LAHYRE IZAETE SILVEIRA GOMES

ETIOLOGY OF SIGATOKA DISEASES IN MINAS GERAIS, GENETIC STRUCTURE OF THE POPULATION OF *Mycosphaerella musicola* AND SENSITIVITY OF BRAZILIAN ISOLATES OF *Mycosphaerella fijiensis* TO FUNGICIDES

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fitopatologia, para obtenção do título de *Doctor Scientiae*.

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LAHYRE IZAETE SILVEIRA GOMES

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APROVADA: 29 de março de 2012.

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(Orientador)

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BIOGRAFIA

LAHYRE IZAETE SILVEIRA GOMES, filha de Joaquim Gomes Barbosa e Joaquina Maria da Silveira Barbosa, nasceu no dia onze de janeiro de 1983, na cidade de Porteirinha-MG.

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Em agosto de 2008, iniciou o curso de Doutorado em Fitopatologia, pela Universidade Federal de Viçosa (UFV), obtendo o título de *Doctor Scientiae* em março de 2012.

SUMÁRIO

ABSTRACT

GOMES, Lahyre Izaete Silveira, D.Sc., Universidade Federal de Viçosa, March, 2012. **Etiology of Sigatoka diseases in Minas Gerais, genetic structure of the population of *Mycosphaerella musicola* and sensitivity of Brazilian isolates of *Mycosphaerella fijiensis* to fungicides.** Adviser: Eduardo Seiti Gomide Mizubuti. Co-advisers: Luiz Antonio Maffia and Robert Weingart Barreto.

The Sigatoka complex is one of the most serious phytosanitary problem affecting banana crops because of the high yield losses associated to it. Among the species of *Mycosphaerella* three are frequently associated with Sigatoka diseases: *Mycosphaerella musicola*, causing yellow Sigatoka; *M. fijiensis*, causing black Sigatoka, and *M. eumusae*, causing eumusae leaf spot. The latter has not been reported in Brazil yet. In the State of Minas Gerais, yellow Sigatoka is found in all banana growing areas, while black Sigatoka has been reported only in the South and the Zona da Mata regions. The objectives of this work were to evaluate the distribution of *Mycosphaerella* spp.; to assess the genetic variability of the most common *Mycosphaerella* species associated with Sigatoka in Minas Gerais state; and to evaluate the sensitivity of *M. fijiensis* to different fungicides. A total of 239 isolates were obtained from different regions of Minas Gerais and all belonged to *M. musicola*. No isolate of *M. fijiensis* or *M. eumusae* was found. The isolates of *M. musicola* were characterized through the use of nine microsatellites markers and mating type. The population reproduces sexually and the isolates of each mating type are distributed at a 1:1 ratio. Additionally high haplotype and genetic diversity were observed, but no population structure was revealed by the Bayesian assignment method. The sensitivity of *M. fijiensis* to the fungicides thiophanate-methyl, tebuconazole, chlorothalonil and mancozeb was assessed using 45, 45, 41 and 48 isolates respectively, obtained from different regions. No isolate was insensitive to tebuconazole. The highest values of EC_{50} to thiophanate-methyl and chlorothalonil were 8.22 μg mL^{-1} and 53.7 μg mL^{-1}, respectively. Approximately 50% of the isolates grew on culture medium amended with 1000 μg of mancozeb mL^{-1}. There was no correlation between EC_{50} and geographic region. Yellow Sigatoka disease is the prevalent foliar disease of banana in Minas Gerais and the pathogen population has high genetic variability. No evidence of resistance of *M. fijiensis* to the fungicides was detected.

RESUMO

GOMES, Lahyre Izaete Silveira, D.Sc., Universidade Federal de Viçosa, março de 2012. **Etiologia de doenças de Sigatoka em Minas Gerais, estrutura genética da população de *Mycosphaerella musicola* e sensibilidade de isolados de *Mycosphaerella fijiensis* do Brasil a fungicidas** Orientador: Eduardo Seiti Gomide Mizubuti. Coorientadores: Luiz Antonio Maffia e Robert Weingart Barreto.

O complexo Sigatoka é um dos problemas fitossanitários mais graves para a cultura da banana, pois são doenças foliares que resultam em grandes perdas de produção. Dentre as espécies fúngicas do gênero *Mycosphaerella*, três são frequentemente relatadas estar associadas ao complexo Sigatoka: *Mycosphaerella musicola*, causadora da Sigatoka Amarela; *M. fijiensis*, causadora da Sigatoka Negra e *M. eumusae* que causa a mancha foliar eumusae. Esta última ainda não foi relatada no Brasil. Em Minas Gerais, a Sigatoka Amarela é encontrada em todos os locais onde se cultiva banana, enquanto a ocorrência de Sigatoka Negra foi relatada em alguns municípios das regiões do Sul de Minas e da Zona da Mata. O objetivo deste trabalho foi avaliar a distribuição de *Mycosphaerella* spp. em Minas Gerais, determinar a espécie predominante e realizar um estudo populacional desta espécie em Minas Gerais; e avaliar a sensibilidade de *M. fijiensis* a diferentes fungicidas. Os 239 isolados obtidos de diferentes regiões de Minas Gerais, foram identificados com base nos caracteres morfológicos e moleculares, como pertencendo a *M. musicola*. Nenhum isolado de *M. fijiensis* ou de *M. eumusae* foi encontrado. O estudo da população de *M. musicola* foi realizado com nove marcadores microssatélites e *mating type*. Os resultados obtidos demonstram que em Minas Gerais *M. musicola* reproduz sexuadamente e a distribuição de isolados dos dois *mating types* foi de 1:1. Além disso, foi observada alta diversidade haplotípica e genética, além da ausência de estruturação da população revelada pela análise Bayesiana. Avaliaram-se 45, 45, 41 e 48 isolados de *M. fijiensis* coletados em bananais de diferentes regiões do Brasil quanto à sensibilidade aos fungicidas tiofanato metílico, tebuconazole, clorotalonil e mancozebe, respectivamente. Nenhum isolado foi insensível ao fungicida tebuconazole. Os maiores valores de EC_{50} para tiofanato metílico e clorotalonil foram 8,22 μg mL^{-1} e 53,7 μg mL^{-1} respectivamente. Cerca de 50% dos isolados cresceram em meio de cultura com mais de 1000 μg de mancozebe mL^{-1}. Não houve correlação entre os valores de EC_{50} e região geográfica. Sigatoka amarela é a doença foliar prevalente em Minas Gerais e a população de *M. musicola* tem alta variabilidade genética. Não foram encontradas evidências de resistência de *M. fijiensis* aos fungicidas.

GENERAL INTRODUCTION

The Sigatoka diseases of banana are caused by species of the *Mycosphaerella* genus which are often considered as destructive plant pathogens (Arzanlou et al., 2007; Arzanlou et al., 2008; Crous, 2009; Crous and Mourichon, 2002; Zapater et al., 2008). *Mycosphaerella* spp. that infect banana induce necrosis of leaf tissue leading to a reduction in the photosynthetic capacity of the plant and also cause physiological changes that contributes to premature ripening of the fruits (Marin et al., 2003). The main *Mycosphaerella* species that infect banana are: *Mycosphaerella musicola*, *M. fijiensis* and *M. eumusae*. Yellow Sigatoka is caused by *M. musicola* R. Leach ex J. L. Mulder (anamorph *Pseudocercospora musae*) (Zimm.) Deighton, and is present in all continents (Jones, 2003). In Brazil, the disease was detected in 1944 in the state of Amazonas and is currently present in all states (Cordeiro et al., 2005).

Black leaf streak disease (BLSD), also known as Black Sigatoka, is caused by *M. fijiensis* M. Morelet (anamorph *Pseudocercospora fijiensis*) (M. Morelet) Deighton and was first reported in the state of Amazonas in Brazil in 1998 (Pereira et al., 1998). BLSD causes major yield losses in banana, ranging from 20 to 80%, when fungicides are not used (Cordeiro and Matos, 2003). In Minas Gerais the occurrence of BLSD was first reported in 2005 in the Zona da Mata and South regions (Castro et al., 2005). *Mycosphaerella eumusae* Crous and Mourichon (anamorph *Pseudocercospora eumusae* Crous and Mourichon), the most recent species recognized as also involved in the Sigatoka complex, causes the eumusae leaf spot and was first reported in the early 1990s in Southeast Asia (Carlier et al., 2000). To date, *M. eumusae* is not known to be present in Brazil.

Mycosphaerella musicola was already widely distributed before *M. fijiensis* was first reported, but when introduced into a new area the latter generally displaces the former (Jones, 2003). However, in many regions where both diseases are known to occur *M. musicola* remained the major problem, particularly in regions with high altitudes and cooler temperatures. *Mycosphaerella fijiensis* causes more severe leaf spot at lower and warmer regions (Churchill, 2011; Moulion-Pefoura et al., 1996).

The traditional diagnosis of Sigatoka complex is based on disease symptoms (Marin et al., 2003). The morphological characters of the teleomorphs of the three species of *Mycosphaerella* are similar, but the anamorphs are distinct (Churchill, 2011; Crous and Morichon, 2002). Species-specific primers designed to amplify the internal transcribed spacer region of rDNA made it possible to differentiate *M. musicola* from *M. fijiensis* (Johanson et al., 1994). More recently, a new set of species-specific primers based on the actin gene and the use of real-time PCR TaqMan diagnostic probes based on the sequence of the B-tubulin gene also contributed to sorting *M. musicola*, *M. fijiensis* and *M. eumusae* (Arzanlou et al., 2007). Overall, molecular tools have been helpful in elucidating key issues of the Sigatoka complex.

The study of genetic diversity of fungal populations is important to the development of strategies for plant propagation and management of disease resistance (Churchill, 2011). There are not many studies conducted to investigate the genetic structure of *M. musicola*, and most used RAPD and RFLP markers (Hayden et al., 2003; Hayden et al., 2005; Moreira et al., 2003). In 2003 isolates of *M. musicola* from four geographic populations representing Africa, Latin America and Caribbean, Australia and Indonesia were examined using RFLP and moderate levels of genetic diversity were observed for most populations. Genetic differentiation between populations was high when pairwise comparisons were made except for the Africa and Latin America-Caribbean populations, suggesting the occurrence of migration of the pathogen between these regions (Hayden et al., 2003). A total of 363 isolates collected along the Australian coast was studied by Hayden et al. (2005) using RFLP, and the population displayed moderate levels of gene diversity and low gene flow at a continental scale. In Brazil a study with few isolates using RAPD was made and revealed high genetic diversity among isolates (Moreira et al., 2003). Microsatellites markers were also developed to study the genetic diversity of *M. musicola* (Molina et al., 2001; Zapater et al., 2008), however no thorough population genetics study using these markers were conducted yet.

Management practices like the use of resistant cultivars, cultural methods and quarantine measures have been recommended to control Sigatoka diseases, but the most reliable tool has been the use of chemical products, mainly site-specific fungicides (Gasparotto et al., 2006; Jones, 2000; Marín et al., 2003). There is one forecasting system for Sigatoka diseases that has been adapted and implemented in several regions where bananas are planted. Nevertheless, disease control depends on fungicide application . Different groups of fungicides such demethylation inhibitors (DMI), amine fungicides, Qo inhibitors (QoI), anilinopyrimidines (AP), benzimidazoles (BCM), succinate dehydrogenase inhibitors (SDHI) fungicides and guanidines are used to control the BLSD in banana plantations (FRAC, 2010). In Brazil the fungicides registered for use in the control of BLSD are DMI, QoI and dithiocarbamates (Agrofit, 2012).

Due to its short generation time and high levels of genetic variation, *M. fijiensis* has a high potential for development of fungicide resistance (Brent and Hollomon, 1998). Since 1987 there is a working group of banana industry representatives participating in the Fungicide Resistance Action Committee (FRAC) that searches for ways to minimize the risk of the development of resistance. For Sigatoka disease the intensive use of fungicides has imposed selective pressures that have been promoting the development of resistant populations (Ma and Michailides, 2005). This serious issue can be exemplified by several reports of changes in the sensitivity of *M. fijiensis* populations to fungicides (Amil et al., 2007; Cañas-Gutíerrez et al., 2009; Chin et al., 2001; Knight et al., 2002; Marin et al., 2003; Romero and Sutton, 1997).

The aims of the present study were:

- To identify the predominant species of *Mycosphaerella* in banana fields in Minas Gerais state using molecular and traditional tools.
- To describe the genetic structure of *M. musicola* populations in Minas Gerais using microsatellites markers.
- To assess the sensitivity of *M. fijiensis* to fungicides commonly used to control BLSD in Brazil.

REFERENCES

AGROFIT. 2012. Available at: http://extranet.agricultura.gov.br/agrofit_cons/principal_agrofit_cons [Assessed January 12, 2012].

Amil, A., Heaney, S., Stanger, C., and Shaw, M. 2007. Dynamics of QoI sensitivity in *Mycosphaerella fijiensis* in Costa Rica during 2000 to 2003. Phytopathology, 97: 1451-1457.

Arzanlou, M., Abeln, E., Kema, G., Waalwijk, C., Carlier, J., de Vries, I., Guzman, M., and Crous, P. 2007. Molecular diagnostics for the sigatoka disease complex of banana. Phytopathology, 97: 1112-1118.

Arzanlou, M., Groenewald, J., Fullerton, R., Abeln, E., Carlier, J., Zapater, M., Buddenhagen, I., Viljoen, A., and Crous, P. 2008. Multiple gene genealogies and phenotypic characters differentiate several novel species of *Mycosphaerella* and related anamorphs on banana. Persoonia, 20: 19-37.

Brent, K. J., and, Hollomon, D.W. 1998. Fungicide resistance: the assessment of risk. FRAC Monogr. No. 2, Global Prot. Fed. 48 pp.

Canas-Gutierrez, G., Angarita-Velasquez, M., Restrepo-Florez, J., Rodriguez, P., Moreno, C., and Arango, R. 2009. Analysis of the CYP51 gene and encoded protein in propiconazole-resistant isolates of *Mycosphaerella fijiensis*. Pest Management Science, 65: 892-899.

Carlier, J., Zapater, M. F., Lapeyre, F., Jones, D. R., and Mourichon, X. 2000. Septoria Leaf Spot of Banana: A newly discovered disease caused by *Mycosphaerella eumusae* (Anamorph *Septoria eumusae*). Phytopathology, 90: 884-890.

Castro, M. E. A., Pereira, J. C. R., and Gasparotto, L. 2005. First report of black-sigatoka in the state of Minas Gerais, Brazil. Fitopatologia Brasileira, 30: 668-668.

Chin, K. M., Wirz, M., and Laird, D. 2001. Sensitivity of *Mycosphaerella fijiensis* from banana to Trifloxystrobin. Plant Disease, 85: 1264-1270.

Churchill, A. C. L. 2011. *Mycosphaerella fijiensis*, the black leaf streak pathogen of banana: progress towards understanding pathogen biology and detection, disease development, and the challenges of control. Molecular Plant Pathology, 12:307-328.

Cordeiro, Z. J. M., and Matos, A. P. de. 2005. Doenças da banana. Informe Agropecuário. Belo Horizonte, 26: 12-16.

Cordeiro, Z. J. M., and Matos, A. P. de. 2003. Impact of *Mycosphaerella* spp in Brazil. In: Workshop on *Mycosphaerella* leaf spot diseases held in San Jose, 2002, Costa Rica. Mycosphaerella leaf spot diseases of bananas: present status and outlook. Montpellier: INIBAP, 2003. p. 91-97.

Crous, P. W. 2009. Taxonomy and phylogeny of the genus *Mycosphaerella* and its anamorphs. Fungal Diversity, 38: 1-24.

Crous, P. W., and Mourichon, X. 2002. *Mycosphaerella eumusae* and its anamorph *Pseudocercospora eumusae* spp. nov.: causal agent of eumusae leaf spot disease of banana. Sydowia, 54: 35-43.

FRAC. 2010. Fungicide resistance action committee. Available at: http://www.frac.info/frac/index.htm [Assessed September 12, 2011].

Gasparotto, L., Pereira, J. C. R., Hanada, R. E., and Montarroyos, A. V. V. 2006. Sigatoka-negra da bananeira. Manaus: Embrapa Amazônia Ocidental. 177p.

Hayden, H. L., Carlier, J., and Aitken, E. A. B. 2003. Population differentiation in the banana leaf spot pathogen *Mycosphaerella musicola*, examined at a global scale. Plant Pathology, 52: 713-719.

Hayden, H., Carlier, J., and Aitken, E. A. B. 2005. The genetic structure of Australian populations of *Mycosphaerella musicola* suggests restricted gene flow at the continental scale. Phytopathology, 95: 489-498.

Johanson, A., Crowhurst, R. N., Rikkerink, E. H. A., Fullerton, R. A., and Templeton, M. D. 1994. The use of species-specific DNA probes for the identification of *Mycosphaerella fijiensis* and *M. musicola*, the causal agents of Sigatoka disease of banana. Plant Pathology, 43: 701-707.

Jones, D. R., 2000. Sigatoka. In Diseases of Banana, Abacá and Enset, D.R. Jones (ed.), pp. 79-92. Wallingford, UK: CABI Publishing.

Jones, D. R. 2003. The distribution and importance of the Mycosphaerella leaf spot diseases of banana. In: Mycosphaerella Leaf Spot Diseases of Bananas: Present Status and Outlook. Proceedings of the Workshop on Mycosphaerella Leaf Spot Diseases, San José, Costa Rica, 20-33 May 2002 (Jacome, L., Lepoivre, P., Marin, D., Oriz, R., Romero, R. and Escalant, J.V., eds), pp. 25-41. Montpellier: The International Network for the Improvement of Banana and Plantain.

Knight, S., Wirz, M., Amil, A., and Cook, A. 2002. Fungicide resistance in *Mycosphaerella fijiensis* Morelet: Current status and outlook.In Acorbat. Memorias XV reunion, Realizada en Cartagena de Indias, Colombia. 27 de octubre al 02 Noviembre 2002. Medellín. (COL): Asociacion de Bananeros de Colombia AUGURA, 2002.

