±0.02 after 12 hours

Suspensions were adjusted to an absorbency of 1.1 by adding sterile nutrient medium. Cell-free extracts were obtained by centrifugation at 5000 rpm for 40 minutes followed by vacuum filtration on 0.22 mm membranes. Extracts were kept at 4°C in sterile flasks and protected from the light.

A suspension of $2x10^5$ conidia/ml of *M. fijiensis* in 700 ml of sterile distilled water was agitated at 100 cycles/min for 48 hours in darkness and then filtered using ethamine and Whatman paper. The residue was lyophilized to obtain 0.341 mg of powder, which was diluted in 70 ml sterile distilled water to obtain a final 20x concentration and filtered through 0.22 µm Nalgene Disposable Filterware filters. The filtrate was protected from light and kept at 4°C until its utilization.

Samples of plantain leaves with black leaf streak disease were transferred to La Montaña farm, which belongs to CATIE. Using a magnifying glass, fragments of viable perithecia were removed and transferred to the laboratory in paper bags. The samples were checked using a stereomicroscope, and sections with abundant sporulating lesions were cut into 2x2 cm pieces. Two to four of these pieces were stapled to pieces of paper and incubated in a humid chamber for 24 hours at room temperature. They were then transferred to sterile distilled water for 5 minutes to hydrate the perithecia. Ascospores discharged on water agar (4% w/v). Treatments were 0.5, 0.1 and 0.01 ppm dilutions of microbiological filtrates and the controls were without microbiological filtrate or with fungicide.

Diluted culture filtrates were mixed with 15 ml of V8 medium, with constant agitation and then transferred to Petri dishes; there were 3 replicates per treatment. Seven-day-old sub-cultures of a strain of *M. fijiensis* isolated from La Montaña were used. Twenty colonies of 1–1.5 cm in diameter were excised with a scalpel and transferred to an assay tube with 3 ml of 0.05% (v/v) Tween 20 and agitated in a vortex. The assay tubes were left to rest for 10 minutes and 10 drops of liquid were transferred to each Petri dish and spread with a glass handle. Dishes were sealed, and incubated in darkness for 5 days at 26°C.

After 5 days of incubation, the diameters of *M. fijiensis* colonies were measured using a micrometer in the 4x field of a microscope. Thirty readings were taken,

frequency intervals of amplitude 10 were done and only the 10 data from the interval with higher frequency were registered to conduct the analysis of the inhibiting effect of the microbiological filtrates.

The longest germination tubes were measured using a micrometer in the 10x or 40x field of a microscope. One hundred readings were taken per treatment. Since bacteria were cultured in nutrient medium, bioassays included a treatment with this medium.

Initial data were multiplied by a correction factor to transform the values to microns, 10 and 2.5 for 10x and 40x, respectively. The difference in germ tube length between the treatment and the control was used to calculate the inhibiting effect of the microbiological filtrates.

Results and discussion

Figure 3A shows preliminary results obtained for growth inhibition of *M. fijiensis* ascospores discharged onto different concentrations of microorganism filtrates. The percentage of inhibition was almost 50% and in some cases higher when the medium included filtrates of bacteria with glucanolytic activity (GBC2) and chitinolytic activity (SE/PO2 and White).

Regarding colony diameter (Figure 3B) the general tendency remained similar except that another bacterium with glucanolytic activity (GC1) showed a higher inhibition which was not fully revealed during the sexual phase of ascospore development.

The crude *M. fijiensis* filtrate (FCMf) also revealed, a clear inhibiting effect on ascospore and colony growth at a concentration of 0.5 ppm but not at the other concentrations. This poses the question as to whether the toxin(s) produced by *M. fijiensis* spores during the germination process can inhibit the pathogen in a "suicidal" type of action.

After five days of incubation, physical growth had stopped in the cultures with the crude filtrates of GBC2 and SE/PO2 at the 0.1 ppm concentration in comparison to the absolute control (water). Electronic transmission microscopy revealed modifications at the level of cell organelles with a strong presence of electrodense osmophylic globules that were not found neither in the control nor in the transversal longitudinal cut (Figure 4).

The preliminary results suggest that liquid culture filtrates of four bacterial strains with glucanolytic or chitinolytic activity, and the liquid filtrate of germinating spores of a Costa Rican strain of *M. fijiensis* inhibited the growth of *M. fijiensis* germ tubes and colonies.

Crude liquid preparations diluted from antibiotic(s) or toxin(s) and without bacterial or fungal cells had similar effects as the fungicide Tilt®.

The promising microbiological filtrates need to be evaluated under greenhouse and field conditions with or without adjuvant applications.

