MARIA ALVES FERREIRA

ESTRUTURA GENÉTICA DE POPULAÇÕES DE *Ceratocystis fimbriata* E PADRÃO ESPAÇO-TEMPORAL DA MURCHA-DE-CERATOCYSTIS

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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Ao mestre, exemplo de profissionalismo e dedicação à Patologia Florestal
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BIOGRAFIA

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RESUMO

FERREIRA, Maria Alves, D.Sc., Universidade Federal de Viçosa, agosto de 2009. **Estrutura genética de populações de *Ceratocystis fimbriata* e padrão espaço-temporal da murcha-de-ceratocystis**. Orientador: Acelino Couto Alfenas. Coorientadores: Luiz Antonio Maffia, Waldir Cintra de Jesus e Eduardo Seiti Gomide Mizubuti.

Ceratocystis fimbriata é um patógeno que causa murcha vascular e tem ocasionado perdas em muitas culturas importantes no Brasil, incluindo *Eucalyptus* spp., *Mangifera indica*, *Ficus carica*, *Colocasia esculenta* e *Gmelina arborea*. Suspeita-se que algumas populações de *C. fimbriata* no Brasil sejam espécies crípticas, hospedeiro-especializadas. Foram usados 15 marcadores polimórficos para comparar a diversidade genética entre 13 populações de *C. fimbriata* coletadas em diferentes espécies hospedeiras, em seis Estados brasileiros. Adicionalmente, foram examinadas 20 populações de *C. fimbriata* coletadas nos plantios clonais de eucalipto nos Estados de São Paulo (SP), Bahia (BA) e Minas Gerais (MG), usando seis marcadores polimórficos. Também foi avaliado o padrão espacial da doença em 35 parcelas em diferentes plantios clonais de eucalipto, entre estas, quatro áreas de coleta de brotações para enraizamento. Os valores de diversidade gênica da maioria das populações em eucalipo e mangueira dos Estados de MG, BA, Rio de Janeiro (RJ) e SP foram similares aos de populações possivelmente naturais de *C. platani* e *C. cacaofunesta*, espécies pertencentes ao complexo *C. fimbriata*. Valores de índice de associação evidenciaram desequilíbrio de ligações entre as populações do fungo originárias de eucalipto e mangueira. As populações e os genótipos do fungo isolados de eucalipto e mangueira aparentemente estão ligados entre si de acordo com as análises de UPGMA. Em contraste, a população de *G. arborea* do Pará e *F. carica* de SP tiveram baixos níveis de diversidade e foram diferentes entre si, e de todas as populações estudadas, sugerindo que elas têm origens diferentes. Algumas populações apresentaram um único genótipo e podem ter sido introduzidas na região por mudas infectadas. Constatou-se, com base nas análises genéticas e de fertilidade, que as populações brasileiras de *C. fimbriata*

pertencem a uma única espécie biológica. Dos 177 isolados analisados em 20 populações do fungo isoladas de plantios clonais de eucalipto em SP, BA e MG, foram identificados 79 genótipos. As diversidades genéticas nas populações de MG e em lgumas da BA foram similares ao esperado para populações nativas do patógeno. Entretanto, algumas das populações apresentaram baixa ou ausência de diversidade genética. Alta diversidade genética foi encontrada nas áreas em que havia pequena propriedade ou floresta do tipo Cerrado antes da cultura do eucalipto, sugerindo que *C. fimbriata* estava estabelecido no solo antes do plantio dessa cultura. Assim, *strains* de *C. fimbriata* isolados de eucalipto parecem ser nativos de algumas áreas de MG e BA. Em contraste, um ou somente poucos genótipos foram encontrados em áreas em que havia pastagens anteriormente ao eucalipto. Adicionalmente, esses genótipos foram encontrados em viveiros ou áreas de coleta de brotações. As populações de *C. fimbriata* de SP foram relacionadas a algumas das populações da BA, e muitos genótipos encontrados em SP também foram observados na BA. Desse modo, o patógeno pode ter sido introduzido em novas áreas por meio de mudas contaminadas. O padrão espaço-temporal da murcha-de-ceratocysits foi avaliado em 35 parcelas em plantios de eucalipto. A maioria dos plantios estudados apresentou padrão agregado da doença. Assim, pode-se inferir que os plantios podem ter sido infectados via inóculo do solo. Entretanto, em alguns casos, a distribuição de mudas contaminadas pode ter influenciado o padrão espacial da doença no campo.

ABSTRACT

FERREIRA, Maria Alves, D.Sc., Universidade Federal de Viçosa, August, 2009. **Genetic structure of *Ceratocystis fimbriata* populations and spatial-temporal patterns of Ceratocystis wilt**. Advisor: Acelino Couto Alfenas. Co-advisers: Luiz Antonio Maffia, Waldir Cintra de Jesus and Eduardo Seiti Gomide Mizubuti.