Ma, Z., and Michailides, T. J. 2005. Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. Crop Protection, 24: 853-863.

Marín, D. H., Romero, R. A., Guzmán, M., and Sutton, T. B. 2003. Black Sigatoka: An increasing threat to banana cultivation. Plant Disease, 87: 208-222.

Molina, C., Kaemmer, D., Aponte, S., Weising, K., and Kahl, G. 2001. Microsatellite markers for the fungal banana pathogen *Mycosphaerella musicola*. Molecular Ecology Notes, 1: 137-139.

Moreira, R. F. C., Cordeiro, Z. J. M., and Vilarinhos, A. D. 2003. Caracterização genética de isolados de *Mycosphaerella musicola* por RAPD. Summa Phytopatologica, 29: 275-277.

Mouliom-Pefoura, A., Lassoudière, A., Foko, J., and Fontem, D. A. 1996 Comparison of development of *Mycosphaerella fijiensis* and *Mycosphaerella musicola* on banana and plantain in the various ecological zones in Cameroon. Plant Disease, 80: 950-954.

Pereira, J. C. R., Gasparotto, L., Coelho, A. F. S., and Urben, A. 1998. Ocorrência da sigatoka-negra no Brasil. Fitopatologia Brasileira, 23: 295.

Romero, R. A., and Sutton, T. B. 1997. Sensitivity of *Mycosphaerella fijiensis*, causal agent of black Sigatoka of banana, to propiconazole. Phytopathology, 87: 96-100.

Zapater, M., Duchemin, M., Dussart, J., Coste, D., Brottier, P., and Carlier, J. 2008. Microsatellite markers for the fungal banana pathogens *Mycosphaerella fijiensis*, *Mycosphaerella musicola* and *Mycosphaerella eumusae*. Molecular Ecology Resources, 8: 1121-1125.

1. Etiology of Sigatoka Diseases in Minas Gerais State, Brazil

1.1 Abstract

A thorough assessment of the distribution of *Mycosphaerella* spp. in Minas Gerais state, Brazil, was conducted after the first report of the occurrence of *Mycosphaerella fijiensis,* published in 2005. From 2009 to 2011, 80 fields located in 20 municipalities including the same fields where the disease was first reported were sampled. A total of 800 samples of leaf tissue with symptoms similar to those of yellow or black leaf streak Sigatoka diseases were examined, and 239 isolates were obtained. The identification of the fungi was based on morphological characters combined with DNA sequences obtained after amplification with species-specific primers and phylogeny inferred from the ITS region of *Mycosphaerella* strains from banana. All 239 isolates were identified as *M. musicola*. The absence of *M. fijiensis* in the examined samples may have been due to the no displacement of *M. musicola* by *M. fijiensis* or no occurrence of this species in Minas Gerais. Yellow Sigatoka is the prevailing leaf spot disease of bananas in Minas Gerais state and regulatory/legislative control measures need to be revised.

1.2 Introduction

There are three leaf spots commonly referred to as Sigatoka diseases of banana which are caused by three distinct species of *Mycosphaerella*. All species cause necrosis of leaf tissues which leads to defoliation that result in yield loss and premature or uneven ripening of the fruit. Yellow Sigatoka (YS) and black Sigatoka, also known as black leaf streak disease (BLSD), are the two major leaf spot diseases that affect banana (*Musa* spp.) production in many tropical countries (Stover, 1972). *Mycosphaerella musicola* R. Leach ex J. L. Mulder (anamorph *Pseudocercospora musae*) (Zimm.) Deighton is the most frequently reported causal agent of YS and was the first 'Sigatoka' foliar pathogen to be identified in 1902 (Jones, 2003). This pathogen is globally distributed and causes crop losses wherever bananas are grown. In 1963, *Mycosphaerella fijiensis* M. Morelet (anamorph *Pseudocercospora fijiensis* (M. Morelet) Deighton) was identified as the causal agent of BLSD (Rhodes, 1964). *Mycosphaerella fijiensis* is not as widely distributed as *M. musicola* but due to its more aggressive nature has often displaced populations of *M. musicola* where the diseases co-occur (Romero, 2003) and BLSD requires more intensive fungicide spray schedules to be controlled (Carlier et al., 1999a). When the species co-occur within a region, their distribution appears to be affected by weather conditions, mainly temperature and humidity (Cintra et al., 2008; Mouliom-Pefoura, 1996; Romero and Gauhl, 1988). More recently, *Mycosphaerella eumusae* Crous & Mourichon (anamorph *Pseudocercospora eumusae* Crous & Mourichon) was reported in Asia (Carlier et al., 2000) and is currently distributed in South-East Asia and parts of Africa (Arzanlou et al., 2010).

Diagnosis of the fungal species that cause Sigatoka diseases has traditionally been based on disease symptoms and morphology of the fungi (Fortune et al., 2005; Gasparotto et al., 2006; Ploetz, 2004; Stover, 1972). However, all three *Mycosphaerella* species cause similar symptoms in banana leaves, which makes disease diagnosis very challenging. Due to the conserved teleomorph morphology, there are no differences between perithecia and ascospores among the three species that infect banana (Meredith and Lawrence, 1969). The major morphological differences between *M. musicola*, *M. fijiensis* and *M. eumusae* is in the anamorphic state (Crous and Mourichon, 2002). Conidiophores of *M. musicola* produce dense fascicles (sporodochia) on a dark brown or black stroma while the conidiophores of *M. fijiensis* emerge singly or in small groups and sporodochia and stromata are absent (Stover, 1972). *Mycosphaerella musicola* is morphologically very similar to *M. eumusae*, and reports suggest that these two pathogens have commonly been misidentified (Crous and Mourichon, 2002). Sporodochia of *M. eumusae* have a similar shape to those of *M. musicola* showing epiphyllous sporodochia formed on dark brown substomatal stromata. Moreover, even when the pathogens are isolated from diseased tissues, identification of the various *Mycosphaerella* species is often difficult due to the overlapping and limited morphological characters (Arzanlou et al., 2007). Reliable diagnosis is essential since all three diseases require unique management strategies to achieve satisfactory levels of disease control (Johanson and Jeger, 1993). *M. fijiensis,* for example, infects younger leaves on susceptible banana clones than those affected by *M. musicola* (Jones, 2003) and symptoms develop faster on banana infected with *M. fijiensis* and *M. eumusae* than with *M. musicola* (Balint-Kurti et al., 2001). Therefore, chemicals are to be applied at different times, perhaps in different targets, and at variable number of applications to control each disease.

To overcome difficulties in disease diagnosis, polymerase chain reaction (PCR) methods have been developed to aid in proper species identification as has been done for many other important plant pathogens (Arzanlou et al., 2007; Frederick et al., 2002; Johanson and Jeger, 1993). Specifically, primers designed to amplify the internal transcribed spacer regions of rDNA have made it possible to differentiate *M. musicola* from *M. fijiensis* (Johanson and Jeger, 1993). More recently, species-specific PCR primers were developed based on the actin locus and the development of TaqMan real-time quantitative PCR assay based on the beta-tubulin locus has made it possible to differentiate *M. musicola*, *M. fijiensis* and *M. eumusae* (Arzanlou et al., 2007).

In Brazil, YS occurs in all regions where banana is cultivated and BLSD was identified as occurring in Amazonas state in 1998 (Pereira et al., 1998). BLSD spread rapidly to other areas throughout the North of Brazil, but has not been reported in the state of Tocantins. Afterwards, the reports of occurrence of BLSD followed a North-South of the country pathway. The disease spread to the central-west regions (Mato Grosso and Mato Grosso do Sul) and was also reported to be present in the states of São Paulo and Minas Gerais, both in the Southeast region of Brazil (Castro et al., 2005; Gasparotto et al., 2006; Hanada et al., 2007) and in other states of the South region (Figure 1A).

Banana is the second most important fruit crop for Minas Gerais state (IBGE, 2010). However, this economic activity is currently facing difficulties due to the restrictions to commercialization after BLSD was officially reported to occur in the state

(Castro et al., 2005; Ferrari et al., 2005). *Mycosphaerella fijiensis* is a quarantine pathogen in some places in Brazil and regulatory mechanisms prohibiting the movement of fruits and other plant materials are reinforced to avoid the spread of the pathogen. This regulatory measure has resulted in serious economic implications to some banana producing states since fruits or plantlets produced in states where the disease has been recorded cannot be sold in states which are free of BLSD. In October 2006, one year after the first report of BLSD in Minas Gerais, a phytosanitary survey was conducted across the main banana regions of the state and a regulatory document was issued by the Ministry of Agriculture of Brazil declaring some municipalities in the North, Northwest, Triângulo and Vale do Jequitinhonha regions as free from BLSD (Maciel, 2006). After the abovementioned survey, no change in the intensity of necrotic leaf spots affecting banana leaves was observed by growers in areas where BLSD was originally reported; nor has the usage of fungicide increased to control the disease. The disease is considered to be “under control” and probably restricted or absent to the areas where it was initially found. Apparently, in the state of Minas Gerais, putative epidemics of BLSD, are not as severe as those recorded in others regions of Brazil. This is a controversial scenario which remains unresolved. A clear response to whether *M. fijiensis* have been rightfully reported in Minas Gerais must be reached. Therefore, the objectives of this work were to (i) determine the etiological agent of the Sigatoka diseases in the main banana producing regions of Minas Gerais state, including those fields where the BLSD was first reported and (ii) to clarify the present status of the disease in Minas Gerais.

1.3 Materials and methods

Sampling scheme and isolate recovery

Leaf pieces (20 by 20 cm) with symptoms of Sigatoka disease were collected, packed in plastic bags and taken to the laboratory. After arriving at the laboratory the samples were kept in the refrigerator until examination and pathogen isolation. Symptomatic leaves were collected from plants of different ages, but no adjacent plants were sampled in a field. Leaves of a minimum of 10 symptomatic plants were collected per field. For each sample, geographic coordinates were obtained using a portable GPS device (Magellan GPS Color TRAK, Magellan).

Diseased plant material was collected from 80 fields in 20 municipalities within four regions of Minas Gerais state: South, North, Triângulo Mineiro (located to the West) and Zona da Mata (located to the East) (Figure 1B and Table 1). The sampling started in January 2009 in the North region of Minas Gerais. From January 2010 until March 2011 a more intensive sampling was performed at different locations. Fields were chosen such as to represent the diversity of farming practices within the regions from small local farms (0.5 ha) with limited input to large (280 ha), well-managed banana plantations. All samples were collected during the summer, period of more intense rainfall and higher temperature, favorable for the occurrence of the diseases. In some fields from Zona da Mata, South and North of Minas Gerais state sampling was conducted in two different years (Table 1).

All plant samples were first tentatively diagnosed by critically observing the Sigatoka disease symptoms in the field. The sampling procedure was biased towards the detection of BLSD as leaves with symptoms resembling those of BLSD were sought more carefully and all suspicious plants were sampled. A subset of the samples, mainly comprised of leaf pieces with BLSD-like symptoms, were subjected to more detailed analysis using microscopic characters.

Under a compound microscope and using a sterile fine needle, conidia present on abaxial surface of the symptomatic leaves were picked and transferred to water-agar medium. Single conidium was then taken from the water-agar medium and transferred to be cultured on V8 agar medium (300 mL V8 juice, 3 g of $CaCO_3$ and 20 g of agar per liter of medium). The single-spore isolates were incubated at 25^oC under 12 h of daily white light regime for 10 days prior to examination and DNA extraction (Figure 2).

Discharge of ascospores from necrotic leaf material was used for leaf samples when no conidium was observed. Pieces of leaves with lesions were cut and were incubated in high humidity for 24 h. After that the leaves were soaked for 5-10 min in sterile water. The pieces of tissue were fixed on filter paper and set on the internal part of the top of petri plates with 2% water-agar for 2 h for ascospore discharge. Single ascospores ejected from perithecia were transferred to fresh V8 medium plates using a fine needle and incubated at 25^oC with 12 h of light for 15 days. After incubation mycelial discs were transferred to V8 brothand the DNA was extracted. Pieces of tissue containing multiple lesions with symptoms of Sigatoka that failed both direct isolation (conidia) and isolation based on ascospores discharge were subjected to to direct extraction of DNA using the DNeasy Plant Mini Kit (Qiagen Germany) according to the manufacturer´s recommendation.

Species identification

Morphological characters

Due to the difficulties to produce large quantities of asexual spores *in vitro*, four isolates of each region were randomly chosen for the morphometric assessments and characterization. The conidia were transferred to microscope slides previously prepared with 50% glycerin and then observations were made under a light microscope. The morphological characters examined were: conidium dimension (length and diameter), pattern of conidia, septation, basal hilum and conidial shape and presence of sporodoquia (Stover, 1972). The identification was derived from approximately 50 observations of conidium from each region.

DNA extraction

DNA was extracted from every single-spore culture. Four mycelium plugs from colonies growing on V8 agar were transferred to 50 mL of V8 broth (250 mL of V8 juice and 3 g $CaCO_3$ per liter of medium, pH 6.5) in a 250 mL flask and incubated at $25^{o}C$ for 10 days at 25°C at 120 rpm in a shaker. The mycelium was then washed with sterile water and the excess water was removed using sterile filter paper. The mycelium was ground to powder using liquid nitrogen in a porcelain mortar and pestle. DNA was extracted using the cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1990). An aliquot of 50 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) was added to each DNA sample. RNA was digested with RNase A for 2h at $37^{o}C$ before storage at -20°C. The concentration of DNA was quantified in a spectrophotometer (NanoDrop 2000 Thermo Scientific) and working solutions were standardized to 20ηg/µL of DNA.

Species specific primers

The primer pairs Mfact/ACTR, MmactF2/MmactRB, and MEactR/ACTF specific for *M. fijiensis*, *M. musicola* and *M. eumusae*, respectively, were used to identify each isolate. PCR conditions were as described by Arzanlou et al. (2007), except that the DNA concentration used was 20ηg/µL. All amplifications were performed in a thermal cycler MJ PTC-100. After the PCR reaction, 5µl of each PCR product was subjected to electrophoresis in 2.0% agarose gel in 1x TBE and viewed under UV on gel stained with GelRed (Biotium). Fragments were compared with a 100bp DNA ladder and scored.

Amplifications controls were made to validate the results. Thus the specific primer used to detect *M. fijiensis* was tested with DNA from naturally infected banana leaves collected in Manaus, Amazonas state, where BLSD is known to occur and the expected diagnostic bands were present. The specific primer sets (ACTR/MFactF, MmactF2/MmactRb and MEactR/ACTF) were also used for DNA amplification from healthy banana leaves (controls). The primers MEactR/ACTF were not tested as control due the absence of *M. eumusae* in Brazil, but were tested with DNA from leaf tissue with symptoms of yellow Sigatoka.

DNA phylogeny

Partial sequences of the internal transcribed spacer (ITS) region was PCR-amplified using fungal specific primers ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990). PCR amplifications were done in 20 µl reaction volumes containing 1X PCR buffer (Invitrogen, Carlsbad, California), 2.5 mM $MgCl_2$, 0.2 mM each dNTP (Invitrogen), 0.375 mM each primer and 0.5 U Taq polymerase (Invitrogen). Thermocycling conditions consisted of an initial melt at 94^o C for 3 min, followed by 30 cycles of 94^o C (30 s), 55^o C (30 s), and 72^o C (1 min), and a final hold of 72^o C for 8 min. All amplifications were performed in a MyCycler thermocycler (Bio-Rad Laboratories Inc., Hercules, California). For each PCR amplification, 5 µl were subjected to electrophoresis in 1.8% agarose gels stained with SYBR Green I nucleic acid stain (Invitrogen) and viewed with UV light. PCR products were cleaned with ExoSap-IT (USB, Cleveland, Ohio) following the manufacturer’s instructions. Sequencing was performed at the Core Instrumentation Facility (CIF) of the University of California’s (UC) Institute of Integrative Genome Biology at UC Riverside.

The nucleotide sequences were edited with Sequencher 4.6 (Gene Codes Corp., Ann Arbor, Michigan) and aligned using the Clustal W in MEGA 5.05 software (Tamura et al., 2011). Two sets of alignments were made. One alignment contained all isolates obtained in the present survey and the second contained only representatives isolates of the present survey plus sequences of *M. fijiensis* (accession EU114250, AY266152, AF181705, EU514252), *M. eumusae* (AY923760, AY923759, AY923757, EU140340, GU168036), *M. musicola* (AY646473, AY646466, AY646505, AY646507), *Pseudocercospora indonesiana* (EU514283, EU514283), *P. basiramifera* (AF309595), *P. paraguayensis* (DQ267602), *P. assamensis* (EU514281), and *P. longispora* (EU514284, EU514285) which were downloaded from GenBank. The first set was used to estimate the number of haplotypes in the sample and the second set was used for phylogenetic inferences. Sequences of the second set were subjected to Bayesian phylogenetic analysis using MrBayes v.3 (Ronquist and Huelsenbeck, 2003). Nucleotide substitution model was selected using MrModeltest v. 2.2 (Nylander, 2004) and the HKY+I evolution model was used for the analysis. The Markov Chain Monte Carlo (MCMC) analysis of four chains started from a random tree topology and lasted 10.000.000 generations. Trees were saved at each 100 generations, resulting in 75.000 saved trees. Burn-in of 25% was made and posterior probabilities (PP's) were calculated.

Maximum parsimony (MP) analysis was made with the heuristic searches with 1000 random-addition sequence replicates and tree bisection reconnection (TBR) branch swapping using PAUP* (4.0 beta 10) (Swofford, 2002). Gaps were treated as missing data.

1.4 Results

Sampling and fungal isolation

A total of 800 samples of leaf tissue were examined and most had typical symptoms of the yellow Sigatoka: elongated streaks, elliptical in shape and chlorotic areas around the necrotic spots with dark brown borders, gray color center where regular lines of sporodochia could be seen. Symptoms of five samples collected in the Zona da Mata region were similar to those of BLSD (Figure 3), but no conidia of *M. fijiensis* were found. In total, 239 isolates were obtained from the four regions of Minas Gerais state (Table 1). Twenty-two isolates were obtained from the Triângulo Mineiro, 76 from the North, 80 from the South, and 61 from the Zona da Mata region.