Acknowledgements
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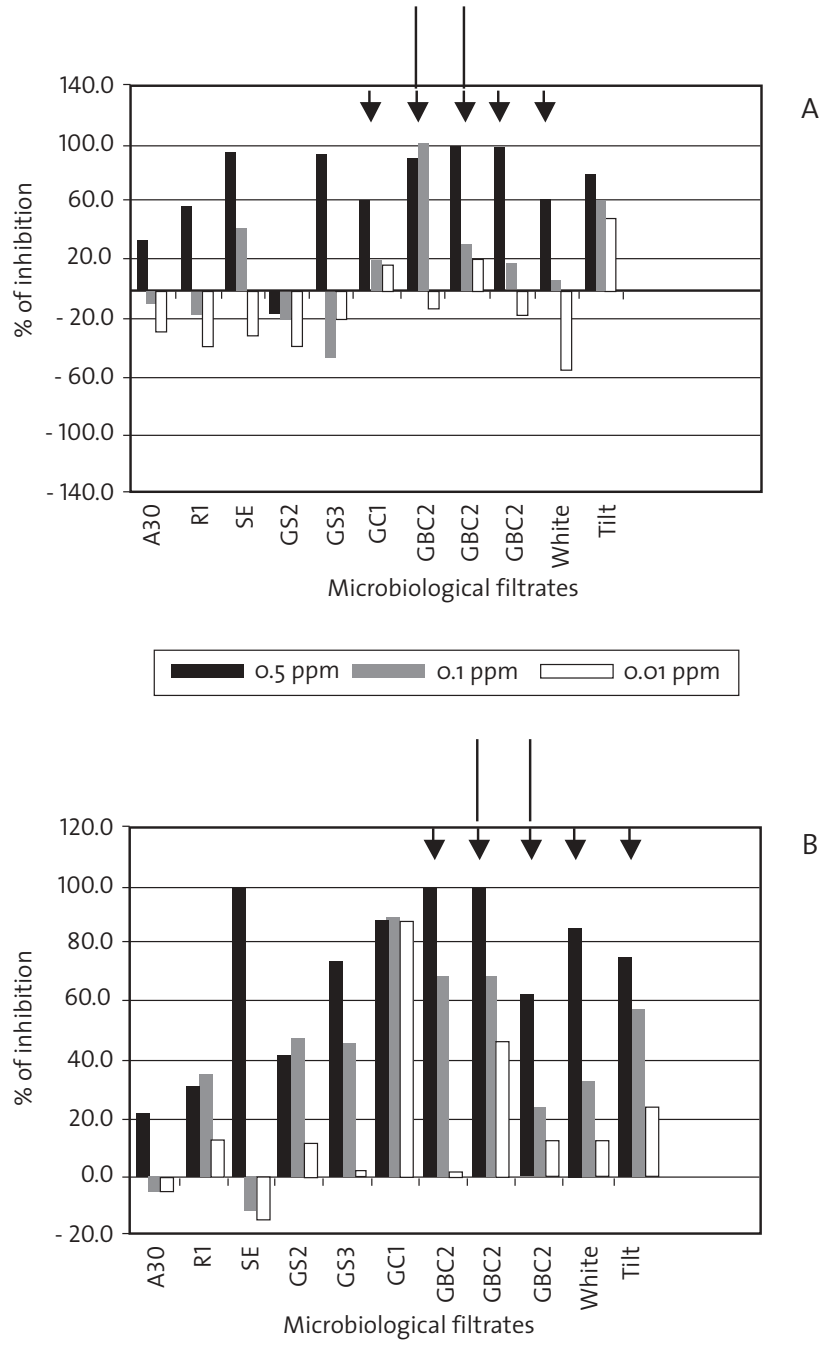

A30:*Bacillus cereus*; R1:*Serratia marcescens*; SE: *Serratia entomophila*; GS2, GS3, GC1 and GBC2: bacteria with glucanolytic activity; SEPO2 and White: bacteria with chitinolytic activity; FCMf: conidial fluid; Tilt: triazole fungicide.

Figure 3. Effect of three dilutions of nine antagonistic microbiological filtrates on A) germ tube growth and B) colony diameter of *M. fijiensis* . Arrows indicate where crude filtrates affected growth.

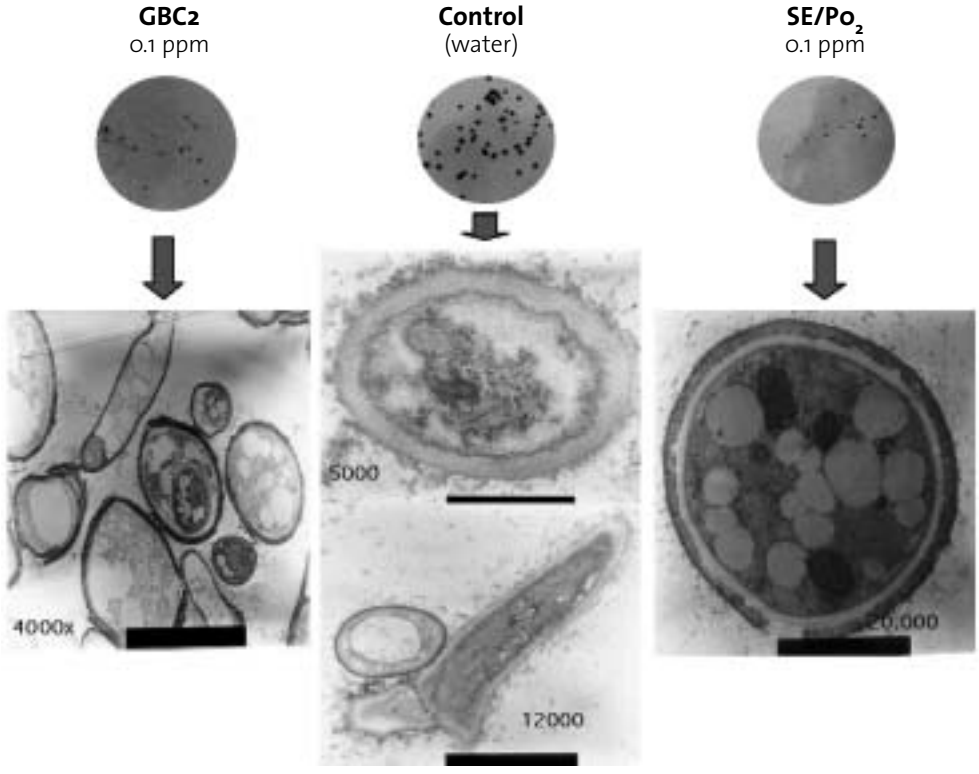

Figure 4. Cytological changes revealed by transmission electron microscopy of *M. fijiensis* hyphae tissues treated with crude filtrates of bacteria with glucanolytic (CBC2) and chitinolytic (SE/PO2) activity.

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Precision agriculture to improve management decisions and field research

E. Spaans and L. Quiros

Abstract

Precision agriculture helps farmers to improve management decisions. Since standard management practices do not take into account variability in the environment, resources may be wasted at certain stages of production and insufficient in others. Instead of calculating an average over the whole farm, there is a need for a detailed profit analysis of agricultural enterprises. It should be done at a high spatial resolution in order to make decisions that take into account local conditions within a field. At the Commercial Farm of EARTH University, we are mapping harvests at a spatial resolution of 4 ha within a 110 ha plantation. The network of railways in the plantation is used to georeference the origin of the fruit which, together with the weight of the fruits, is stored in a database. The costs are divided into fixed and variable costs, and subtracted from the income to produce a map of profits. The spatial variability of the harvest was enormous, with a greater than 300% difference within the field. To investigate the possible causes of the variability, we are monitoring parameters in the field that affect the growth of banana, e.g. soil fertility, plant nutrition, functional roots and age of plantation. Correlation coefficients between production and each of the parameters were calculated. The coefficients can be used to decide exactly what needs to be done in the areas not generating sufficient profit. This scheme of intensive and systematic data acquisition, and interpretation provides opportunities for field research and hence to improve management practices including the control of Sigatoka disease. The project is in progress and we present the preliminary results we have obtained.

Resumen - Agricultura de precisión como base para mejorar las decisiones de manejo e investigación en la finca

La agricultura de precisión es una herramienta eficaz para ayudar a los agricultores a tomar mejores decisiones de manejo. Debido a que las prácticas de manejo normalizadas no reconocen la variabilidad del ambiente, se gastan recursos en algunas áreas, mientras que en otras no se invierte lo suficiente. Este hecho requiere realizar un análisis de ganancias detallado de nuestra empresa agrícola; de esta manera, no es el promedio global de toda la finca, sino una resolución

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espacial alta, la que permite hacer tomas de decisiones que respondan a las condiciones locales en el campo. Los ingresos provienen de la cosecha, así, que hacemos un mapa de la cosecha con una resolución espacial de 4 ha dentro de una plantación de 110 ha en la Finca Comercial de la Universidad EARTH. La red de ferrocarril en la plantación sirve para hacer referencia geográfica al origen de la fruta, que, junto con su peso, medido en la planta empacadora, se almacena en una base de datos. Los costos se dividen en costos fijos y variables, y se restan del ingreso para producir un mapa de ganancias. Se descubrió que la variabilidad espacial de la cosecha fue enorme, con más de 300% de diferencias dentro del campo. Para investigar las causas posibles de esta variabilidad, empezamos a monitorear varios parámetros en el campo que afectan el crecimiento de los bananos, como la fertilidad del suelo, nutrición de la planta, raíces funcionales y la edad de la plantación, y luego calculamos los coeficientes de correlación entre la productividad por un lado y cualesquiera de los parámetros del campo por otro. Este coeficiente puede ser utilizado como una guía para decidir las necesidades exactas que deben ser cumplidas en cada una de las áreas que no generan ganancias suficientes. Este esquema de adquisición de datos intensivo y sistemático y su interpretación también permiten que las investigaciones en el campo mejoren eficazmente las prácticas de manejo, incluyendo el control de la Sigatoka. Este proyecto se encuentra en progreso y se discutirán los resultados preliminares.