Species identification

Morphological characters

Two hundred conidia of isolates obtained from the four regions (50 conida/region) were morphologically characterized. Conidia were typically cylindric to obclavate in shape, pale brown to olivaceous in color, 2-7 septa predominating 3-6, straight or curved, indistinct basal hilum and dimensions 30-70 x 3-5 µm (Table 2). Sporodochia were detected in the majority (>90%) of leaf tissue samples with sporulating lesions, suggesting that most lesions were caused by *Pseudocercospora musae*.

Species-specific PCR analysis

DNA of all isolates was screened PCR-amplified with species-specific primers based on the partial sequence of the actin gene. The 200bp amplicons were detected only for *M. musicola* specific primer. No amplification with *M. fijiensis* and *M. eumusae* specific primers was observed.

ITS phylogeny and haplotype diversity

Seven haplotypes were identified based on the ITS sequences of 186 isolates.The haplotype 1 was shared by most isolates (161) and was detected in all sampling regions. Haplotype 2 consisted of seven individuals, three from the North and four from the Triângulo regions. Haplotype 3 consisted of two individuals from Zona da Mata and haplotypes 4 and 5 had one individual from the Zona da Mata and South regions, respectively. Haplotype 6 had two individuals from the North and Triângulo regions. The haplotype 7 was the second most frequently found and consisted of 12 individuals, five from the Triângulo, four from the South and three from the North. Haplotype 1 differs from haplotypes 2, 3, 4, and 5 by a single base pair (bp); it differs by 9 bp and 1 indel from haplotype 6; and by 11 bp and 1 indel from haplotype 7.

Results of BLAST analyses revealed that four out of seven haplotypes found in this study were also identified in other countries: haplotype 1 has been identified in Saint Lucia, Martinique, Guadeloupe, Guinea, Cameroon and Australia, haplotype 2 in Colombia; haplotype 6 in Australia; haplotype 7 in Australia, Venezuela, Martinique, Guadeloupe, Costa Rica and Colombia. No sequences were found in GenBank that matched haplotypes 3, 4 and 5.

Thirty three sequences were used in the phylogenetic analysis based on ITS which included representative haplotypes (n=7) detected in this study and representative sequences downloaded from GenBank. A total of 486 bp were subjected to the analyses.

The maximum parsimony analysis resulted in 1 tree of length 72, consistency index (CI) 0.847, and retention index (RI) 0.971.

The MP and Bayesian analyses based on ITS sequence resulted in a tree with five well-supported clades. Clade I, IV and V consisted of *P. longispora*, *M. eumusae* and *M. fijiensis* species had bootstrap/posterior probability (PP) values of 96/0.94, 94/0.98 and 100/1.00 respectively. Members of *M. musicola* were divided into two clades with bootstrap/PP values of 97/0.92 and 100/1.00 (Figure 4).

1.5 Discussion

The establishment of BLSD in banana producing areas located in the tropical regions usually leads to severe crop losses. However, in Minas Gerais State no changes in disease impact or in disease management were recorded as a result of the occurrence of BLSD as reported in Castro et al. (2005) and Ferrari et al. (2005). Therefore, it was hypothesized that *M. fijiensis* did not establish in the State; the pathogen is not fully adapted yet to the environmental conditions of these areas or the first report of BLSD was not correct. Based on classical and molecular diagnostic techniques, only *M. musicola* was detected in all samples examined in the current study.

It is generally pointed out that once *M. fijiensis* has been introduced into an area *M. musicola* is rapidly displaced. This has been observed in the Pacific Islands, Latin America and Africa (Carlier et al., 1999b; Zandjanakou-Tachin et al., 2009). However, in other places such as The Philippines and Vietnam this has not occurred and *M. musicola* is still found after the appearance of BLSD in 1965 and 1993, respectively (Carlier et al., 1999b; Jones, 2003). Thus, the rate of displacement seems to be influenced by genetic and/or ecological factors (Marin et al., 2003). Displacement of species of plant pathogenic fungi as the result of the introduction of more aggressive genotypes is well documented (Day and Shattock, 1997). Unfortunately, no comparison of aggressiveness between isolates of *M. musicola* and *M. fijiensis* was possible due to the fact that isolates of the latter species were not detected in the sampled fields.

The environmental conditions, mainly temperature, may have been playing an important role in determining the distribution of *Mycosphaerella* spp. in the state of Minas Gerais. In some countries, both species have been found and they are thought to co-exist (Romero, 1988; Johanson, 1995). The co-existence and distribution of both species is apparently linked with temperature, which in turn is markedly influenced by altitude (Churchill, 2011; Marín et al., 2003). In two studies carried out to investigate the distribution of *Mycosphaerella* spp., *M. musicola* was the dominant species at high altitudes and *M. fijiensis* developed better at low altitudes (Mouliom-Pefoura et al., 1996; Romero, 1988). The analyses of conidia germination, growth of germ tubes, incubation period and life cycle of both *M. fijiensis* and *M. musicola* under different ecological conditions revealed that in low altitude zone (80 m elevation) with temperature around 21 and 33°C both diseases were found in the area, but BLSD was dominant. On the other hand, in highland areas (> 1300 m elevation) with temperatures around 15 and 18 °C, *M. musicola* had shorter incubation period than *M. fijiensis* and was more prevalent (Mouliom-Pefoura et al., 1996). Interestingly, studies have also suggested that *M. fijiensis* may be adapting to higher elevations (Arzanlou et al., 2007; Carlier et al., 2000). Despite these facts, species prevalence according to altitude seems not to occur in all areas where both diseases co-occur. In Colombia, both pathogens are equally severe at altitudes of 1500 m (Marín et al., 2003) and low temperatures and high altitude are not limiting factors for *M. fijiensis* infections, also in Costa Rica where the pathogen has been detected affecting banana plants at 1360 m of (Arzanlou et al., 2007). Inherent characteristics of the populations need to be investigated in more details, mainly those related to the aggressiveness of the individuals.

The topographic conditions of Minas Gerais State where samples were collected vary considerably regarding altitude and temperature. This seems to be the case for most areas of South and Southeast regions of Brazil. Overall, however, there are favorable conditions for the development of BLST in all regions where it has been reported in Brazil. The annual average mean temperature ranges from 20 °C in the South and in Zona da Mata to 26°C in the North regions of Minas Gerais state. Usually, temperature in São Paulo and Santa Catarina states are cooler than those recorded in the south and Zona da Mata regions of Minas Gerais but severe epidemics of BLSD are recorded in the first two states (Ferrari and Nogueira, 2011; Volpato, 2005). The average values of relative humidity in the South and Zona da Mata are suitable for BLSD. In these regions, the annual average relative humidity ranges from 75 to 80 % (INMET, 2011). Despite the favorable temperatures and suitable humidity conditions in the South and Zona da Mata, BLSD has not become widespread and currently it is not regarded to be of any relevance by banana growers. Additionally, based on the results of this study, if BLSD were correctly diagnosed in the state it might have not established in these areas.

Another intriguing aspect of the putative occurrence of BLSD in Minas Gerais is the restriction of the disease to the South and Zona da Mata regions. The North of Minas Gerais is the most important banana producing region and approximately 15,000 ha are cultivated (IBGE, 2010). Based on the reports from other countries, one would expect that BLSD should have easily spread throughout the state. Ascospores of *M. fijiensis* may not spread more than 100-200 kilometers when there is a combination of strong winds and heavy clouds, or at night (Jones, 2003). The producing areas in the North region are located approximately 750 kilometers from the South and Zona da Mata regions. Likewise, the Triângulo Mineiro region is located 600 kilometers from the areas where BLSD occur. Aerial dissemination to these areas would be expected, particularly since banana are widely cultivated as a subsistence crop and even in great areas in the state. The availability of susceptible host plants would have contributed to the spread of the pathogen in “small jumps”, mediated by the dispersal of airborne ascospores of *M. fijiensis* that could have spread slowly to the more distant regions.

In warmer areas of Brazil such as Rondônia and Mato Grosso states the establishment of BLSD was fast. Within a 3 year-period, the disease spread from 9 to 28 municipalities in Rondônia (Fernandes et al., 2007). In Mato Grosso state, BLSD was reported to occur in more than 18 municipalities in 2010 (Souza and Feguri, 2010).

Other possible but unlikely explanation for the absence of *M. fijiensis* would be that the emergency control measures adopted when BLSD was reported for the first time were effective in preventing the spread of the recently detected pathogen. When the disease was reported in Minas Gerais, plant pathologists from several institutions were gathered to assess the epidemiology of the disease and provide technical guidance to farmers. Additionally, abandoned banana fields were destroyed as part of prevention to avoid the dispersal of the pathogen to other areas of the state. These practices might have prevented dissemination of the disease to unaffected areas. Nevertheless, considering the nature of an airborne pathogen having a broad availability of host populations this would be of low likelihood.

The analyses of all samples collected in this study regarding symptoms and fungal morphology supported only the occurrence of yellow Sigatoka in Minas Gerais. The morphological characteristics of the conidia of *M. musicola* (length, diameter, number of septa, basal hilum and form) sampled in Minas Gerais match those described for this species by Meredith and Lawrence (1969). Some of the critical morphological characters of the conidia of *M. fijiensis* overlap with those of *M. musicola* and can make their distinction somewhat confusing. Conidia of *M. fijiensis* are longer and the number of septa is higher than those of *M. musicola* and there are some slight differences in the shape of the conidia and in the basal hilum. However, in addition to the characteristics of the conidia, the presence of sporodoquia, which are not produced by *M. fijiensis* and were constantly found on the samples examined in the present study support the identification of *M. musicola* as the sole fungus in the samples.

The symptoms of BLSD and yellow Sigatoka can be confused and vary depending on the cultivar and whether the plants have been treated with fungicides (Gasparotto et al., 2006). In the municipality of Piau, in the Zona da Mata region, where *M. fijiensis* was first reported (Castro et al., 2005), banana leaves with symptoms resembling those of BLSD were found, but data from molecular tests did not confirm the presence of *M. fijiensis*. The combination of morphological and molecular data helped the identification of more than 20 species of *Mycosphaerella* occurring on banana (Arzanlou et al., 2008). The latter approach was used in the present study and we are confident that *M. fijensis* was not present in the analyzed samples.

The nucleotide variation between the haplotypes ranged between 1 and 11 bp. Similar results were found by Thomas-Hall et al. in 2004 where phylogenetic analysis of the ITS region from 111 samples of Sigatoka disease revealed 7 distinct clades with nucleotide variation ranging from 4 to 14 bp. The split of *M. musicola* sequences into two clades found in this study could suggest the occurrence of more than one closely related or cryptic species in Minas Gerais state, but additional analyses did not support any evidence for this. For example, isolates with different haplotypes from both clades were scored for genetic variation using AFLP and all isolates produced variable banding patterns suggesting there was not enough genome-wide variation to support a speciation event. Additionally, three nuclear loci (β-tubulin, Actin and Elongation factor 1-alpha, EF-1α) and one mitochondrial locus (small subunit rDNA) were also sequenced from representative haplotypes which also did not suggest evidence of cryptic species. The analysis of other genomic regions combined with an assessment of genome-wide variation with microsatellite markers could help to elucidate this and we are currently investing this.

This work confirmed that the yellow Sigatoka is the prevailing leaf spot disease of banana in the state of Minas Gerais. It is difficult to explain why BLSD is not ubiquitous in Minas Gerais because the pathogen was reported in areas with favorable weather conditions and susceptible host materials were and remain available for this fungus. More surveys need to be conducted on a regular basis in which thorough sampling procedures and genetic analyses are employed. This will help address an important question that remained opened and which is difficult to deal with: could the first report of the occurrence of BLSD have been related to the difficulty in identifying the pathogen? If this is the case, regulatory/legislative control measures need to be revised, because the restrictions imposed by phytosanitary laws have been perverse to farms and the state’s economy.

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1.7 References

Arzanlou, M., Abeln, E., Kema, G., Waalwijk, C., Carlier, J., de Vries, I., Guzman, M., and Crous, P. W. 2007. Molecular diagnostics for the sigatoka disease complex of banana. Phytopathology, 97: 1112-1118.

Arzanlou, M., Crous, P. W., and Zwiers, L. H. 2010. Evolutionary dynamics of mating-type loci of *Mycosphaerella* spp. occurring on banana. Eukaryotic Cell, 9: 164-172.

Arzanlou, M., Groenewald, J., Fullerton, R., Abeln, E., Carlier, J., Zapater, M., Buddenhagen, I., Viljoen, A., and Crous, P. W. 2008. Multiple gene genealogies and phenotypic characters differentiate several novel species of *Mycosphaerella* and related anamorphs on banana. Persoonia, 20: 19-37.

Balint-Kurti, P. J., May, G. D., and Churchill, A. C. L. 2001. Development of a transformation system for *Mycosphaerella* pathogens of banana: a tool for the study of host/pathogen interactions. FEMS Microbiology Letters, 195: 9-15.

Carlier, J., El Hadrami, A., Hayden, H., Zapatar, M. F., and Lapere, F. 1999a. Populations study of *Mycosphaerella fijienis* and genetic improvement of bananas for resistance to black leaf streak disease. InfoMusa, 8: 16

Carlier, J., Fouré, E., Gauhl, F., Jones, D. R., Lepoivre, P., Mourichon, X., Pasberg-Gauhl, C., and Romero, R. A. 1999b. Black leaf streak. In: Jones, D. R. (Ed.). Diseases of banana, abacá and enset. Wallingford, UK: CAB Publishing, p. 37-79.

Carlier, J., Zapater, M. F., Lapeyre, F., Jones, D. R., and Mourichon, X. 2000. Septoria leaf spot of banana: A newly discovered disease caused by *Mycosphaerella eumusae* (Anamorph *Septoria eumusae*). Phytopathology, 90: 884-890.

Castro, M. E. A., Pereira, J. C. R., and Gasparotto, L. 2005. First report of black-sigatoka in the State of Minas Gerais, Brazil. Fitopatologia Brasileira, 30: 668-668.

Churchill, A. C. L. 2011. *Mycosphaerella fijiensis*, the black leaf streak pathogen of banana: progress towards understanding pathogen biology and detection, disease development, and the challenges of control. Molecular Plant Pathology, 12: 307-328.

Cintra, W. J., Valadares, R., Avelino, R. C., Bucker, W. M., Xavier, F. R. V., Ramos, A. F., and Anderson, P. P. 2008. Worldwide geographical distribution of Black Sigatoka for banana: predictions based on climate change models. Scientia Agricola, 65: 40-53.

Crous, P. W., and Mourichon, X. 2002. *Mycosphaerella eumusae* and its anamorph *Pseudocercospora eumusae* spp. nov.: causal agent of eumusae leaf spot disease of banana. Sydowia, 54: 35-43.

Day, J. P., and Shattock, R. C. 1997. Aggressiveness and other factors relating to displacement of populations of *Phytophthora infestans* in England and Wales. European Journal of Plant Pathology, 103: 379-391.

Doyle, J. J., and Doyle, J. L. 1990. Isolation of plant DNA from fresh tissue. Focus, 12: 13-15.

Fernandes, C. F., Júnior, J. R. V., Silva, D. S. G., Santiago, V., Miranda, S. L. V., Neto, A. F., Costa, J. N. M., Filho, Z. F. H., and Nunes, A. M. L. 2007. Sigatoka-negra em Rondônia. Comunicado Técnico-Embrapa. Porto Velho, RO.

Ferrari, J. T., Harakava, R., Nogueira, E. M. de C., and Castro, M. E. A. 2005. Ocorrência de sigatoka-negra da bananeira no Sul de Minas Gerais. Summa Phytopathologica, 31: 34.

Ferrari, J. T., and Nogueira, E. M. de C. Sigatoka Negra: como identificar e combater a Sigatoka Negra da bananeira. Available at: http://www.biologico.sp.gov.br/artigos_ok.php?id_artigo=16. [Accessed September, 22 2011].

Fortune, M. P., Gosine, S., Chow, S., Dilbar, A., Hill, A. S., Gibbs, H., and Rambaran, N. 2005. First report of black Sigatoka disease (causal agent *Mycosphaerella fijiensis*) from Trinidad. Plant Pathology, 54: 246.

Frederick, R. D., Snyder, C. L., Peterson, G. L., and Bonde, M. R. 2002. Polymerase chain reaction assays for the detection and discrimination of the soybean rust pathogens *Phakopsora pachyrhizi* and *P. meibomiae*. Phytopathology, 92: 217-222.

Gardes, M., and Bruns, T. D. 1993. ITS primers with enhanced specificity for basidiomycetes application to the identification of mycorrhizae and rusts. Molecular Ecology, 2: 113-118.

Gasparotto, L., Pereira, J. C. R., Hanada, R. E., and Montarroyos, A. V. V. 2006. *Sigatoka-negra da bananeira*. Manaus: Embrapa Amazônia Ocidental.

Hanada, R. E., Gasparotto, L., Resende, J. C. P., and Sedoguchi, E. T. 2007. Panorama atual da sigatoka-negra da bananeira no Brasil. Fitopatologia Brasileira, 32: 159.

IBGE- Cidades- Lavoura Permanente 2010. Available: http://www.ibge.gov.br/cidadesat/topwindow.htm?1. [Accessed: November 29, 2011].

INMET- INSTITUTO NACIONAL DE METEREOLOGIA –. Available: http://www.inmet.gov.br/html/clima/mapas/?mapa=tmax. [Accessed: November 12, 2011].

Johanson, A. 1995. Detection of banana leaf spot pathogens by PCR. Bull. OEPP/EPPO Bull, 25: 99-107.

Johanson, A., and Jeger, M. J. 1993. Use of PCR for detection of *Mycosphaerella fijiensis* and *M. musicola*, the causal agents of Sigatoka leaf spots in banana and plantain. Mycological Research, 97: 670-674.