Résumé – L'agriculture de précision pour améliorer les décisions de gestion et la recherche en champ

L'agriculture de précision aide les agriculteurs à améliorer leurs décisions de gestion. Les pratiques de gestion standard ne prennent pas en compte la variabilité de l'environnement, et des ressources pourraient donc être dilapidées à certains stades de la production mais être insuffisantes à d'autres. Au lieu de calculer une moyenne sur toute l'exploitation, une analyse détaillée du profit est nécessaire pour les entreprises agricoles. Elle devrait être réalisée avec une résolution spatiale élevée afin de prendre des décisions qui tiennent compte des conditions locales au sein du champ. Dans la ferme commerciale de l'Université EARTH, nous cartographions les récoltes avec une résolution spatiale de 4 ha sur une plantation de 110 ha. Le réseau des voies ferrées est utilisé pour géoréférencer l'origine des fruits qui, avec le poids des fruits, est stocké dans une base de données. Les coûts sont divisés en coûts fixes et variables, et soustraits du revenu pour produire une cartographie des bénéfices. La variabilité spatiale de la récolte s'est avérée énorme, avec une différence de plus de 300% à l'intérieur d'un même champ. Afin de rechercher les causes possibles de cette variabilité, nous faisons le suivi des paramètres en champ qui affectent la croissance des bananiers, tels que la fertilité du sol, la nutrition des plants, les racines fonctionnelles et l'âge de la plantation. Les coefficients de corrélation entre la production et chacun des paramètres ont été calculés. Les coefficients peuvent être utilisés pour décider exactement ce qui doit être fait dans les zones qui ne génèrent pas de bénéfices suffisants. Ce schéma intensif et systématique d'acquisition de données, ainsi que son interprétation, offrent des occasions de recherche en champ qui pourraient permettre d'améliorer les pratiques de gestion, y compris la lutte contre les cercosporioses. Le projet est en cours et nous présentons les premiers résultats que nous avons obtenus.

Introduction

Globalization has opened up markets and increased competition between agricultural producers worldwide. In order to continue being competitive, farmers must improve the efficiency of their production systems, i. e. reduce costs while maintaining or even improving the production as well as the social and environmental impact of their agricultural enterprise.

Productivity has increased considerably over the last century due to technological advances (e.g. in plant nutrition and fertilization, genetic improvement, mechaniza-

tion and pest management) that have allowed the agricultural community to respond to the increasing demands for agricultural products. These advances and the improved understanding of plant-environment interactions have resulted in the development of technological packages for major crops. A technological package is a recommended set of agricultural practices that describe all stages of production, e.g. soil preparation, crop planting, pest and disease management, soil fertilization, and harvesting and packing methods. These generalized recommendations were developed for average conditions and do not take into account specific conditions encountered in the field. Inevitably, resources are wasted in some areas, hence raising production costs and increasing the risk of environmental contamination. Similarly, not enough resources may be invested in certain areas, resulting in suboptimal growth and loss of income.

The reason is that resources (soil, weather, water, etc.) are not homogenous throughout the farm and over time. Moreover, the socioeconomic conditions of the enterprise (the nature of the market, prices, policies and standards of certification, amongst others) are also in a constant state of change.

This calls for a more entrepreneurial approach to agriculture. We need to improve decision making, to take more precise ones to fine-tune the management of the resources to what is really needed. That is precisely what precision agriculture is all about: doing the right thing, at the right time and at the right place. To answer the fundamental question of what is the right thing to do, we need to acquire detailed information about the production system at the adequate spatial resolution.

Traditional applications of precision agriculture involve high-tech data acquisition: a Global Positioning System (GPS) for georeferencing the data and monitoring sensors mounted on harvest equipment to gather high resolution data while harvesting. A Geographic Information System (GIS) is used to manage the large amount of data and map them. These requirements have hindered the implementation of precision agriculture in many Latin American production systems where harvest is often done manually and access to and support for technology is sometimes limited. We think it is more useful to reflect on the basic principle of precision agriculture and then creatively develop the implementation, considering the specific conditions of each production system.

This paper presents our interpretation of precision agriculture and its implementation at the commercial banana farm of EARTH University. Its relevance to this workshop is that it improves decision-making and provides a unique opportunity for farm research, including the study of the effectiveness of agricultural practices to control the leaf spot diseases caused by *Mycosphaerella*.

Implementation of precision agriculture

Being competitive means optimizing profits, not harvest. Considering the spatial and temporal variability of resources throughout the farm, it is to be expected that profits vary as well, particularly if the same technological package is used over the entire farm. Thus an overall financial analysis of the farm is not sufficient; we need a detailed analysis of costs and benefit throughout the entire farm, in order to allow for precise management.

As an example we describe banana production on a 110 ha farm located on the campus of EARTH University in the Humid Tropics of the Atlantic Zone of Costa Rica. Since the financial benefit results from the harvested banana, a precise monitoring of the harvest is needed to precisely monitor income. The farm was divided into blocks of 4 ha (100 m wide by 400 m long). The railway that transports the fruits runs through the center of each block, and fruits are collected on a distance of 50 m on each side of the tracks. Each block has an identification, and printed labels are provided to the harvesters to identify the geographical origin of every bunch. At the packing plant, each bunch is weighed and the information, together with the geographical origin (spatial component) and the day of harvest (temporal component), is stored in a database. This mapping system is inexpensive and low-tech, and does not require GPS since the railway network serves as the geographical reference within the plantation.