Jones, D. R. 2003. The distribution and importance of the Mycosphaerella leaf spot diseases of banana. In: Mycosphaerella Leaf Spot Diseases of Bananas: Present Status and Outlook. Proceedings of the Workshop on Mycosphaerella Leaf Spot Diseases, San José, Costa Rica, 20-33 May 2002 (Jacome, L., Lepoivre, P., Marin, D., Oriz, R., Romero, R., and Escalant, J.V., eds), pp. 25-41. Montpellier: The International Network for the Improvement of Banana and Plantain.

Maciel, G. A. 2006. Instrução Normativa N^{o}- 289, de 20 de outubro de 2006. Available: http://www.in.gov.br. [Accessed: January 12, 2011].

Marín, D. H., Romero, R. A., Guzmán, M., and Sutton, T. B. 2003. Black Sigatoka: an increasing threat to banana cultivation. Plant Disease, 87: 208-222.

Meredith, D. S., and Lawrence, J. S. 1969. Black leaf streak disease of banana (*Mycosphaerella fijiensis*): symptoms of disease in Hawaii, and notes on the conidial state of the causal fungus. Transactions of the British Mycological Society, 52: 459-476.

Mouliom-Pefoura, A., Lassoudière, A., Foko, J., and Fontem, D. A. 1996. Comparison of development of *Mycosphaerella fijiensis* and *Mycosphaerella musicola* on banana and plantain in the various ecological zones in Cameroon. Plant Disease, 80: 950-954.

Nylander, J. A. A. 2004. MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

Pereira, J. C. R., Gasparotto, L., Coelho, A. F. S., and Urben, A. F. 1998. Ocorrência da Sigatoka negra no Brasil. Fitopatologia Brasileira, 23: 295.

Ploetz, R. C. 2004 . First report of Black Sigatoka of banana caused by *Mycosphaerella fijiensis* on Grand Bahama Island. Plant Disease, 88: 772.

Rhodes, P. L. 1964. A new banana disease in Fiji. Commonwealth Phytopathological News, 10: 38-41.

Romero, R. A. 2003. The spread, detection and impact of black leaf streak disease and other *Mycosphaerella* species in the 1990s. In: Mycosphaerella Leaf Spot Diseases of Bananas: Present Status and Outlook. Proceedings of the Workshop on Mycosphaerella Leaf Spot Diseases, San José, Costa Rica, 20–23 May 2002 (Jacome, L., Lepoivre, P., Marin, D., Ortiz, R., Romero, R. and Escalant, J.V., eds), pp. 21-24. Montpellier: The International Network for the Improvement of Banana and Plantain.

Romero, R. A., and Gauhl, F. 1988. Efectos de la Sigatoka en las plantas de banano a diferentes altitudes sobre el nivel del mar. Revista ASBANA, 12: 7-10.

Ronquist, F., and Huelsenbeck, J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics, 19: 1572-1574.

Souza, N. S., and Feguri, E. 2010. Ocorrência da Sigatoka Negra em bananeira causada por *Mycosphaerella fijiensis* no Estado de Mato Grosso. Available at: http://www.mt.gov.br/wps.2010. [Acessed September 23, 2011].

Stover, R. H. 1972. Banana, Plantain and Abaca diseases. Commonwealth Mycological Institute, Kew, Surrey, UK. 316pp.

Swofford, D. L. 2002. paup*: Phylogenetic analysis using parsimony (* and other methods). Sunderland, MA: Sinauer Associates.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution, 28: 2731-2739.

Thomas-Hall, S., Porchum, S., Henderson, J. Pattemore, J., and Aitken, E. 2004. Phylogenectic diversity of Mycosphaerella leaf spot disease, 1st International Musa Congress, Penang, Malaysia, July 6-9.

Volpato, O. 2005. Sigatoka Negra, Situação atual. Informativo Gedev, 1:3 Available at: http://www.cidasc.sc.gov.br/html/artigos/Volpato_Sigatoka_Negra_Situa%E7%E3o_atual.pdf. [Assessed November 14, 2011].

White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: A Guide to Methods and Applications (eds. M.A. Innis, D.H. Gelfand, J. J. Sninsky and T. J. White. Academic Press, San Diego: 315-322.

Zandjanakou-Tachin, M., Vroh-Bi, I., Ojiambo, P., Tenkouano, A., Gumedzoe, Y., and Bandyopadhyay, R. 2009. Identification and genetic diversity of *Mycosphaerella* species on banana and plantain in Nigeria. Plant Pathology, 58: 536-546.

Tables:

Table 1. Origin of the isolates of *Mycosphaerella* ssp. collected from banana fields located in 20 municipalities of Minas Gerais state, in 2009 to 2011.

N^o	Municipalilty	Region of Minas Gerais State	Number of isolates	Year
1	Pedralva	South	3	2009
2	Pouso Alegre	South	1	2009
3	Brazópolis	South	11	2009 and 2011
4	Cristina	South	23	2009 and 2011
5	Ipuiuna	South	6	2011
6	Itajubá	South	18	2009 and 2011
7	Maria da Fé	South	18	2011
8	Santa Bárbara do Tugúrio	Zona da Mata	16	2010
9	Piau	Zona da Mata	18	2010
10	Coronel Pacheco	Zona da Mata	22	2010 and 2011
11	Viçosa	Zona da Mata	3	2009 and 2011
12	Muriaé	Zona da Mata	2	2011
13	Araguari	Triângulo	13	2010
14	Monte Alegre de Minas	Triângulo	1	2010
15	Uberlândia	Triângulo	8	2009 and 2010
16	Matias Cardoso	North	16	2011
17	Nova Porteirinha	North	9	2009 and 2011
18	Verdelândia	North	4	2009
19	Jaíba	North	28	2011
20	Janaúba	North	19	2011

Table 2. Dimensions (μm) and characteristics of *Mycosphaerella musicola* conidia from different regions of Minas Gerais.

Source	Length	Diameter	Number of septa	Basal hilum	Form
Minas Gerais	30 - 70 (mean 50.45)	3 - 5 (mean 3.30)	2 - 7 (mostly 3 - 6)	absent	Straight and curved
Meredith and Lawrence (1970)	19 - 94 (mean 59)	2.5 - 3.8 (mean 3.0)	1 - 7 (mostly 3 - 6)	indistinct	Straight and curved

Figures:

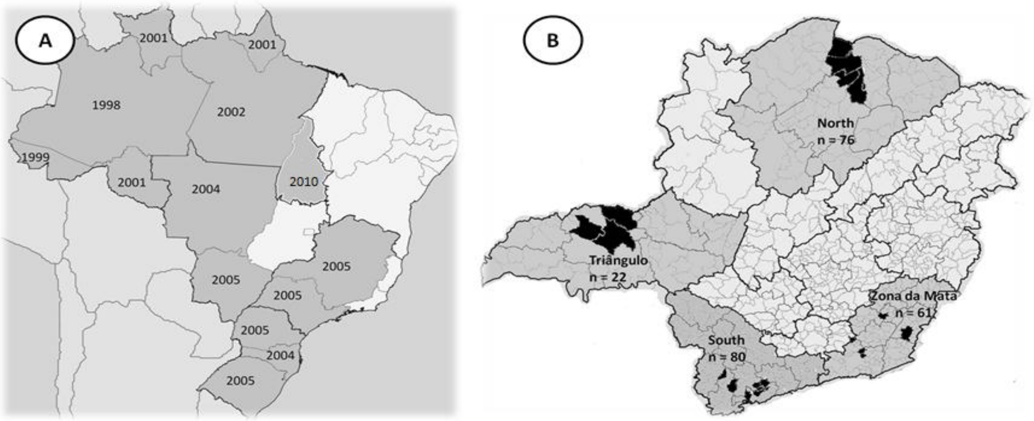

Figure 1. A- A map of Brazil showing the state distribution of *Mycosphaerella fijiensis* (in gray) and year of first report. B- A detailed map of Minas Gerais showing the location of the municipalities where samples were collected (in black).

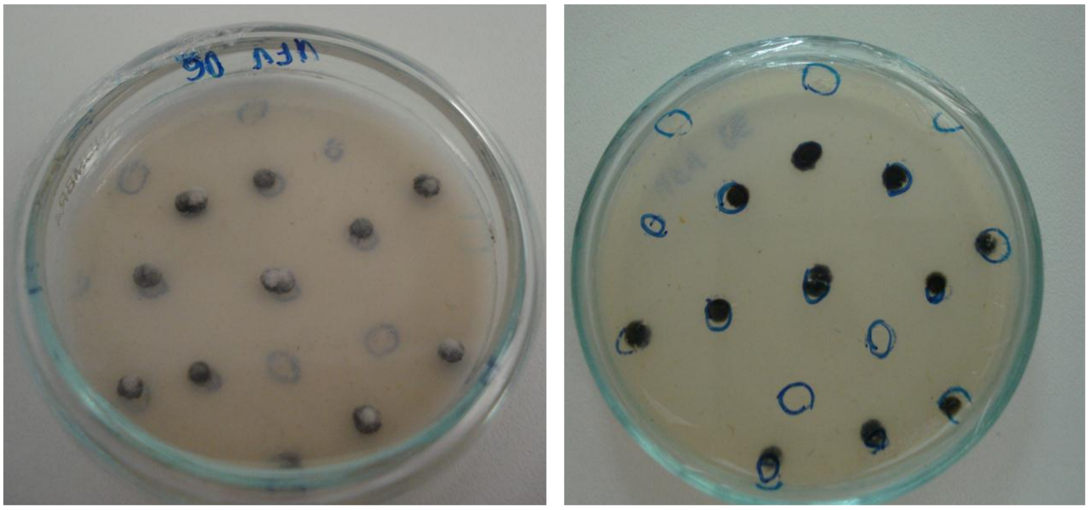

Figure 2. Colonies from single-conidium in medium V8 after 10 days of isolation.

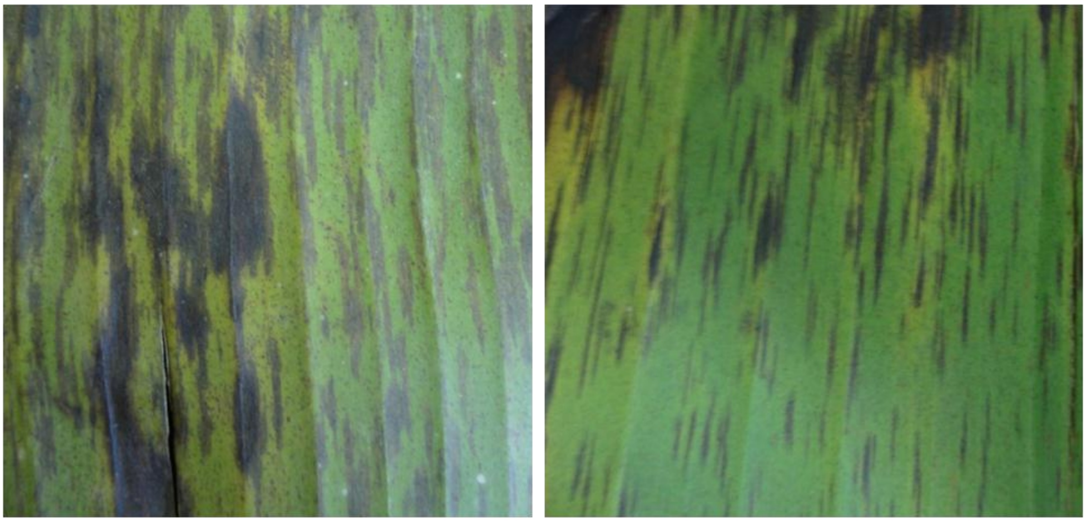

Figure 3. Symptoms similar to BLSD from samples collected in the Zona da Mata, Minas Gerais, Brazil.

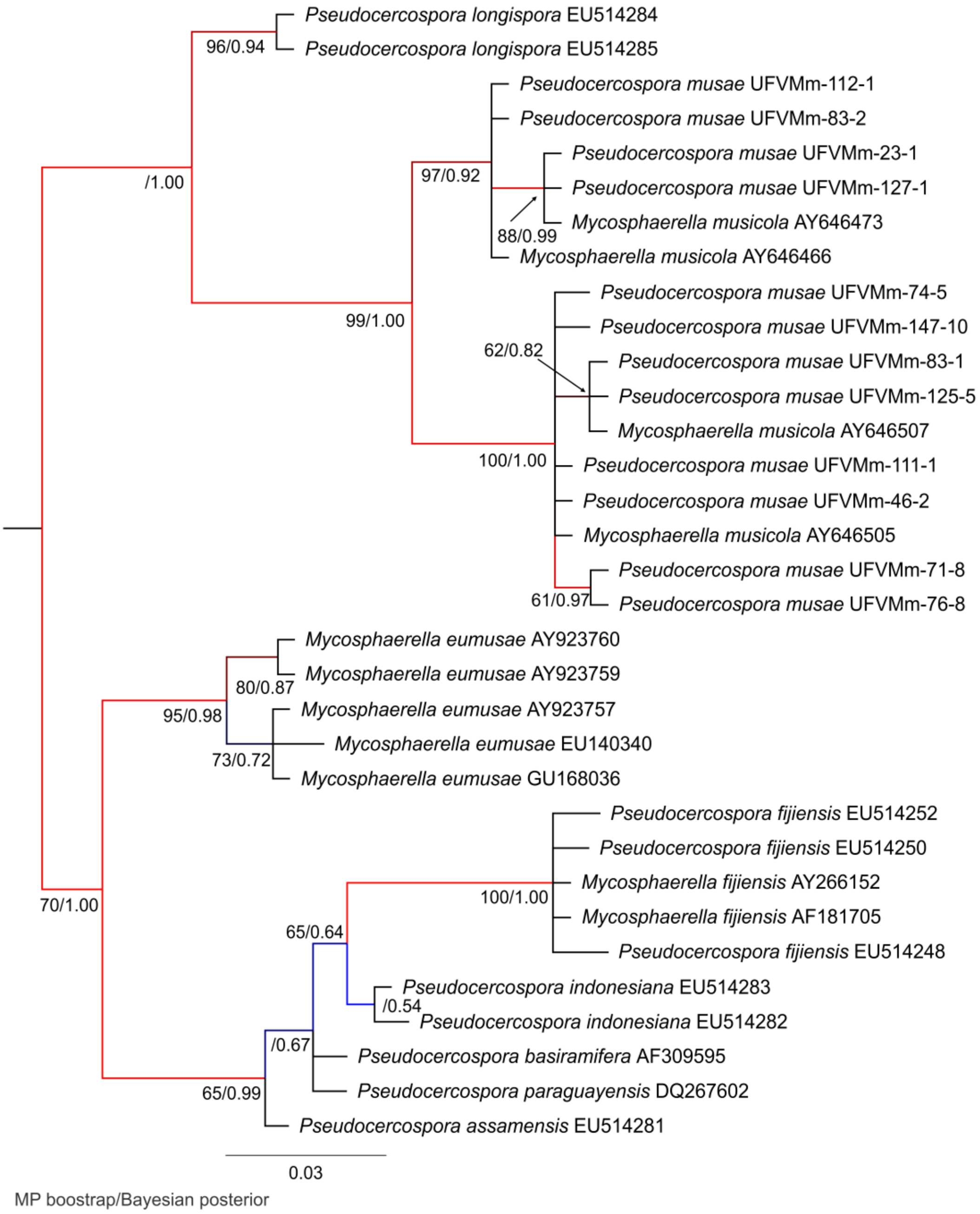

Figure 4. The 50% majority rule tree of 75,000 trees from Bayesian phylogeny based on the ITS sequence alignment. Number at nodes indicates bootstrap support (>50%) of maximum parsimony and Bayesian posterior probabilities and the scale bar shows 0.03 expected changes per site.

2.0 Genetic structure of the population of *Mycosphaerella musicola* in the state of Minas Gerais, Brazil

2.1 Abstract

A total of 215 single-conidium isolates obtained from several regions of Minas Gerais State, Brazil, was used to assess the genetic variability of the population of *Mycosphaerella musicola,* the causal agent of the yellow Sigatoka, a severe leaf spot disease of bananas. Four subpopulations, North, South, Zona da Mata and Triangulo were sampled from sites located at least 280 km apart from each other. Nine SSR markers were used and 89 alleles were revealed. The mating type frequency was also assessed. There is evidence of a mixed mode of reproduction and the mating-type ratio was of nearly 1:1. Haplotypic diversity was high and 209 unique haplotypes were detected. High levels of gene diversity (H= 0.55 to 0.60) were observed within the subpopulations. F_{ST} values ranged from 0.008 for the Zona da Mata and South subpopulations to 0.026 for the Triângulo and South populations. Most genetic variation (96.7%) was detected within subpopulations. Bayesian assignment analysis did not reveal any geographic structuring of the population. The highly variable population of *M. musicola* may constitute in a challenging problem to manage yellow Sigatoka in all banana production areas in Brazil.

2.2 Introduction

Banana (*Musa* spp.) is one the most important fruit crops in the world and is cultivated in more than 4.7 million hectares, and approximately 102 million tons of fresh fruit were produced in 2010. It is the second most important fruit crop produced in Brazil, the fifth largest producer in the world after India, China, Philippines and Ecuador (FAO, 2010). Leaf spot diseases caused by species of *Mycosphaerella* are major limiting factors for banana production worldwide and can have considerable social and agricultural economic impacts due to lowering crop yields. The leaf spot diseases are particularly threatening in tropical areas where banana is a staple food (Crous et al., 2002; Jones, 1999).

Mycosphaerella musicola R. Leach ex J. L. Mulder (anamorph: *Pseudocercospora musae*) (Zimm.) Deighton is a fungal (Ascomycete) pathogen that infects banana and plantain worldwide causing Sigatoka leaf spot disease, also known as yellow Sigatoka. Leaf spots can coalesce and lead to premature death of large areas of leaf tissue reducing the photosynthetic capabilities of the plant. The disease can also disturb the physiology of the fruits, resulting in premature ripening (Jones, 1999; Stover, 1972).