The bananas are still attached to the stalk when they are weighed. To obtain the weight of the bananas, the weight of the bunch is reduced by 10% to account for the weight of the stalk. Finally, a general value of 12% is used to account for the rejected banana (because of inadequate quality), and the resulting weight is divided by 18.14 to obtain the number of boxes exported. Data were collected for the period February to October of the year 2001.

The costs of banana production were divided into fixed and variable costs. Fixed costs were equally divided over the entire productive surface area of the plantation, while the variable costs were equally divided over the weight of the bunches.

Costs are currently recorded for the entire farm, thus we do not have the same spatial resolution as for the production costs. The quantity of rejected bananas is also an average value reported by the packing plant. These factors limit data analysis, but the situation shows how in practice the implementation of precision agriculture is a process of gradual improvements in data acquisition and interpretation as the entire team familiarizes itself with the process. This year, for example, we started recording production cost per block.

The cost and harvest data are used to determine the profit of every block within the plantation. In order to explain and eventually reduce the variability in harvest, we also monitor field parameters that we know affect plant growth, like soil fertility, plant nutritional status, nematode infestation (as expressed in % of functional roots), and age of plantation. This last parameter was included because the plantation was planted about 40 years ago and recently some areas have been renewed. To determine the degree to which each field parameter affects the production of bananas, correlation coefficients were calculated between the harvest and each of the field parameters.

Results and discussion

The productivity of bananas varied widely over the entire farm, ranging from 943 to 3040 boxes/ha/year, with an average of 1538 boxes/ha/year. These numbers translated into a loss of US$2655/ha/year in the least productive block, and a profit of US$2316/ha/year in the most productive block. Overall, the financial loss of the plantation was US$1245/ha/yea. This shows the usefulness of precision agriculture,

since we can now locate the problem areas and quantify their impact on the financial return of the farm.

So what should we do with this information, and where and when? To answer these questions we analysed the data statistically. Although we found a range of soil fertility, plant nutritional status and nematode infestation, none of these parameters were significantly correlated ($P<0.05$) with the harvest, except for foliar Mg ($r = 0.52$) and Cu ($r = 0.50$). This means that when the harvest is good, levels of foliar Mg and Cu are high, and when harvest is bad, levels of foliar Mg and Cu are low. But although significant, the correlation was not very strong. Figure 1 shows what really happened in the plantation. All the areas that produced profit are the ones that had been renewed, and all the areas with old plant material produced losses. From this (preliminary) analysis we recommended that the right thing to do was to speed up the rejuvenation of the plantation, starting with the least productive block.

The next obvious question is when should a plantation be renewed. There is no standard answer. The moment will depend on cultivar, soil conditions and management practices. The answer will be provided by the continuous and precise profit analysis of the plantation over time.

Interestingly, interpretation of the foliar analyses in comparison with optimum published values, suggested that the entire plantation was deficient in N, even the blocks that produced 3040 boxes/ha/year. Without this careful spatial analysis of production and field parameters, the recommendation would have probably been to increase nitrogen fertilization, but this would probably not have solved the problem. Instead, it would have probably increased production costs and enhanced the risk of nitrate leaching to groundwater.

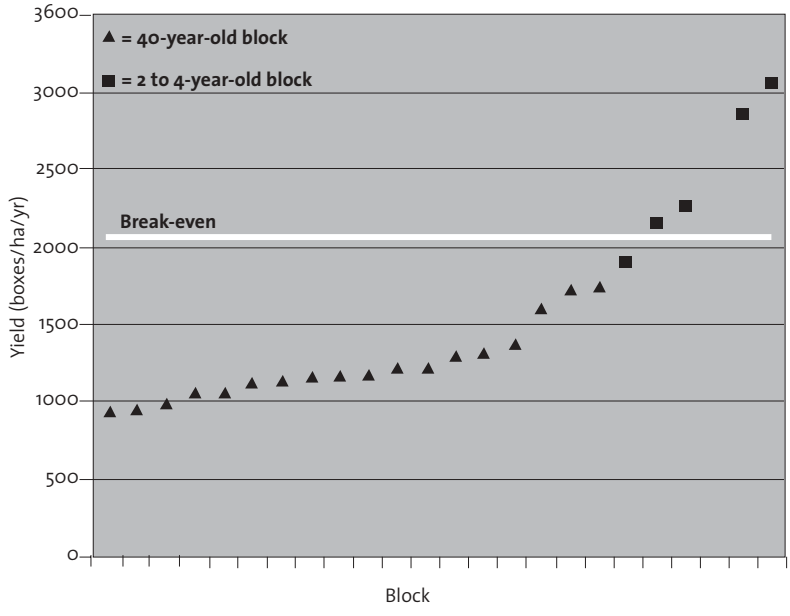

Figure 1. Ranking of blocks in order of increasing productivity. The break-even point represents the production needed to cover the costs (zero profit).