Black Sigatoka, also known as black leaf streak disease (BLSD) is another disease that also affects banana and is caused by *Mycosphaerella fijiensis* M. Morelet (anamorph *Pseudocercospora fijiensis*) (M. Morelet) Deighton. Usually, after the introduction of BLSD in a region the occurrence the yellow Sigatoka becomes a “secondary problem” and a subject of lower relevance (Molina et al., 2001). Nevertheless, in many areas, *M. musicola* remains a destructive pathogen to banana plants mainly where *M. fijiensis* has not become established (Jones, 1999). This seems to be the case in Minas Gerais state, located in southeastern Brazil. The BLSD was first reported in the state in 2005 (Castro et al., 2005), but there was no increase in the severity of leaf spot epidemics or in fungicide usage to control the disease. This puzzling outcome raised several questions including one related to the distribution of the disease. Recently, a thorough survey was conducted aimed at assessing the distribution of ‘Sigatoka’ diseases of banana based on 800 samples collected in four different regions in the main production areas of Minas Gerais, Brazil. In all examined samples, only *M. musicola* was found to be associated with the leaf spots (Gomes et al., unpublished).

The management of banana leaf spots is costly and often requires aerial application of fungicides with aircrafts or big tractor-mounted terrestrial apparatus (Marín et al., 2003). The use of resistant varieties would greatly contribute to disease management, but in Brazil most cultivars that are commercially desirable lack enough level of leaf spot resistance (Dias, 2008). The understanding of the amount and distribution of genetic variation in the population of *M. musicola* would be useful to breeders for developing new varieties and for pathologists for setting strategies to better use resistant materials and prevent resistance breakdown. These issues are particularly important for perennial crops cultivated in large areas.

Molecular markers such as restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) have been used to analyze the genetic structure of *M. musicola* populations at regional, continental and global scales (Hayden et al., 2003; Hayden et al., 2005; Moreira et al., 2003). Hayden et al. (2003) used RFLP markers to analyze the global population structure of *M. musicola* and they reported genetic differentiation among the geographic populations from Indonesia, Australia, Africa, Latin America and the Caribbean. The authors also suggested migration of the pathogen between Africa and Latin America. In another study, moderate genetic differentiation and levels of genetic diversity within the Australian population of *M. musicola* were reported (Hayden et al., 2005). In Brazil, preliminary information was gathered regarding the genetic variability of the population of *M. musicola* (Moreira et al., 2003). Only 24 isolates of *M. musicola* from five states were analyzed, but a high level of genetic variability was revealed using RAPD markers (Moreira et al., 2003). In order to make stronger inferences about the genetic structure of the population of *M. musicola*, it is necessary to implement a better sampling scheme, a much larger sample size, and to use higher resolution genetic markers.

Simple sequence repeats (SSR) are widely used as genetic markers because of their co-dominance, ubiquity, ease to score, reproducibility, assumed neutrality and high level of polymorphism (Jarne and Lagoda, 1996). These markers have been used to investigate the genetic structure of several fungal plant pathogens (Atallah et al., 2010; Fahleson et al., 2009; Gurung et al., 2011; Jarne and Lagoda, 1996; Robert et al., 2012). Recently, a thorough study was conducted to understand the genetic variability of 735 isolates of *M. fijiensis* collected from 37 countries. Variation was assessed using 21 microsatellites and sequence-based markers (Robert et al., 2012). An interesting outcome of this investigation was the determination of South-East Asia as the center of origin and the source of global migrations of this pathogen. SSR markers were developed to assess genetic variation in *M. musicola* (Molina et al., 2001; Zapater et al., 2008), but to date no study aimed at elucidating the genetic structure of the pathogen population has been conducted.

Studies aimed to quantify the genetic variability should be complemented with more detailed analysis of the mode of reproduction of the pathogen. Determining the prevalent mode of reproduction can be both genetically and epidemiologically relevant. *M. musicola* is a heterothallic fungus, and both the MAT1-1 and MAT1-2 mating idiomorphs have been characterized (Arzanlou et al., 2010; Conde-Ferráez et al., 2010). However, no study using a population approach has been conducted to infer the frequency of mating types in Brazilian isolates of *M. musicola*. If both idiomorphs are found in approximately similar frequencies, i.e. 1:1 ratio, this could indicate that recombination can take place and a genetically variable population may be established. From the epidemiological perspective, determining the prevalent inoculum associated with epidemics is important for disease management and policy making. If sexual reproduction can occur then two types of spores may contribute to disease development: ascospores and conidia.

The main objective of this study was to determine the genetic structure of *M. musicola* populations in Minas Gerais State, Southeast Brazil, estimate the level of genetic diversity in each population, gametic disequilibrium, frequency of mating type idiomorphs, genetic differentiation and gene flow based on SSR marker and mating type frequencies.

2.3 Materials and methods

Sampling collection and isolation

Naturally infected leaf sections, with typical symptoms of yellow Sigatoka were collected from 2009 to 2011. Eighty banana fields that varied in size, age and level of technical inputs of the cultural practices were sampled. Most fields were planted to susceptible varieties and the geographic coordinates of all sampling spots were recorded with a portable GPS device (Magellan Color TRAK, Magellan). The fields were distributed in 20 municipalities, located in the main banana-producing regions of Minas Gerais State: South, North, Triângulo Mineiro and Zona da Mata (Figure 1). At least 10 symptomatic leaf sections from non-adjacent plants were collected per field and transported to the laboratory to confirm the initial disease diagnosis and for pathogen isolation. Using a sterile fine needle, conidia present on abaxial surface of the leaves with symptoms were picked and put in a water-agar medium on Petri plates using a stereoscopic microscope. Single conidium was taken with a sterile fine needle using a compound microscope and cultured on V8 agar medium (300 mL V8 juice, 3 g of CaCO3 and 20 g of agar per liter of medium). The single-spore isolates were incubated at 25^{o}C under 12 h of daily white light regime for 10 days prior to examination and DNA extraction.

DNA extraction, quantification and identification

Fungal cultures were grown in flasks containing 50 mL of V8 broth culture medium (250 mL of V8 juice and 3 g $CaCO_3$ per liter of medium, pH 6.5) at 25^{o}C for 10 days at 120 rpm on a shaker. Total genomic DNA was extracted from each isolate using a CTAB protocol as described by Doyle and Doyle (1990). An aliquot of 50 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) was added to each DNA sample. RNA was digested with RNAse A for 2 h at 37^{o}C before storage at –20°C. The concentration of DNA was quantified with a spectrophotometer (NanoDrop 2000 Thermo Scientific) and working solutions at 20 ηg of DNA/µl were prepared from each sample for all polymerase chain reaction (PCR) analyses.

The identification of all isolates was based on the product of PCR analysis using species-specific primers for the actin genes MMactF2 (ACGGCCAGGTCATCACT), MMactRb (GCGCATGGAAACATGA) designed by Arzanlou et al. (2007). The PCR products were subjected to electrophoresis in 2.0% agarose gel in 1×TBE buffer and viewed under UV in gels stained with GelRed (Uniscience).

Mating type characterization

The presence of MAT1-1-1 and MAT1-2-1 idiomorphs was determined by amplicons produced after PCR using procedures and primers (Mat1F/ Mat1R and Mat2F /Mat2R) previously described (Arzanlou et al., 2010). Five microliters of each PCR product was subjected to electrophoresis in 2.0% agarose gel in 1 × TBE buffer and viewed under UV on gel stained with GelRed. Fragments were compared with a 100bp DNA ladder and scored as either mating-type 1 (702 bp) or mating-type 2 (720bp) (Arzanlou et al., 2010). Amplification using both primer sets were attempted for DNA of each isolate. For each subpopulation, defined according to the geographic area from which isolates were obtained, a χ^2 test was performed to evaluate the null hypothesis of the occurrence of both mating types in a 1:1 ratio. Likewise, the frequencies of MAT1-1-1 and MAT1-2-1 for all isolates collected in Minas Gerais State were also assessed.

Genotyping with microsatellite markers

Microsatellites analysis was conducted using 9 primer pairs for loci Mm SSR 01, Mm SSR 07, Mm SSR 10, Mm SSR 15, Mm SSR 24, Mm SSR 30, Mm SSR 31, Mm SSR 44 and Mm SSR 46 (Table 1) (Molina et al., 2001). The forward primer was labeled with fluorescent dye: HEX (hexachloro-6-carboxyfluorescein), NED (N(1-naphytyl) ethylenediamine) or FAM (6-carboxyfluorescein). Cycling conditions were as follows: 95°C for 5 min followed by 28 cycles of 95°C for 30 s, 55°C for 90 s, 72°C for 30 s, and a final extension step of 72°C for 10 min. The PCR products were visualized in agarose gel (2%), before loading the samples in an ABI-3100 capillary system (Applied Biosystems), with a DNA ladder labeled with ROX dye. Alleles were scored using Peak Scanner™ Software v1.0. (Applied Biosystems).

SSR allele size

In order to check the size scoring based on the fragment analysis, each microsatellite was sequenced from 49 isolates chosen to represent the various allele size classes scored. PCR amplifications were done in 20 µl reaction volumes containing 1X PCR buffer (Invitrogen, Carlsbad, California), 2.5 mM $MgCl_2$, 0.2 mM each dNTP (Invitrogen), 0.375 µM each primer and 0.5 U Taq polymerase (Invitrogen). Thermocycling conditions consisted of an initial melt at 95°C for 5 min followed by 28 cycles of 95°C for 30 s, 55°C for 90 s, 72°C for 30 s, and a final extension step of 72°C for 10 min.

All amplifications were performed in a MyCycler thermocycler (Bio-Rad Laboratories Inc., Hercules, California). For each PCR amplification reaction 5 µl was subjected to electrophoresis in 1.8% agarose gels stained with SYBR Green I nucleic acid stain (Invitrogen) and viewed with UV light. PCR products were cleaned with ExoSap-IT (USB, Cleveland, Ohio) following the manufacturer’s instructions. Sequencing was performed at the Core Instrumentation Facility (CIF) of the University of California’s (UC) Institute of Integrative Genome Biology at UC Riverside. The validation of the SSR alleles was based on the comparison of number of repeated sequences of each allele among the different isolates.

Genetic analyses

Multilocus haplotypes were constructed for each isolate based on the alleles detected at all loci. Gene diversity was estimated based on Nei's h index (1978) and the genotypic diversity (G) was calculated for each population using the Stoddart and Taylor index (Stoddart and Taylor, 1988). The rarefaction method was used to compare genotypic richness, which is the proportion of genotypes contained in a population, between populations of different samples sizes. Rarefaction curves yield the number of genotypes expected in a sample corresponding to the smallest sample size (n) of all the population being compared (Grünwald et al., 2003). All analyses of diversity were done with the Vegan package (Oksanen et al. 2010), using the R program (R Development Core Team 2010).

The selective neutrality of the loci was examined with the Ewens-Watterson test in POPGENE version 1.32 (Yeh et al., 1997). Genetic differentiation among populations was compared by means of θ statistic (Weir and Cockerham, 1984), equivalent to Wright’s F_{ST} for haploid data, calculated with the program MULTILOCUS 1.3 (Agapow and Burt, 2001) using 1000 bootstrap sampling to determine the level of significance.

The ARLEQUIN program (Excoffier and Lischer, 2010) was used to assess the population differentiation through the analysis of molecular variance (AMOVA) and Wright's F_{ST}. Genetic distance was calculated according to Nei (1978). Population genetic structure was also evaluated using Bayesian inference of all parameters involved using Markov Chain Monte-Carlo (MCMC) simulations, implemented in the GENELAND version 3.1.4 software (Guillot et al. 2005) and the R program (R Development Core Team, 2010).

The number of clusters was determined by running the MCMC iterations, allowing K to vary, with the following parameters: 5.000.000 MCMC iterations, maximum rate of the Poisson process fixed to 100, k was allowed to vary from 1 to 10 and the default settings were used for the other options. Three independent runs were performed to check for convergence of Markov chains. After inferring the K value in the data set from these three runs, the MCMC was run 30 times with K fixed to the inferred number of clusters, and a burn-in of 25% iterations in the post-processing. The mean logarithm of the posterior probability was calculated for each run and the posterior probability of population membership for each pixel of the spatial domain was then computed for the run with the highest value.

Mode of reproduction

Multilocus linkage-disequilibrium tests were used to investigate the contribution of recombination and clonality in shaping the population. The standardized index of association (I_A) (Smith et al, 1993) and the locus number independent measure rD (Agapow and Burt 2001) were used. The estimates of I_A and rD were compared with the expected distribution of the loci when in random association (null hypothesis), and significance was assessed from the results of 1000 randomized datasets. Both I_A and rD were calculated in MULTILOCUS program version 1.3b (Agapow and Burt, 2001).

2.4 Results

Sampling, isolation and identification

A total of 215 isolates were obtained: 21 isolates from the Triângulo Mineiro, 64 from the North, 73 from the South, and 57 from the Zona da Mata region (Figure 1). Amplification with the *M. musicola* specific primer pair was detected for all isolates (data not shown).

Mating type characterization

The PCR analyses amplification of most isolates (97.7%) produced a single amplicon matching either the MAT1-1 or MAT1-2. Among the 215 isolates analyzed, 123 were MAT1-1 and 87 were MAT1-2. For four isolates there was no amplification with both primer pairs. For the isolate MmUFV_115-3, from the North region both MAT1-1 and MAT1-2 were detected (data not shown). Overall, the frequencies of MAT1-1:MAT1-2 did not significantly vary from a 1:1 ratio. When comparisons were made at each region, the frequency of MAT1-1 (70%) in the Zona da Mata population exceeded that of the MAT1-2 (Table 2).

SSR allele size

Of 49 samples used to check the sizes of 89 alleles found in this study, 77.6% had the same allele size found in alleles scored in Peak Scanner™. The sequences of the alleles present in the SSR 01, SSR 07 and SSR 24 loci were not of suitable quality and were not included in this analysis.

Genetic variability and population structure

All nine SSR markers were polymorphic. The number of alleles ranged from 4 to 23 and the mean across all loci was 9.88 (Table 1). Based on the Ewens-Watterson test all polymorphic loci were selectively neutral (data not shown). Gene diversity (H) values within the four regional populations were similar and varied from 0.55 in the South to 0.60 in the North (Table 3).

High genotypic diversity (G) values were observed for all regions. The lowest value corresponded to approximately 90% of the theoretical maximum and was estimated for the Zona da Mata population. After rarefaction, genotypic richness E(gn) was similar to the number of observed genotypes in all regions. Thus, to a large extent, each individual comprised a unique genotype. Genotypic richness was lowest in the Zona da Mata region. The indices of evenness were high for all populations, ranging from 0.97 to 1.00 (Table 3).

Among the 215 isolates of *M. musicola* analyzed, 209 different multilocus SSR haplotypes were identified. The most frequent haplotype was recorded twice. Isolates from the same region and with the same multilocus SSR haplotype had the same mating type idiomorphs. Just one common haplotype was found between different regions. Isolates UFVMm112-1 from the North and UFVMm152-12 from the South subpopulations shared the same haplotype but had different mating type idiomorphs. The clonal fractions were low and genotypic diversity was near the maximum possible value in all subpopulations.

Analysis of molecular variance (AMOVA) revealed a small but significant (φ=0.03; P<0.000) genetic differentiation among populations. However, the vast majority of genetic variation (96.7%) occurred within subpopulation (Table 4).

The genetic differentiation of each population estimated based on the F_{ST} ranged from 0.008 for Zona da Mata and South populations to 0.026 for Triângulo and South populations (Table 5), and indicates differences between the South and North subpopulations.

The Bayesian analysis showed no population structuring (K = 1). The most likely number of cluster (K) was confirmed by observing the maximal value of average log posterior probability of the three different runs.

Mode of reproduction

The null hypothesis of recombination was accepted for subpopulations by the index of association (I_A) and multilocus linkage disequilibrium (rD), except for the North region where the multilocus measures of association was significant ($P < 0.01$), with I_A value of 0.236 (Table 2).

2.5 Discussion

In Brazil, yellow Sigatoka causes yield loss in all areas where banana is cultivated (Cordeiro et al., 2005) and in Minas Gerais state it is the most destructive foliar disease of this fruit crop. Despite its widespread distribution and destructive effects to banana plants, the genetic variability of the pathogen is poorly understood. The knowledge of the genetic variability of *M. musicola* in Minas Gerais can help in the analysis and to choose effective disease management strategies, mainly in the development of resistant cultivars and the effective use of resistance genes under natural conditions (Sharma, 2003).

High variability was detected in the population of *M. musicola* from Minas Gerais state and 97% of the isolates had distinct haplotypes. The same trend was observed when analyzing a related species, *M. fijiensis*, in which 78% of the isolates had distinct haplotypes from the Australasian-Pacific region (Hayden et al., 2003). The higher resolution of SSR markers used in the current study revealed greater genetic variability of *M. musicola* in Minas Gerais than previously reported by Hayden et al., (2005) using RFLP for isolates from different locations in Australia. Similar (high) level of genetic variability was reported for Brazilian isolates when assessed using RAPD markers (Moreira et al., 2003) however, as pointed out before, the sample size in this study was small.

The high variability found in populations of Minas Gerais is most likely due to the occurrence of sexual reproduction. Similar mating type allele frequencies in most populations indicate that *M. musicola* can reproduce sexually and that mating is not prevented by the absence of a particular mating type. In most cases there was no evidence to reject the null hypothesis that the population are in linkage equilibrium. Only the subpopulations from North and Zona da Mata showed significant linkage disequilibrium among microsatellite loci or differences in the frequency of mating-type idiomorphs, respectively. These populations also had the higher clonal fraction and lower richness and evenness. But even so, there was high genotypic diversity. In the case of Zona da Mata region, the unequal proportions of mating types may be due to the sampling or selection acting upon the individuals. These two factors may have favored one mating type over the other or less frequent sexual reproduction. In the North region one possible explanation is that the populations are reproducing sexually but have not yet reached equilibrium among alleles at the SSR loci scored. The high number of unique haplotypes and low clonal fractions present in the subpopulations support this claim. Conidia were observed in the majority of leaves sampled in our study, thus one would expect the detection of clonal individuals from the same field. Nevertheless, the sampling scheme avoiding the collection of adjacent plants within a field may have reduced the chance of sampling clones present in the surrounding area. Based on the epidemiological information that conidia are dispersed mainly by rain-splash, no leaves were collected nearby a sampling point to minimize clone-biased estimates (Hayden et al., 2005).

Sexual reproduction occurs in the population of *M. musicola* in Minas Gerais, but asexual reproduction is also frequent. The high number of lesions with sporodochia corroborates the results of the genetic analysis. In a recent study on the aerobiology of *M. musicola* conducted in the municipality of Coronel Pacheco, in the Zona da Mata of Minas Gerais state both ascospores and conidia were trapped year-round. This work revealed two periods of greatest severity of yellow Sigatoka, the first during the rainy season presumably influenced by conidia and the second during the driest season caused mainly by ascospores (Rocha et al., 2012). In the current study, samplings were done in the rainy season which explains the large amount of sporodochia found in the lesions. Nevertheless, even when asexual reproduction takes place, genetic variation can be high due to the occurrence of mutation. This is an important evolutionary mechanism contributing to increase DNA variation and to the formation of new alleles (McDonald and Linde, 2002). Mutation is directly connected with recombination and DNA repair mechanisms (Strand et al., 1993). Thus, mutation could also have contributed to the high variability of the population. When assessments of genetic variability are made based on microsatellite markers, the mechanism of the polymerase slippage at DNA replication can increase or decrease the number of repeat units (Jarne and Lagoda, 1996).

Mycosphaerella musicola appears to constitute a unique population in Minas Gerais. Most of the genetic variation occurred within each population and the low level of genetic differentiation among populations indicates a high degree of homogenization over larger areas. The occurrence of gene flow over long distances mediated by the dispersal of airbone ascospores may contribute to homogenize the population. Ascospores of *M. musicola* can be released from pseudothecia and be carried by air currents over long distances (Meredith, 1970). Contrary to what was found in the present study, in Australia restricted gene flow was recorded resulting in a high level of genetic differentiation between populations (Hayden et al., 2005). In general the low genetic differentiation between these subpopulations indicates some degree of gene flow, suggesting high migration in Minas Gerais state. Nevertheless, the results of this work did not support this hypothesis, because of the high number of unique haplotypes found among the different regions. Maybe due to the occurrence of sexual reproduction, there is a high number of genotypes being produced in each subpopulation and gene flow is not easily detected.

Pathogens with mixed reproduction systems and genotype flow pose the highest risk of evolution, creating new genotypes which may include new resistance genes to fungicides and selection of isolates that may be evolving into a new population that could overcome plant resistance genes (McDonald and Linde, 2002). These facts have a direct impact on disease-management strategies to control yellow Sigatoka in Minas Gerais, due to the fact that fungicide usage is the main control measure and that major resistance genes of cultivars used as genetic control may be quickly overtaken.

2.6 Ackowledgments

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2.7 References

Agapow, P., and Burt, A. 2001. Indices of multilocus linkage disequilibrium. Molecular Ecology Notes, 1: 101-102.

Arzanlou, M., Abeln, E., Kema, G., Waalwijk, C., Carlier, J., de Vries, I., Guzman, M., and Crous, P. 2007. Molecular diagnostics for the sigatoka disease complex of banana. Phytopathology, 97: 1112-1118.

Arzanlou, M., Crous, P. W., and Zwiers, L. H. 2010. Evolutionary dynamics of mating-type loci of *Mycosphaerella* spp. occurring on banana. Eukaryotic Cell, 9: 164-172.

Atallah, Z. K., Maruthachalam, K., du Toit, L., Koike, S. T., Michael Davis, R., Klosterman, S. J., Hayes, R. J., and Subbarao, K.V. 2010. Population analyses of the vascular plant pathogen *Verticillium dahliae* detect recombination and transcontinental gene flow. Fungal Genetics and Biology, 47: 416-422.

Castro, M. E. A., Pereira, J. C. R., and Gasparotto, L. 2005. First report of black-sigatoka in the State of Minas Gerais, Brazil. Fitopatologia Brasileira, 30: 668-668.

Conde-Ferráez, L., Grijalva-Arango, R., Canto-Canche, B., Manzo-Sanchez, G., Canul-Salazar, M., and James, A. 2010. The development of mating type-specific primers for *Mycosphaerella fijiensis*, the causal agent of black Sigatoka of banana, and analysis of the frequency of idiomorph types in Mexican populations. Australasian Plant Pathology, 39: 217–225.

Cordeiro, Z. J. M., and Matos, A. P. de. 2005. Doenças da banana. Pages 12-16 In: Informe Agropecuário. Vol. 26. Belo Horizonte.

Crous, P. W., and Mourichon, X. 2002. *Mycosphaerella eumusae* and its anamorph *Pseudocercospora eumusae* spp. nov.: causal agent of eumusae leaf spot disease of banana. Sydowia, 54: 35-43.

Dias, M. S. C. 2008. Doenças da bananeira. Pages 47-48 In: Informe agropecuário: Bananicultura irrigada: inovações tecnológicas Vol. 29. Belo Horizonte: EPAMIG.

Doyle, J. J, and Doyle, J. L. 1990. Isolation of plant DNA from fresh tissue. Focus, 12:13-15.

Excoffier, L., and Lischer, H. E. L. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources, 10: 564-567.

Fahleson, J., Nakyanzi, M., Okori, P., Seal, S., Kenyon, L., and Dixelius, C. 2009. Genetic analysis of *Mycosphaerella fijiensis* in the Ugandan Lake Victoria region. Plant Pathology, 58: 888-897.

FAO. (2010) Food Agriculture Organization of United Nations. Available at http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor [Assessed March 17, 2012].

Grünwald, N. J., Goodwin, S. B., Milgroom, M. G., and Fry, W. E. 2003. Analysis of genotypic diversity data for populations of microorganisms. Phytopathology, 93: 738-746.

Guillot, G., F. Mortier., and A. Estoup, 2005. Geneland: a computer package for landscape genetics. Molecular Ecology Notes, 5: 708-711.

Gurung, S., Goodwin, S. B., Kabbage, M., Bockus, W. W., and Adhikari, T. B. 2011. Genetic differentiation at microsatellite loci among populations of *Mycosphaerella graminicola* from California, Indiana, Kansas, and North Dakota. Phytopathology, 101:1251-1259.

Hayden, H. L., Carlier, J., and Aitken, E. A. B. 2003. Population differentiation in the banana leaf spot pathogen *Mycosphaerella musicola*, examined at a global scale. Plant Pathology, 52: 713-719.

Hayden, H., Carlier, J., and Aitken, E. A. B. 2005. The genetic structure of Australian populations of *Mycosphaerella musicola* suggests restricted gene flow at the continental scale. Phytopathology, 95: 489-498.

Jarne, P., and Lagoda, P. J. L. 1996. Microsatellites, from molecules to populations and back. Trends in Ecology and Evolution, 11: 424-429.

Jones, D. R. 1999 . Diseases of Banana, Abaca and Enset (First.). CABI.

Marín, D. H., Romero, R. A., Guzmán, M., and Sutton, T. B. 2003. Black Sigatoka: an increasing threat to banana cultivation. Plant Disease, 87: 208-222.

McDonald, B. A., and Linde, C. 2002. Pathogen population genetics, evolutionary potential, and durable resistance. Annual Review of Phytopathology, 40: 349-379.

Meredith, D. S. 1970. Banana leaf spot disease (Sigatoka) caused by *Mycosphaerella musicola* Leach. (Commonwealth Mycological Institute, Kew, Surrey, England).

Molina, C., Kaemmer, D., Aponte, S., Weising, K., and Kahl, G. 2001. Microsatellite markers for the fungal banana pathogen *Mycosphaerella musicola*. Molecular Ecology Notes, 1: 137-139.

Moreira, R. F. C., Cordeiro, Z. J. M., and Vilarinhos, A. D. 2003. Caracterização genética de isolados de *Mycosphaerella musicola* por Rapd. Summa Phytopathologica, 29: 275-277.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics, 89:583.

Oksanen, J., Blanchet, F. G. , Kindt, R., Legendre, P., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., and Wagner, H. 2010. Vegan: community ecology package. R package. version 1.17-4. Available at: http://CRAN.R-project.org/package=vegan [Assessed December, 2011].

R DEVELOPMENT CORE TEAM. 2010. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. Available at: http://www.R-project.org [Assessed December, 2011].

Robert, S., Ravigne, V., Zapater, M., Abadie, C., and Carlier, J. 2012. Contrasting introduction scenarios among continents in the worldwide invasion of the banana fungal pathogen *Mycosphaerella fijiensis*. Molecular Ecology, 21: 1098-1114.

Rocha, H. S., Pozza, E. A., Uchôa, C. D. N., Cordeiro, Z. J. M., De Souza, P. E., Sussel, Â. A. B., and De Rezende, C. A. 2012. Temporal progress of yellow Sigatoka and aerobiology of *Mycosphaerella musicola* spores. Journal of Phytopathology, 160: 277-285.

Sharma, T. R. 2003. Molecular diagnosis and application of DNA markers in the management of fungal and bacterial plant diseases. Indian Journal of Biotechnology, 2: 99-109.

Smith, J. M., Smith, N. H., O'Rourke, M., and Spratt, B. G. 1993. How clonal are bacteria? Proceedings of the National Academy of Sciences, 90: 4384-4388.

Stoddart, J. A., and Taylor, J. F. 1988. Genotypic diversity: estimation and prediction in samples. Genetics, 118: 705-711.

Stover, R. H. 1972. Banana, plantain and abaca diseases. Key: Commonwealth Mycological Institute.

Strand, M., Prolla, T. A., Liskay, R. M., and Petes, T. D. 1993. Destabilization of tracts of simple repetitive DNA in yeast by mutations affecting DNA mismatch repair. Nature, 365: 274-276.

Weir, B. S. and Cockerham, C. C. 1984. Estimating F-statistics for the analysis of population structure. Evolution, 38: 1358-1370.

Yeh, F., and Yang, R. 1999. Popgene version 1.31. Available at: http://www.ualberta.ca/~fyeh/ [Assessed December, 2011].

Zapater, M., Duchemin, M., Dussart, J., Coste, D., Brottier, P., and Carlier, J. 2008. Microsatellite markers for the fungal banana pathogens *Mycosphaerella fijiensis*, *Mycosphaerella musicola* and *Mycosphaerella eumusae*. Molecular Ecology Resources, 8: 1121-1125.

Figure:

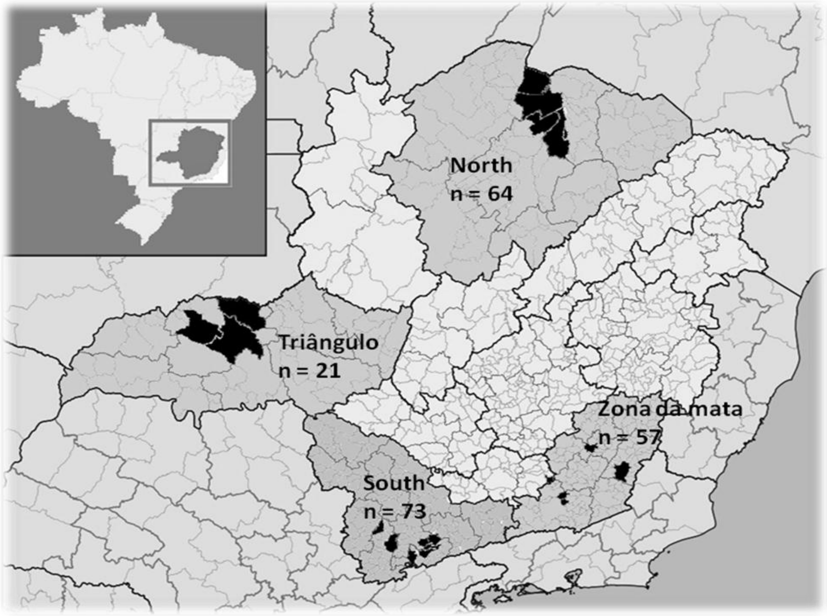

Figure 1. Map of Minas Gerais showing the municipalities where fields were sampled (black) in the different regions of the State and the number of isolates from each region.

Tables:

Table 1. Characteristic of microsatellite loci of *Mycosphaerella musicola* used in this study.

Locus	Primer sequence (5'-3')	Label	Allele size range (bp)	Number of alleles
SSR 01	F:TAGTTGCAACCGAACAGG R:CTCCGTAGGTATGATGGTGT	HEX	120-190	8
SSR07	F:ACGAGGTTTCAGAAGCAATA R:TCTTTCACCGAAGAAACCT	NED	216-278	20
SSR10	F:GAGAGCATGAAAAGTGGAAA R:CGTGACACTCGTCAGTTACA	HEX	145-171	7
SSR15	F:CTACTGAGGCAGTCGCTAAC R:GGAGAGGTGGAAAAAGAAGT	6-FAM	179-214	5
SSR24	F:TCAAGAGGAGGAGAAGTTGA R:GGTTCTGATCAAGAGGAGGA	6-FAM	201-207	4
SSR30	F:TGATGTTAAGTTGACGGACA R:CTAAGCCAAACCTCAATCAG	HEX	132-209	8
SSR31	F:AACCACATCTTCGATCAGG R:CACATGGAATATCCTTGGTC	NED	167-219	9
SSR44	F:CCTCACTCTCGCTCATACA R:AGAATGGACGAAAAACACTG	6-FAM	135-143	5
SSR46	F:CGTGGACCTATTGTCAACTC R:TGGGTTACATTTACGAGAGAA	HEX	235-298	23

The nine loci are described in Molina et al. (2001).

Table 2. Association and mating type frequencies and test of multilocus of *Mycosphaerella musicola* populations.

		Nº of isolates		Mating Frequency				
Population	**N**	**Mat1-1**	**Mat1-2**	**Mat1-1**	**Mat1-2**	X^{2a}	I_A^b	$\bar{r}_d^c$
North	64	34	29	0.54	0.46	0.396	0.236*	0.031*
Triângulo Mineiro	21	10	11	0.48	0.52	0.05	0.007	0.001
South	73	42	31	0.42	0.31	1.65	-0.031	-0.004
Zona da Mata	57	37	16	0.70	0.30	8.32*	0.024	0.003
Overall	215	123	87	0.58	0.42	3.47	0.044	0.006

[a] $\chi 2$ value based on 1:1 ratio and 1 degree of freedom. *P-value indicates mating type frequencies which is significant at $P<0.05$. [b] I_A index of association . [c] $\bar{r}_d$ multilocus association . *Indicates significant I_A and $\bar{r}_d$ values at $P <0.01$.

Table 3. Genetic diversity parameters for four subpopulations of *Mycosphaerella musicola* from Minas Gerais, examined at nine SSR loci.

Population	**Sample size (n)**	g_{obs}^a	**Clonal fraction**^{b}	H^c	G^d	N^e	**E(gn)**^{f} **for smallest n (n=21)**	E_5^g
North	64	62	0.03	0.60	60.23	61.28	20.79	0.98
South	73	73	0.00	0.55	73.00	73.00	21.00	1.00
Zona da Mata	57	54	0.05	0.56	51.60	52.98	20.60	0.97
Triângulo	21	21	0.00	0.58	21.00	21.00	21.00	1.00
Total	215	209	0.03	0.58	203.63	206.84	20.94	0.98

[a] g_{obs} genotypes observed; [b] Estimated as 1 – [(number of different genotypes)/(total number of isolates)]; [c] Gene diversity (H) within populations was calculated based on clone-corrected data at 9 microsatellite loci;[d] Genotypic diversity - Stoddart and Taylor index; [e] Hill's index; [f] Genotypic richness for smallest n; [g] E_5 index of evenness calculated by $(G\text{-}1)/(N_1\text{-}1)$.

Table 4. Analysis of molecular variance (AMOVA) among 215 individuals of *Mycosphaerella musicola*, collected from four population in Minas Gerais, based on 9 SSR loci.

Source of variation	df	Sum of squares	Variance components	% Variation	φ^a	*P*
Among populations	3	34.16	0.086	3.29	0.0329	0.000
Within populations	426	1078.58	2.531	96.71		
Total	429	1112.74	2.618			

a ϕ is a statistic analogous to Wright's F_{ST} value

Table 5. F_{ST} ^{a} value differentiation estimator between *Mycosphaerella musicola* populations in four geographical regions in Minas Gerais.

Population	North	South	Zona da Mata
South	0.018*		
Zona da Mata	0.023	0.008	
Triângulo	0.020	0.026	0.013

[a]Pairwise population θ values calculated from the Weir and Cockerham (1984) (Equivalent Wright's F_{ST}). Asterisks (*) indicate significant at 1%.

3.0 Sensitivity of Brazilian isolates of *Mycosphaerella fijiensis* to fungicides

3.1 Abstract

Black leaf streak disease or Black Sigatoka is a severe foliar disease that affects banana plants throughout the world and large amounts of fungicides are required to prevent crop losses. Intensive fungicide applications can lead to selection for fungicide-resistant isolates of *Mycosphaerella fijiensis* and the establishment of resistant population. The objective of this study was to assess the sensitivity of isolates of *M. fijiensis* collected in Brazil to four fungicides namely: thiophanate-methyl, tebuconazole, chlorothalonil and mancozeb. Sensitivity was assessed by measuring the mycelium growth of the isolates in culture medium amended with different concentrations of the fungicides. The effective concentration needed to reduce mycelium growth by 50% (EC_{50}) was determined for each fungicide. The point mutation G143A in the cytochrome b gene was assayed for 46 isolates to detect resistance to strobilurin, but no mutation was detected. The EC_{50} values for tebuconazole and thiophanate-methyl ranged from 0.02 to 1.39 and from 0.008 to 8.22 µg mL^{-1}, respectively. For chlorothalonil, the lowest and highest EC_{50} values were 0.39 µg mL^{-1} and 53.7 µg mL^{-1}, respectively. For mancozeb, approximately 50% of the isolates had EC_{50} values greater than 1000 µg mL^{-1}. There was no correlation between EC_{50} values for all fungicides and geographic region.