We propose that this type of on-farm research also be used to identify farm-specific optimum values or tolerance levels, which could lead to a better interpretation of field data. Furthermore, this implementation of precision agriculture could be used to rapidly and effectively evaluate alternative practices, by selecting those blocks that meet certain criteria and applying different treatments to them. The monitoring system provides us with continuous and timely feedback about the plant response to the different treatments. We are now including the monitoring of leaf spot diseases in the plantation, which will allow us to investigate the interaction between the disease and other field parameters, and determine the tolerance levels for the desired production.

Conclusion

In this paper we presented the principles of precision agriculture, and discussed a case where we implemented precision agriculture on a commercial banana plantation. The implementation is a gradual process, and we are still far away from a precisely managed farm. However, we have shown that the system has already provided valuable information which we have been able to use to take effective management decisions. The continuous and precise data collection inherent to precision agriculture should make it possible to rapidly and effectively investigate alternative practices.

Poster

The role of managing resistance to fungicides in maintaining the effectiveness of integrated strategies to control black leaf streak disease

S. Knight[1], M. Wirz[1], A. Amil[2], A. Hall[2] and M. Shaw[3]

Abstract

Fungicide programmes are essential for commercial production of banana in all regions where *Mycosphaerella fijiensis* is prevalent. A key factor influencing the design of fungicide programmes is the importance of following resistance management principles, in order to preserve long-term effectiveness. Resistance management is based on the appropriate limitation and alternation of products that have a site-specific mode of action. The introduction in 1997 of the first strobilurin fungicide, azoxystrobin, represented a significant step forward in the integrated control of black leaf streak disease, because of its efficacy and favourable environmental and toxicological profile. In recognition of its site-specific mode of action, anti-resistance management guidelines were developed by the Fungicide Resistance Action Committee (FRAC) before its commercial introduction. The first strobilurin-resistant individuals were documented in 2000 in Costa Rica, and resistance to strobilurin has reached high levels on some farms in the main banana production zones of Costa Rica. Molecular characterization of resistant isolates has identified the cause of resistance as a single point mutation in the fungal target protein, cytochrome b. A large-scale population dynamics study is in progress to examine the evolution of resistance in the field, using molecular techniques (PCR). The factors that influence this evolution (disease pressure, climate, fungicide spray programme) will be examined, and the extent of migration of the resistant population will be estimated. The study will enable recommendations to be validated or improved, and will support efforts to limit the proliferation of resistance to this important group of fungicides.

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Resumen – Papel del manejo de la resistencia a funguicidas en el mantenimiento de la eficacia de las estrategias integradas para el control de la Sigatoka negra

Los programas de funguicidas siguen siendo esenciales para la producción bananera comercial en todas las regiones donde prevalece *Mycosphaerella fijiensis*. Un factor clave que influye sobre el diseño de los programas de aplicación de funguicidas es la importancia de seguir los principios del manejo de la resistencia, con el fin de preservar la eficacia a largo plazo. El manejo de la resistencia se basa en la limitación y alternación apropiadas de los productos que tienen un modo de acción específico en el sitio. La introducción en 1997 del primer funguicida strobilurina, azoxistrobina, representó un significativo paso hacia adelante en el control integrado de la Sigatoka negra, debido a su excelente eficacia y perfil ambiental y toxicológico favorable. En reconocimiento de este modo de acción específico del sitio, se desarrollaron guías de manejo anti-resistencia por el Comité de Acción de Resistencia a los Fungicidas (*Fungicide Resistance Action Committe*e (FRAC) antes de su introducción comercial. Los primeros individuos resistentes a la strobilurina fueron documentados en 2000 en Costa Rica, y la resistencia a la strobilurina ha alcanzado niveles altos en algunas fincas en las principales zonas productoras de banano de Costa Rica. La caracterización molecular de los aislados resistentes ha identificado como la causa de la resistencia una mutación puntual individual en la proteína diana fungosa, citochromo b. Un estudio a gran escala de las dinámicas de la población se está llevando a cabo actualmente para examinar la evolución de la resistencia en el campo, utilizando técnicas moleculares (PCR). Se examinarán los factores que influyen sobre esta evolución (presión de la enfermedad, clima, programa de rociado de fungicidas), y se estimará la extensión de migración de la población resistente. El estudio permitirá validar o mejorar las recomendaciones para el manejo de la resistencia, y apoyará los esfuerzos que se realizan para limitar la proliferación de la resistencia en este importante grupo de fungicidas.