3.2 Introduction

Black leaf streak disease (BLSD), also known as black Sigatoka, is caused by the ascomycete *Mycosphaerella fijiensis* M. Morelet (anamorph *Pseudocercospora fijiensis*) (M. Morelet) Deighton and is the most devastating foliar disease of bananas. Disease control depends mainly on the application of systemic and protectant fungicide that are alternated as part of a strategy to avoid fungicide resistance (Churchill, 2011; Marín et al., 2003). Systemic fungicides provide better control of BLSD than protectant fungicides, but are at a higher risk due to the potential for resistance to develop in the pathogen population (Marín et al., 2003). The main chemical classes of fungicides used to control BLSD are the demethylation inhibitors (DMIs), amines, outer quinone inhibitors (QoI; strobilurins), anilinopyrimidines (APs), benzimidazoles (BCMs), succinate dehydrogenase inhibitors (SDHIs) and guanidines (FRAC, 2010). However, resistance to each of these classes of compounds has evolved in *M. fijiensis* and in cases where a resistant population has established the chemical control became more difficult and the number of sprays required for disease control increased with time (Marín et al., 2003).

Populations of *M. fijiensis* resistant to systemic fungicides are known to occur in several regions. Field isolates of *M. fijiensis* insensitive to benomyl were reported in 1979 (Stover, 1979) and as recently as in 2006 (Cañas-Gutierrez et al., 2006). Although benzimidazoles are highly effective on BLSD control and relatively inexpensive, their use was limited or prohibited in some countries due to the emergence of fungal populations resistant to the fungicides of this group (Marín et al., 2003). Several studies conducted with different fungal species have identified single nucleotide polymorphisms (SNP) associated with resistance to benzimidazoles. For *M. fijiensis* it was demonstrated that a change from cytosine to adenine in codon 198 of the b-tubulin gene was associated with a change in the phenotype and the mutant isolates acquired medium or high resistance to benomyl (Canas-Gutierrez et al., 2006).

Resistance to strobilurin, an outer quinone inhibitor, and sterol demethylation inhibitors are known in several areas. Strobilurin fungicides inhibit the mitochondrial respiration of fungi by binding to the cytochrome bc1 enzyme complex and several studies have been published reporting resistance to these fungicides (Amil et al., 2007; Chin et al., 2001; Heaney et al., 2000; Sierotzki et al., 2000). Resistance to strobilurins was found in high levels in populations of *M. fijiensis* in banana plantations in some areas in Costa Rica. The isolates analyzed had the G143A mutation in the cytochrome b gene, which confers resistance to the fungicide (Chin et al., 2001). The sterol demethylation inhibitors (DMIs) fungicides inhibit the biosynthesis of sterol C-14 α-demethylation of 24-methylenedihydrolanosterol, a precursor of ergosterol in fungi (Brent and Hollomon, 2007). Resistance to DMIs is also known and resistant isolates of *M. fijiensis* to propiconazole were reported (Romero and Sutton, 1997).

Given that fungicide resistance in *M. fijiensis* seems to be frequent and ubiquitous, monitoring pathogen populations becomes a key component for the strategic management of BLSD. In Brazil, banana crops are spread throughout the country and BLSD is a major phytosanitary issue in many areas, particularly in the North part of the country. Nevertheless, no study was ever conducted in Brazil aimed at assessing the sensitivity of isolates of *M. fijiensis* to fungicides used in banana fields. The aim of the current study was to assess the sensitivity of *M. fijiensis* to fungicides commonly used to control black leaf streak disease in Brazil.

3.3 Materials and methods

Isolates

Isolates of *M. fijiensis* collected in 2008 and 2009 in banana fields in seven Brazilian states were stored at Embrapa Amazônia Ocidental – CPAA and utilized in this study (Table 1).

Mycelium growth assays

Isolates were tested for sensitivity to tebuconazole (Folicur 200 CE, Bayer CropScience Ltda), mancozeb (Manzate 800, Du Pont do Brasil S.A.) chlorothalonil (Daconil–BR, Iharabras S.A.) and thiophanate-methyl (Cercobin 700 WP, Iharabras S.A.).

The procedures recommended by the Fungicide Resistance Action Committee (FRAC) as monitoring methods to investigate the possible development of resistance (FRAC, 2012) were adjusted to assess the sensitivity to tebuconazole and thiophanate-methyl. A total of 45 isolates were grown in the M4 liquid medium (Junqueira et al.,1984) for 10 days in a rotary shaker at 25^o C and 120 rpm. A portion of 0.35g of the fresh mycelium mass was transferred to 10 ml of 2X potato dextrose (PD) liquid medium and the Politron ® apparatus was used to grind the mycelial mass (set a speed 4 for 1 min) and obtain uniform suspension of hyphal fragments. Fifty microliters of the suspension was transferred to wells of sterile microtitre plates (ELISA) and mixed with 50 µl of PD amended with fungicides at different concentrations. Tebuconazole was dissolved in dimethyl sulfoxide (DMSO) to the following final concentrations of the active ingredient (a.i.) 0; 0.01; 0.10; 1.0; 10; and 100 µg mL^{-1}, and the fungicide thiophanate-methyl was dissolved in acetone to the following final concentrations, 0; 0.1; 1.0; 10.0; 100; and 1000 a.i µg $mL^{- 1}$. PD with DMSO (1%) and acetone (1%) was used as controls.

Plates were sealed using PVC film to prevent evaporation and contamination, and incubated for 7 days at 25°C and 12 h photoperiod. After incubation, fungal growth was estimated based on mycelium density. This variable was measured indirectly as absorbance at 450 ηm using a microtiter plate reader (Thermo Scientific). Two readings were carried out to assess fungal mass, one before incubation and another after the incubation time. The first reading was subtracted from the second and mycelial growth was estimated based on a regression model previously fitted to the data (data not shown). The EC_{50} for each fungicide was estimated using linear regression of absorbance reads as a function of the logarithm of fungicide concentration.

The sensitivity of 41 and 48 isolates to the protectant fungicides chlorothalonil and mancozeb, respectively, was assessed based on the radial growth on culture medium amended with fungicides. The isolates were grown in V8 agar medium for 15 days at 25° C. Four-mm diameter V8 plugs containing growing mycelium of each isolate were excised from colonies and placed upside down in the center of PDA plates amended with different concentrations of fungicides as formulated commercial products dissolved in 1% dimethyl sulfoxide (DMSO). The final concentrations of chlorothalonil and mancozeb in the medium were 0; 0.1; 1; 10; 100; 1000 µg a.i. $mL^{- 1}$. The control was amended with DMSO (1%) without fungicide. After 15 days of incubation at 25°C, radial mycelial growth of each culture was measured using a ruler in two perpendicular directions and the original plug diameter was subtracted.

Experimental design and statistical analysis

Experiments of mycelial growth were set in a completely randomized design with 5 replicates for each isolate-fungicide concentration combination. All statistical analyses were conducted with the R software (R Development Core Team 2008). The effective concentration to reduce growth by 50% (EC_{50} value) was determined by linear regression of the values of growth inhibition by the logarithm of fungicide concentration.

A distance matrix of geographic location of the isolates was generated using Euclidian distance. Likewise, a distance matrix of the values of EC_{50} was also constructed and a matrix correlation analysis was carried out using the Mantel's test.

The occurrence of the point mutation G143A in the cytochrome b gene was assayed in 46 isolates using the primers MFcytFor_1 5'CTCAATACTGCCTCAGC-3', MFcytRev_1 (R1) 5'-CCGTAATGTGGTTCATC-3' and MFcytRev_S 5' GTTATAACTGTAGCTCC3' (Garcia, 2009).

The DNA was extracted from each isolate using a CTAB method as described by Doyle and Doyle (1990). An aliquot of 50 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) was added to each DNA sample. Samples were treated with RNAse A for 2 h at 37^{o}C before storage at –20°C. A spectrophotometer (NanoDrop 2000 Thermo Scientific) was used to quantify the concentration of nucleic acids and working solutions of DNA at 20 ηg µL $^{-1}$ were prepared from each sample for all polymerase chain reaction (PCR) analyses. Each PCR reaction was performed in 20 µL total volume, containing 20 ηg of template genomic DNA, 2 mM $MgCl_{2,}$ 600 µM dNTPs, 5µM each primer and 0.4 U of Taq-DNA polymerase (Fermentas). PCR was performed in a thermocycler (PTC-100) with an initial denaturation of 94^{o}C (2min), 40 cycles of 94 ^{o}C (60s), 70 ^{o}C (30s) and 72 ^{o}C (60s), and final elongation at 72 ^{o}C (10min). Five microliters of each PCR product was subjected to electrophoresis in 2.0% agarose gel in 1x TBE and viewed under UV on gel stained with GelRed (Biotium). Fragments were compared with a 100bp DNA ladder and scored.

3.4 Results

Mycelium growth inhibition

Significant variation in the sensitivity to fungicides measured by mycelial growth was observed. For tebuconazole the EC_{50} values ranged from 0.02 to 1.39 µg mL^{-1} (Table 2). Only 13% of the isolates had EC_{50} greater than 1 µg mL^{-1} (Figure 1) and the mean EC_{50} value was 0.36 µg mL $^{-1}$ (standard deviation – S.D. ± 0.4).

The EC_{50} values for thiophanate-methyl ranged from 0.008 to 8.22 µg mL^{-1}. The mean value was 0.70 µg mL^{-1} (SD±1.74) (Table 2). Most isolates (57.8%) had EC_{50} lower than 0.1 µg mL^{-1}, 28.9 % of the isolates had EC_{50} values between 1 and 10 µg

mL^{-1} and for 13.3% of the isolates the EC_{50} values were greater than 1 µg mL^{-1} (Figure 1).

The EC_{50} values for chlorothalonil ranged from 0.39 to 53.7 µg mL^{-1} (Table 2). Of the 41 isolates of *M. fijiensis* tested with this fungicide 75.6% of isolates had EC_{50} values between 1 and 10 µg mL^{-1}, 14.6% of isolates had EC_{50} values less than 1 µg mL^{-1} and 9.76% of the isolates had EC_{50} values greater than 10 µg mL^{-1}.The mean EC_{50} was 5.35 µg mL^{-1} (Figure 1).

The EC_{50} values for mancozeb had higher amplitude as compared with the other fungicides tested. The EC_{50} values ranged from 0.09 to more than 1000 µg mL^{-1} (Table 2). Fifty percent of the isolates had EC_{50} greater than 1000 µg mL^{-1} (Figure 1).

Correlation between geographic distance and mean EC_{50} values

There was no correlation between EC_{50} values for all fungicides and geographic region based on Mantel's test (Table 2). There was no evidence of clustering of isolates regarding fungicide sensitivity according to geographic region.

Detection of the G143A mutation that confers strobilurin resistance

The G143A point mutation in the cytochrome b was not found in any of the isolates tested. The amplicon of approximately 200 bp was present in all isolates. Thus, there is evidence that all isolates tested are sensitive to strobilurin.

3.5 Discussion

According to the Fungicide Resistance Action Committee (FRAC) *M. fijiensis* is at a high risk for developing of resistance to site-specific fungicides (FRAC, 2010) and this has been supported by the results of several studies conducted worldwide (Amil et al., 2007; Cañas-Gutíerrez et al., 2009; Chin et al., 2001; Knight et al., 2002; Marin et al., 2003; Romero and Sutton, 1997). Commonly, *M. fijiensis* populations are exposed to many sprays of the same fungicide. To make matters worse, growers inadvertently use several fungicides of the same chemical group that can rapidly increase selection pressure favoring insensitive genotypes.

As fungal growth is a characteristic that allows easier detection of resistant isolates, the mycelium growth inhibition methodology was used in this study. The method used to determine the EC_{50} values for *M. fijiensis* to tebuconazole and thiophanate-methyl fungicides was adapted to use mycelium growth instead of conidia germination assay. This method is recommend by FRAC to monitoring populations of *Mycosphaerella graminicola*, *Phytophthora infestans*, *Fusarium graminearum*, *Botrytis cinerea*, *Pyrenophora tritici-repentis* and other fungi (FRAC, 2012). After the adjustment of the methodology, the method proved to be an advantageous substitute for the *in vitro* growth inhibition test. The new method uses small amounts of medium, is faster than the conventional method, and takes less space. However, the method requires extra care to avoid contamination. For large-scale population monitoring the procedures described here should be preferred.

There were clear differences in the levels of sensitivity of the isolates to the site-specific fungicides. The lack of information regarding the baseline sensitivity of Brazilian isolates of *M. fijiensis* to the fungicides used in this study limits our ability to properly infer the magnitude of the shift with respect to the sensitivity of this population. However, the mean EC_{50} value for tebuconazole (0.36 µg mL^{-1}) was lower than the estimated for isolates of *M. fijiensis* collected in the Ivory Coast (0.73 µg mL^{-1}) (Koné et al., 2008) and for Danish isolates of *M. graminicola* (0.60 µg mL^{-1}) (Thygesen et al., 2009). Comparing tebuconazole with other azoles routinely applied to control BLSD, the average EC_{50} found in this study was higher than the estimated for a population of *M. fijiensis* in Costa Rica (0.06 µg mL^{-1}) (Romero and Sutton, 1997). Resistance to DMI-based fungicides is commonly reported. Point mutation causes insensitivity to the fungicides and cross-resistance is common among the different products (Beresford, 1994; Brent and Holloman, 1998; Knight et al,. 2002, Stergiopoulos et al., 2003). Therefore, monitoring programs for DMI resistance should be conducted on a regular basis in Brazil. Loss in sensitivity to DMI of *M. fijiensis* has been demonstrated to correlate with several distinct point mutations in the CYP51 gene (Cañas-Gutíerrez et al., 2009). Besides point mutations other molecular mechanisms as overexpression of protein and ATP-binding cassette (ABC) transporters encoding efflux pumps can affect resistance to DMI fungicides (Schnabel et al., 2001; Zwiers et al., 2002).

There was no evidence for the lack of sensitivity of Brazilian isolates of *M. fijiensis* to thiophanate-methyl. Resistance to this fungicide has been reported for a number of foliar fungal plant pathogens (Koch et al., 2009; Luo et al., 2007, Ma and Michailides, 2005; May-De Mio et al., 2011, Tanaka et al., 2000) and it is considered a fungicide of high risk for resistance (Ghini and Kimati, 2000). Even though thiophanate-methyl is not registered to control BLSD in Brazil, it is commonly used to control of the yellow Sigatoka. Thus, the fungicide is also used for the control of BLSD where both diseases occur and resistant isolates of *M. fijiensis* could be selected. Isolates of *Monilinia fructicola* and *Botrytis cinerea* were considered sensitive when EC_{50} values based on mycelial inhibition data were <1.0 µg mL^{-1} (May-De Mio et al., 2011; Yourman et al., 2000). Most isolates of *M. fijiensis* had EC_{50} values lower than 1.0 µg mL^{-1} therefore they were considered as sensitive.

Chlorothalonil and mancozeb are multi-site and broad-spectrum protectant fungicides (Pscheidt, 2006). These fungicides are key for resistance management strategies because they have low resistance risk (Gullino et al., 2010). However, some species of fungi have reduced sensitivity to this fungicide (Chang et al., 2007; Daayf and Platt, 2002). The development of resistance to systemic fungicide has been reported in commercial plantations of banana, and this has led to an increase in application of the contact fungicides mancozeb and chlorothalonil. Up to 40 to 45 applications per year may be required to control BLSD (de Lapeyre de Bellaire et al., 2007). In this work, isolates of *M. fijiensis* showed high EC_{50} values to mancozeb fungicide. However, according to Chang et al. (2007) the insensitivity in laboratory studies may not necessarily mean insensitivity under field conditions because of the differences between the extreme selection pressure in amended media and field conditions. Otherwise insensitive isolates can be insensitive mutants selected in the laboratory that are not representative of the naturally occurring population (Chang et al., 2007; Koenraadt et al., 1992). The resistance to mancozeb probably involves mutations within multiple genes like polygenic resistance that is associated to a gradual decline in efficacy over time, however there is no evidence that this has occurred for mancozeb and the more likely potential resistance mechanism is detoxification of the fungicide (Gullino et al., 2010). *In vitro* tests using mycelial growth to assess mancozeb sensitivity have also been reported for *Ascochyta rabiei*, causal agent of ascochyta blight of chickpea (Chang et al., 2007), and *Colletotrichum gloeosporioides* isolates that causes anthracnose in mango (Kumar et al., 2007). Mancozeb inhibits spore germination (Wicks and Lee, 1982) in *Plasmopara viticola*, and this kind of test needs to be done to clearly demonstrate the insensibility to this fungicide in *M. fijiensis*.

There is no evidence of insensitivity among isolates regarding chlorothalonil in BLSD, however, variability in sensitive to chlorothalonil has been been reported in the isolates of *Phytophthora infestans* in potato and tomato (Daayf and Plantt, 2002) and

Ascochyta rabiei, the cause of ascochyta blight of chickpea growing in higher concentrations than 300 ppm (Chang et al., 2007).

Analysis of single nucleotide polymorphism in the cyt b gene leading to a change at amino acid position 143 from glycine to alanine (G143A) suggest that isolates are sensitive to strobilurin. However, other point mutations conferring resistance can occur in cyt b gene as the substitution of phenylalanine to leucine at position 129 (F129L) reported in resistant isolates of *Alternaria solani* (Pasche et al., 2005). Thus, it is possible that point mutations in other positions can occur in *M. fijiensis* isolates from Brazil and confer resistance to strobilurin.

The population of *M. fijiensis* tested in this study showed variable levels of fungicide sensitivity, however these results do not provide any basis for developing fungicide management recommendation to be used under field conditions. Instead, the profile of sensitivity will allow the appropriate implementation of field monitoring programs to assess the effectiveness of fungicides and to understand the evolution of fungicide resistance in populations of *M. fijiensis*. Due to the fact that isolates collected in the state of Amazonas were never exposed to fungicides and the lack of evidence of clustering of isolates regarding fungicide sensitivity according to geographic region, the EC_{50} values estimated in this study can serve as baseline values for future comparison of sensitivity of the population of *M. fijiensis* to fungicides.

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3.7 References

Amil, A., Heaney, S., Stanger, C., and Shaw, M., 2007. Dynamics of QoI sensitivity in *Mycosphaerella fijiensis* in Costa Rica during 2000 to 2003. Phytopathology, 97: 1451-1457.

Beresford, R. 1994. Understanding fungicide resistance**.** Auckland: University in England. Available: http://www.hortnet.co.nz/publications/science/orch94.htm. [Assessed April 12, 2012].

Brent, K. J., and Hollomon, D. W. 2007. Fungicide resistance in crop pathogens: How can it be managed? FRAC monogr. 1, 2nd, revised ed. Fungicide Resistance Action Committee.

Brent, K. J. and Hollomon, D.W. 1998. Fungicide resistance: the assessment of risk. Global Crop Protection Federation, Brussels, Belgium.48p.

Cañas-Gutierrez, G., Angarita-Velasquez, M., Restrepo-Florez, J., Rodriguez, P., Moreno, C., and Arango, R. 2009. Analysis of the CYP51 gene and encoded protein in propiconazole-resistant isolates of *Mycosphaerella fijiensis*. Pest Management Science, 65:892-899.

Cañas-Gutierrez, G. P., Patiño, L. F., Rodríguez–Arango, E., and Arango, R. 2006. Molecular characterization of benomyl resistant isolates of *Mycosphaerella fijiensis*, collected in Colombia. Journal of Phytopathology, 154: 403-409.

Chang, K. F., Ahmed, H. U., Hwang, S. F., Gossen, B. D., Strelkov, S. E., Blade, S. F., and Turnbull, G. D. 2007. Sensitivity of field populations of *Ascochyta rabiei* to chlorothalonil, mancozeb and pyraclostrobin fungicides and effect of strobilurin fungicides on the progress of ascochyta blight of chickpea. Canadian Journal of Plant Science, 87:937-944.

Chin, K. M., Wirz, M., and Laird, D. 2001. Sensitivity of *Mycosphaerella fijiensis* from banana to trifloxystrobin. Plant Disease, 85: 1264-1270.

Churchill, A. C. L. 2011. *Mycosphaerella fijiensis*, the black leaf streak pathogen of banana: progress towards understanding pathogen biology and detection, disease development, and the challenges of control. Molecular Plant Pathology, 12: 307-328.

Daayf, F., and Platt, H. 2002. Variability in responses of US-8 and US-11 genotypes of potato and tomato isolates of *Phytophthora infestans* to commercial fungicides *in vitro*. American Journal of Potato Research, 79: 433-441.

de Lapeyre de Bellaire, L., Ngando, J. E., Abadie, C., Chabrier, C., Blanco, R., Lescot, T., and Cote. F. 2007. Is chemical control of *Mycosphaerella* foliar diseases of banana sustainable? In: ISHS/ProMusa symposium Recent advances in banana crop protection for sustainable production and improved livelihoods Greenway Woods Resort, White River, South Africa September 10-1.

Doyle, J. J., and Doyle, J. L. 1990. Isolation of plant DNA from fresh tissue. Focus, 12:13-15.

FRAC. 2010. Fungicide Resistance action Committee. Available at: http://www.frac.info/frac/index.htm [Assessed September 12, 2011].

FRAC. 2012. Fungicide Resistance action Committee. FRAC Monitoring Methods Status: January 2012. Available at: http://www.frac.info/frac/index.htm [Assessed March 21, 2012].

Garcia, S. A. L. 2009. Identification validation and use of est-derived molecular markers from the genomes of *Mycosphaerella fijiensis* and *Musa* spp. Dissertação. UFLA.

Ghini, R., and Kimati, H. 2000. Resistência de fungos a fungicidas. Jaguariúna: Embrapa Meio Ambiente. 78p.

Gullino, M. L., Tinivella, F., Garibaldi, A., Kemmitt, G. M., Bacci, L., and Sheppard, B. 2010. Mancozeb: Past, present, and future. Plant Disease, 94: 1076-1087.

Heaney, S. P., Hall, A. A., Davies, S. A., and Olaya, G. 2000. Resistance to fungicides in the QoI-STAR cross-resistance group: current perspectives. Proceedings of the BCPC Conference – Pests & Diseases, 2: 755-762.

Junqueira, N. T. V., Chaves, G. M., Zambolin, L., Romeiro, R. S., and Gasparotto, L. 1984. Isolamento, cultivo e esporulação de *Microcyclus ulei*, agente etiológico do mal das folhas da seringueira. Revista Ceres, 31: 322-331.

Knight, S., Wirz, M., Amil, A., and Cook, A. 2002. Fungicide resistance in *Mycosphaerella fijiensis* Morelet: Current status and outlook.In Acorbat. Memorias XV reunion, Realizada en Cartagena de Indias, Colombia. 27 de octubre al 02 Noviembre 2002. Medellín. (COL): Asociacion de Bananeros de Colombia Augura.

Koch, P. L., Grau, C. R., Jo, Y. K., and Jung, G. 2009. Thiophanate-methyl and propiconazole sensitivity in *Sclerotinia homoeocarpa* populations from golf courses in Wisconsin and Massachusetts. Plant Disease, 93: 100-105.

Koenraadt, H., Somerville, S. C., and Jones, A. L. 1992. Characterization of mutations in the beta-tubulin gene of benomyl-resistant field strains of *Venturia inaequalis* and other plant pathogenic fungi. Phytopathology, 82: 1348-1354.

Koné, D., Badou, O. J., Bomisso, E. L., Camara, B., and Aké, S. 2008. Activités in vitro de différents fongicides sur la croissance chez *Mycosphaerella fijiensis* var. *difformis* Stover et Dickson, *Cladosporium musae* Morelet et *Deightoniella torulosa* (Syd.) Ellis, parasites isolés de la phyllosphère des bananiers en Côte-d'Ivoire. Comptes Rendus Biologies, 332: 448-455.

Kumar, S. A., Reddy, N. P. E., Reddy, K. H., and Devi, M. C. 2007. Evaluation of fungicidal resistance among *Colletotrichum gloeosporioides* isolates causing mango anthracnose in Agri Export Zone (AEZ) of Andhra Pradesh, India. Plant Pathology Bulletin, 16: 157-160.

Luo, Y., Ma, Z., Reyes, H. C., Morgan, D. P., and Michailides, T. J. 2007. Using real-time PCR to survey frequency of azoxystrobin-resistant allele G143A in *Alternaria* populations from almond and pistachio orchards in California. Pesticide Biochemistry and Physiology, 88: 328-336.

Ma, Z., and Michailides, T. 2005. Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. Crop Protection, 24: 853-863.

Marín, D. H., Romero, R. A., Guzmán, M., and Sutton, T. B. 2003. Black Sigatoka: an increasing threat to banana cultivation. Plant Disease, 87: 208-222.

May-De Mio, L. L., Luo, Y., and Michailides, T. J. 2011. Sensitivity of *Monilinia fructicola* from Brazil to tebuconazole, azoxystrobin, and thiophanate-methyl and implications for disease management. Plant Disease, 95: 821-827.

Pasche, J. S., Piche, L. M., and Gudmestad, N. C. 2005. Effect of the F129L mutation in *Alternaria solani* on fungicides affecting mitochondrial respiration. Plant Disease, 89: 269–278.

Pscheidt, J. W. 2006. Fungicide theory of use and mode of action. In J. W. Pscheidt and C. M. Ocamb, eds. Plant disease control. Available: http://plant-disease.ippc.orst.edu/article_ index.aspx?article_id=22 [Assessed March 21, 2012].

R Development Core Team. 2008. R: A language and environment for statistical computing. R foundation for statistical computing. Available at: http://www.R-project.org. [Assessed January 10, 2012].

Romero, R. A., and Sutton, T. B. 1997. Sensitivity of *Mycosphaerella fijiensis*, causal agent of black Sigatoka of banana, to propiconazole. Phytopathology, 87: 96-100.

Schnabel, G., and Jones, A. L. 2001. The 14α-demethylasse (CYP51A1) gene is overexpressed in *Venturia inaequalis* strains resistant to myclobutanil. Phytopathology, 91: 102-110.

Sierotzki, H., Parisi, S., Steinfeld, U., Tenzer, I., Poirey, S., and Gisi, U. 2000. Mode of resistance to respiration inhibitors at the cytochrome bc1 enzyme complex of *Mycosphaerella fijiensis* field isolates. Pest Management Science, 56: 833-841.

Stergiopoulos, I., Van Nistelrooy, J. G. M., Kema, G. H. J., and De Waard, M. A. 2003. Multiple mechanisms account for variation in base-line sensitivity to azole fungicides in field isolates of *Mycosphaerella graminicola.* Pest Management Science, 59: 1333-1343.

Stover, R. H. 1979. Field observations on benomyl tolerance in ascospores of *Mycosphaerella fijiensis* var. *difformis*. Transactions of the British Mycological Society, 72: 518-519.

Tanaka, S., Kamegawa, N., Ito, S., and Kameya-Iwaki, M. 2000. Detection of thiophanate-methyl-resistant strains in *Diplocarpon mali*, causal fungus of apple blotch. Journal of General Plant Pathology, 66: 82-85.

Thygesen, K., Jørgensen, L., Jensen, K., and Munk, L. 2009. Spatial and temporal impact of fungicide spray strategies on fungicide sensitivity of *Mycosphaerella graminicola* in winter wheat. European Journal of Plant Pathology, 123: 435-447.

Wicks, T., and Lee, T. C. 1982. Evaluation of fungicides applied after infection for control of *Plasmopara viticola* on grapevine. Plant Disease, 66: 839-841.

Yourman, L. F., Jeffers, S. N., and Dean, R. A. 2000. Genetic analysis of isolates of *Botrytis cinerea* sensitive and resistant to benzimidazole and dicarboximide fungicides. Phytopathology, 90: 851-859.

Zwiers, L.-H., Stergiopoulos, I., Van Nistelrooy, J. G. M., and De Waard, M. A. 2002. ABC transporters and azole susceptibility in laboratory strains of the wheat pathogen *Mycosphaerella graminicola*. Antimicrobial Agents and Chemotherapy, 46: 3900-3906.

Tables:

Table 1. Description of *Mycospherella fijiensis* isolates used in this study.

Isolate	Thiophanate-methyl	Tebuconazole	Chlorothalonil	Mancozeb	City	State [a]	Year
1	x	x		x	Presidente Figueiredo	AM	2008
9			x	x	Manacapuru	AM	2008
24	x	x			Rio Preto da Eva	AM	2008
31	x	x	x	x	Rio Preto da Eva	AM	2008
32	x	x	x	x	Rio Preto da Eva	AM	2008
38	x	x	x	x	Rio Preto da Eva	AM	2008
44	x	x	x	x	Manaus	AM	2008
46			x	x	Manaus	AM	2008
47	x	x	x	x	Manaus	AM	2008
62	x	x	x	x	Manacapuru	AM	2008
63	x	x			Manacapuru	AM	2008
68			x	x	Manacapuru	AM	2008
69	x	x	x	x	Manacapuru	AM	2008
83	x	x	x	x	Cáceres	MT	2008
91	x	x			Jangada	MT	2008
99			x	x	Iranduba	AM	2008
100	x	x			Iranduba	AM	2008
102	x	x	x	x	Iranduba	AM	2008
118	x	x	x	x	Caroebe	RR	2008
120	x	x	x	x	Caroebe	RR	2008
121	x	x		x	Caroebe	RR	2008
124	x	x			Caroebe	RR	2008
126	x	x	x	x	Rio Preto da Eva	AM	2008
129	x	x	x	x	Presidente Figueiredo	AM	2008
131	x	x			Caroebe	RR	2008

134	x	x	x	x	Atalaia do Norte	AM	2008
135	x	x			Benevides	PA	2008
138	x	x	x	x	Manaus	AM	2008
139	x	x	x	x	Marituba	PA	2008
141	x	x	x	x	Autazes	AM	2008
150	x	x	x	x	Itacoatiara	AM	2008
155				x	Autazes	AM	2008
158	x	x	x	x	Autazes	AM	2008
160	x	x	x	x	Careiro Castanho	AM	2008
169	x	x	x	x	Porto Velho	RO	2008
172	x	x		x	Rio Branco	AC	2008
189	x	x	x	x	Itacoatiara	AM	2008
190			x	x	Itacoatiara	AM	2008
193	x	x			Itacoatiara	AM	2008
195	x	x	x	x	Itacoatiara	AM	2008
196	x	x			Rio Branco	AC	2008
200				x	Itariri	SP	2008
202			x	x	Pedro de Toledo	SP	2008
207			x	x	Sete Barras	SP	2008
208	x	x	x	x	Eldorado	SP	2008
210	x	x	x	x	Eldorado	SP	2008
216			x	x	Jacupiranga	SP	2008
218	x	x			Jacupiranga	SP	2008
219	x	x	x	x	Pariquera-Açu	SP	2008
220	x	x			Pariquera-Açu	SP	2008
222	x	x			Pariquera-Açu	SP	2008
224				x	Iranduba	AM	2008
225			x	x	Iranduba	AM	2008
300	x	x	x	x	Manaus	AM	2009
302	x	x	x	x	Manaus	AM	2009
303			x	x	Manaus	AM	2009

304			x	x	Manaus	AM	2009
306			x	x	Manaus	AM	2009
307	x	x		x	Manaus	AM	2009
308	x	x	x	x	Manaus	AM	2009

[a] States of Brazil: AM = Amazonas, AC = Acre, SP = São Paulo, RR = Roraima, RO = Rondônia and MT = Mato Grosso. x = isolates tested for sensitivity to the fungicide.

Table 2. Sensitivity of isolates of *Mycosphaerella fijiensis* to the fungicides chlorothalonil, mancozeb, tebuconazole and thiophanate-methyl and the results of the Mantel's test of correlation between geographic distance and mean EC$_{50}$ values.

Fungicides	**EC$_{50}$ values (µg mL^{-1})**			**Mantel Test (EC$_{50}$ Vs Geographic distance)**
	Minimum	**Maximum**	**Mean±SD**	**r (P value)**
chlorothalonil	0.39	53.7	5.35±8.53	-0.02 (0.31)
mancozeb	0.09	>1000	>1000±1000	-0.05 (0.32)
tebuconazole	0.02	1.39	0.36±0.40	-0.11 (0.82)
thiophanate-methyl	0.008	8.22	0.70±1.74	0.26 (0.10)

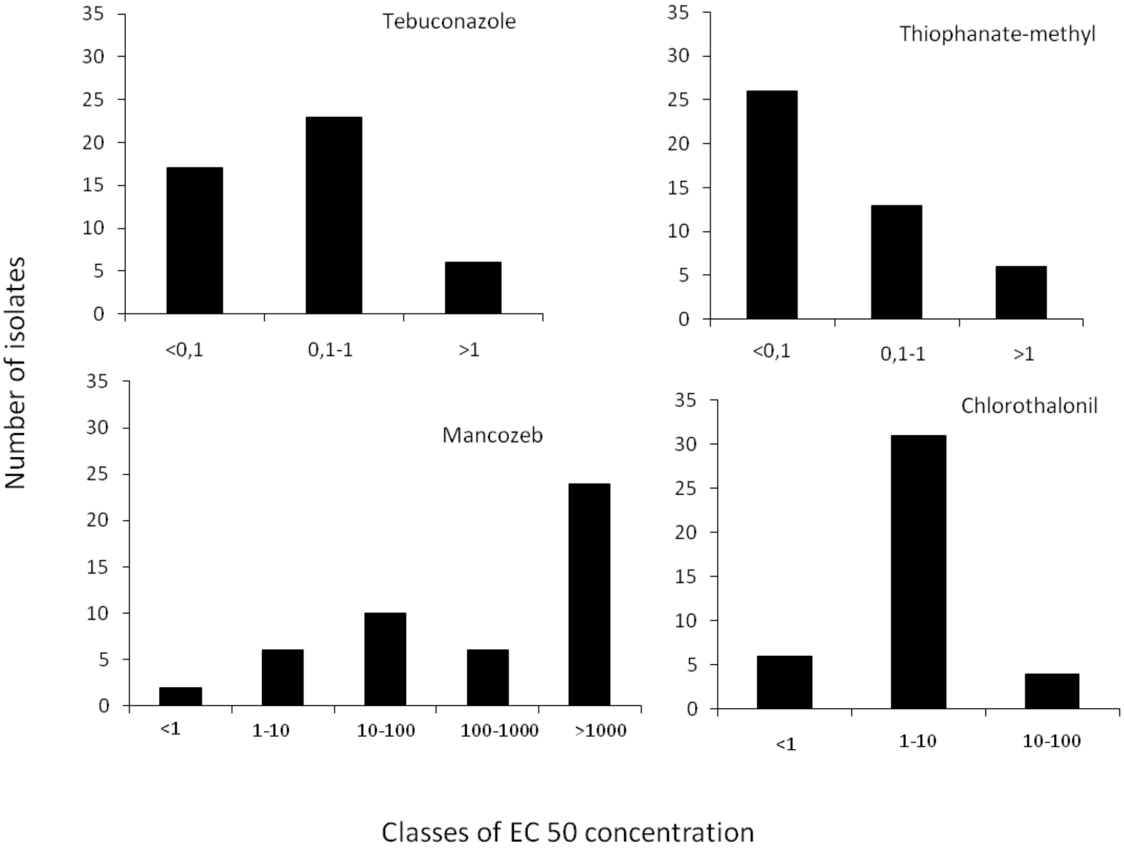

Figure 1. Frequency of isolates of *Mycosphaerella fijiensis* in different classes of EC_{50} values estimated for the fungicides. Values on the x-axis are classes of EC_{50} concentrations; values on the y-axis are number of isolates of *M. fijiensis* in each EC_{50} class.

GENERAL CONCLUSIONS

- All isolates sampled in Minas Gerais state were of *M. musicola*.

- There is high genetic variability in the population of *M. musicola* from Minas Gerais state.

- There is evidence of a mixed mode of reproduction and the mating-type ratio was similar to 1:1.

- There is no evidence of insensitivity of *M. fijiensis* populations to tebuconazole, chlorothalonil and thiophanate-methyl fungicides.

- There is no evidence of the G143A point mutation on cyt b that confers strobilurin resistance in the *M. fijiensis* population.

- There was no correlation between EC_{50} values for all fungicides and geographic region.