Résumé – Le rôle de la gestion de la résistance aux fongicides dans le maintien de l'efficacité des stratégies de lutte intégrée contre la maladie des raies noires

Les plantations commerciales de bananes sont extrêmement dépendantes des applications de fongicides partout où *Mycosphaerella fijiensis* prévaut. Un facteur important qui influence la conception des programmes d'arrosage est le respect des principes de gestion de la résistance aux fongicides afin de préserver leur efficacité. La gestion de la résistance repose sur la restriction de l'usage et l'alternance de produits qui ont un mode d'action spécifique. L'introduction en 1997 du premier fongicide à base de strobilurine, l'azoxystrobine, représente une avancée importante dans la lutte à la maladie des raies noires étant donné son efficacité et son profil environnemental et toxicologique favorable. En reconnaissance de son mode d'action spécifique, des lignes directrices pour empêcher le développement de résistance ont été élaborées par le *Fungicide Resistance Action Committee* (FRAC) avant sa distribution commerciale. Les premiers cas de résistance à la strobilurine ont été documentés en 2000 au Costa Rica, et les niveaux de résistance ont atteint des taux très élevés sur certaines fermes des principales zones de production bananière au Costa Rica. La caractérisation d'isolats résistants a permis de remonter à la source de la résistance, une mutation isolée dans une protéine ciblée du champignon, le cytochrome b. Une étude de population à grande échelle utilisant des techniques moléculaires (PCR) est en cours afin de suivre l'évolution de la résistance en champ. Les facteurs qui influencent cette évolution (la pression de la maladie, le climat, le programme d'application des fongicides) seront examinés et l'étendue de la migration de la population résistante estimée. L'étude permettra de valider ou améliorer les recommandations et participera à limiter la prolifération de la résistance à ce groupe important de fongicides.

Introduction

A key factor influencing the design of programmes to control *Mycosphaerella fijiensis* is the importance of following resistance management guidelines to ensure the long term effectiveness of site-specific fungicides.

The introduction in 1997 of the first strobilurin fungicide, Bankit (azoxystrobin), represented a significant step forward in the integrated control of black leaf streak disease, due to its excellent efficacy and favourable environmental and toxicological profile. It has a site-specific mode of action, the inhibition of electron transport at the Qo site of cytochrome bc_1. Anti-resistance management guidelines were developed by the Fungicide Resistance Action Committee (FRAC) before commercial introduction, and a global sensitivity monitoring programme was initiated.

The first strobilurin-resistant individuals were documented in 2000 in Costa Rica, and resistance to strobilurin has reached high levels on some farms in the main banana production zones of Costa Rica. Molecular characterization of resistant isolates has identified the cause of resistance as a single point mutation in the fungal target protein, cytochrome b, known as $G_{143}A$. Resistant isolates can be detected via quantitative polymerase chain reaction analysis.

A large-scale population dynamics study was initiated to examine the evolution of resistance in the field by using molecular analysis. The factors that influence this evolution (disease pressure, climate, fungicide spray programme) will be examined, and the extent of migration of the resistant population will be estimated.

Materials and methods

In vitro bioassay

Sensitivity of *M. fijiensis* to azoxystrobin, was evaluated using the *in vitro* methodology recommended by the FRAC.

Sporulating tissue collected from the field was allowed to discharge onto fungicide-amended agar, and elongation of ascospore germ tubes was measured at 1 ppm and 10 ppm of azoxystrobin. A 75% growth or more at the discriminating rate of 10 ppm relative to the control indicated resistance to strobilurin.

Molecular detection of strobilurin resistance

The presence of the $G_{143}A$ mutation was detected using a diagnostic primer for $G_{143}A$. This primer is extended only when the $G_{143}A$ mutation is present in the sample.

Field population dynamics in Costa Rica

Repeated sampling of infected foliar tissue by using a multilayered hierarchical sampling structure is in progress in Costa Rica. Plantations were selected to meet the following criteria:

- Presence/absence of strobilurin selection pressure;
- Presence/absence of resistance mutation;
- High/low levels of relative disease pressure.

Results and discussion

Global resistance monitoring results

The results of the global sensitivity monitoring for azoxystrobin are shown in Table 1.

Table 1. Prevalence of resistance to azoxystrobin (measured as >75% growth at 10 ppm relative to control) in Central and South America (Number of subpopulations tested given in brackets).

Country	Prevalence of resistance (% of resistant spores)		
	1999	2000	2001
Mexico	-	0.0 (2)	-
Belize	-	-	-
Honduras	0.0 (2)	0.0 (3)	0.0 (30)
Guatemala	0.0 (4)	0.0 (4)	0.0 (25) *
Nicaragua	-	-	0.0 (3)
Costa Rica	-	11.3 (33)	11.1 (78)
Panama	-	0.0 (5)	3.3 (15)
Colombia	-	0.0 (4)	0.03 (25)
Ecuador	-	-	0.01 (28)**
Cameroon	-	-	0.0 (12)

*Some resistant individuals were detected in two farms through PCR analysis.

**False positive (PCR analysis demonstrated that resistance was not present).

Molecular analysis

A high degree of correlation ($P \leq 0.01$) was observed between extensive germ tube growth at 10 ppm of zoxystrobin and detection of the $G_{143}A$ resistance mutation (Figure 1). The correlation between the PCR data and bioassay data at 1 ppm was lower (0.8 compared to 0.94). In other words, extensive germ tube growth, normally associated with resistance, is occasionally detected on 1 ppm agar in the absence of $G_{143}A$. This may indicate an alternative mechanism of resistance, and studies are in progress to address this question.

Table 2 gives the prevalence of resistance in populations of *M. fijiensis* sampled from 16 different sites in Costa Rica. Ten samples showed varying levels of $G_{143}A$ detection, whilst 6 sample populations remained sensitive.

Conclusion

The ongoing population dynamics study will enable the validation or improvement of the management guidelines regarding resistance to strobilurin, and will support efforts to limit the proliferation of resistance to this important group of fungicides.

Fungicide programmes are expected to remain indispensable for commercial banana production for the foreseeable future. A significant increase in the number of alternative chemical modes of action to control of Sigatoka diseases is unlikely within the next 5-10 years. Therefore resistance management will remain a prime consideration in the design of control programmes, underpinned by appropriate monitoring efforts and a judicious revision of the guidelines.

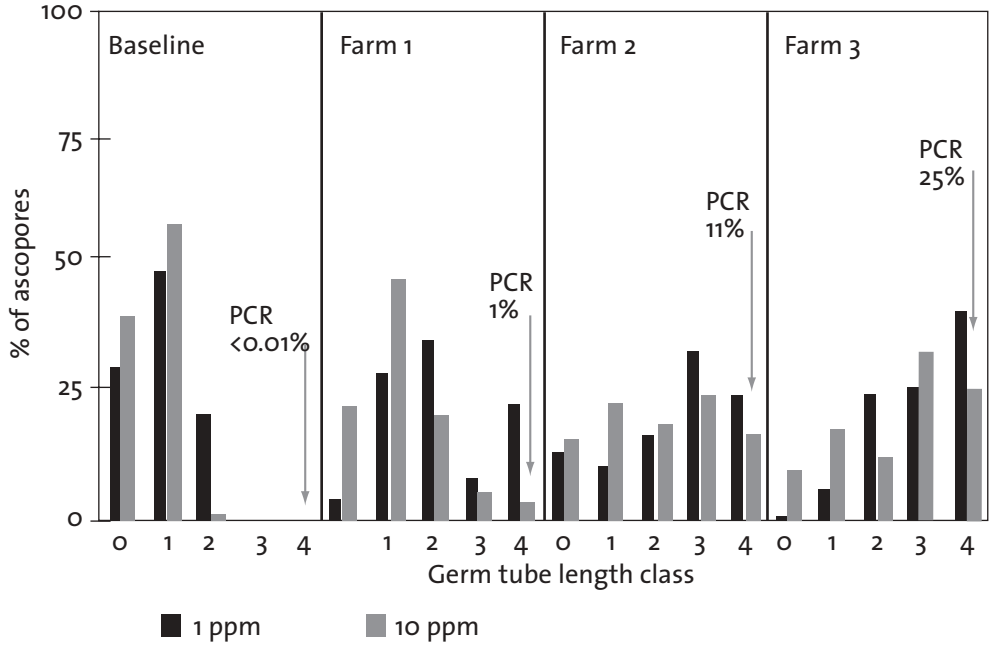

Figure 1. Correlation between *in vitro* growth in the presence of azoxystrobin and detection of the $G_{143}A$ resistance mutation. (Germ tube length class: 0 means not germinated, 1 means <25% growth relative to control, 2 means between 25 and 50% growth, 3 means between 50 and 75% growth, and 4 means over 75% growth).

Table 2. Prevalence of resistance in populations of *M. fijiensis* estimated by determining the presence of the $G_{143}A$ resistance mutation at sixteen different sites in Costa Rica.

Farm number	Region	Prevalence of resistance (%)
1	Guápiles	1
2	Guápiles	3
3	Guápiles	25
4	Limón	0
5	Guápiles	27
6	Siquirres	0
7	Siquirres	2
8	Limón	0
9	Sarapiquí	6
10	Siquirres	0.6
11	Guápiles	5
12	Siquirres	0.6
13	Siquirres	0
14	Siquirres	0
15 (untreated area)	Siquirres	0
16	Guápiles	11

Recommendations of session 5

Integrated disease management

Strategies to control black leaf streak disease and Sigatoka disease can, according to the country and the scale of production, include not only chemical and cultural practices but also the use of mixed crops or resistant clones. The important inhibitory effect of some natural substances derived from microorganisms antagonistic to fungi, have also been reported as effective in reducing, *in vitro,* the development of *M. fijiensis.*

It was recommended to integrate working groups from different disciplines to develop an achievable IPM approach to manage Sigatoka diseases.

Chemical strategies and/or the use of improved hybrids should always be used jointly with adequate agricultural practices to maximize yield and efficacy of the management practices.

It was recommended to study natural/synthetic substances capable of promoting or activating systemic acquired resistance in the broad sense.

It was recommended to evaluate the feasibility of precision agriculture farming to optimize disease management.

It was recommended to assess different crop systems with potential positive impact on disease management.

It was recommended to include the FRAC's Banana Working Group Guidelines (http://www.gcpf.org/frac) for fungicide resistance management in order to broaden the knowledge of such guidelines.

It was recommended to develop alternative/improved methods/equipments for ground-based applications that can be used by smallholders.

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