Ceratocystis fimbriata is an insect-associated wilt pathogen that causes serious losses on many important crops in Brazil, including *Eucalyptus* spp. (eucalyptus), *Mangifera indica* (mango), *Ficus carica* (fig), *Colocasia esculenta* (inhame), and *Gmelina arborea*. It was suspected that some populations of *C. fimbriata* in Brazil are host-specialized, cryptic species, but mating studies showed that isolates of *C. fimbriata* from these hosts and sweet potato are interfertile, and progeny from those crosses showed normal segregation for microsatellite markers. We used 15 highly-polymorphic microsatellite markers to compare genetic diversity among 13 populations of *C. fimbriata* collected from six states in Brazil. The gene diversity values of most eucalyptus and mango populations from Minas Gerais, Bahia, Rio de Janeiro, and São Paulo were similar to putatively-natural populations of *C. platani* and *C. cacaofunesta*, two other species in the *C. fimbriata complex* that are homothallic. Index of association values indicated substantial asexual reproduction or selfing in populations on mango and eucalyptus. Most of these eucalyptus and mango populations were not highly differentiated from each other, there was evidence of gene flow among them, and the populations and genotypes appeared to be related to each other by UPGMA analyses. In contrast, the *Gmelina* population from Pará and the fig and inhame populations from São Paulo had relatively low levels of diversity and were highly differentiated from each other and all other studied populations, suggesting that they were from different origins and had gone through genetic bottlenecks. Twenty populations of *C. fimbriata* from clonal plantations of eucalyptus in São Paulo, Bahia and Minas Gerais were examined using six polymorphic microsatellite markers. We identified 79 genotypes among the 177 isolates of *C. fimbriata* from individual trees in the 20 plantations. The

gene and genotypic diversity values of eucalyptus populations (plantations) from Minas Gerais and some populations in Bahia were similar to those expected for natural, soilborne populations. However, some of the other populations showed little or no genetic diversity. Populations of high genetic diversity data were found in plantations that were formerly farmland or forest, suggesting that *C. fimbriata* was established in the soil before eucalyptus was planted. In contrast, one or only a few genotypes were found in some plantations on previous pastureland (with no woody hosts for *C. fimbriata*), and these same genotypes were found in nurseries or plantations that were the source for rooted cuttings. São Paulo populations of *C. fimbriata* were closely related to some of the Bahia populations, and many of the genotypes found in São Paulo were also found in Bahia, often on the same eucalyptus clones. Thus, although eucalyptus strains of *C. fimbriata* appear to be native to some areas of Minas Gerais and Bahia, the pathogen apparently can be introduced to new areas in infected eucalyptus cuttings from diseased mother plants taken from plantations or hedges in nurseries. The spatial-temporal patterns of Ceratocystis wilt were evaluated in 35 plots of eucalyptus plantations. In the majority of plantations studied, Ceratocystis wilt occurred in clusters. In many of the plantations, it appeared that the eucalyptus trees were infected from soilborne inoculum. However, the distribution of contaminated rooted in the field can influence the spatial partern of the disease.

INTRODUÇÃO GERAL

Das doenças do eucalipto, a murcha-de-ceratocystis causada por *Ceratocystis fimbriata* Ellis & Halsted é uma das mais importantes, em virtude do difícil controle e da natureza sistêmica das infecções, dos danos causados e das características do patógeno que dificultam o controle. A murcha-de-ceratocystis, em eucalipto, foi constatada primeiramente no sudeste da Bahia, em 1997 (Ferreira *et al.*, 1999). Atualmente, já foram registradas ocorrências no Espírito Santo, Mato Grosso do Sul, em Minas Gerais, em São Paulo (Alfenas *et al.*, 2004) e, recentemente, no Maranhão e Pará (Alfenas, A.C., UFV, informação pessoal). Diversas espécies florestais e agronômicas são suscetíveis à doença, entre elas *Eucalyptus* spp. (Zauza *et al*., 2004), *Acacia mearnsii* (Santos & Ferreira, 2003), *Cassia fistula* (Galli, 1958), *Cassia renigera* (Ribeiro *et al*., 1987), *Calocasia esculenta* (Harrington *et al.*, 2005), *Ficus carica* (Valarini & Tokeshi, 1980), *Gmelina arborea* (Muchovej *et al.*, 1978) e *Mangifera indica* (Batista, 1960).

A doença pode apresentar maior importância em determinados locais dependendo da cultura afetada. Por exemplo, em café há maior impacto econômico na Colômbia (Pontis, 1951), enquanto no Brasil é mais importante no Estado de São Paulo para a cultura da mangueira (Rossetto & Ribeiro, 1990; Ribeiro *et al*., 1995) e gmelina somente na Amazônia (Baker *et al.*, 2003). No Brasil, causa sérios prejuízos para determinados clones de eucalipto (Ferreira *et al.*, 1999). No Congo e na Uganda a doença causou alta mortalidade em determinados clones (Roux *et al*., 2000).

Harrington (2000) delineou três clados dentro do complexo *C. fimbriata* com centros na América Latina, Ásia e América do Norte, sendo o último o centro de diversidade genética. Dentro do clado da América Latina, Baker *et al*. (2003) estudaram diferentes isolados de *Theobroma cacao* (cacau), *Herrania* sp., *Ipomoea batatas* (batata-doce), *Platanus* sp., café, *Xanthosoma* sp., *M. indica* (mangueira), *Annona* sp., *Eucalyptus* sp. e *Gmelina* sp. e verificaram, com base nas análises de sequências de ITS, que todos os isolados de *Platanus* sp., cacau e batata-doce, formaram linhagens fortemente suportadas pela similaridade das sequências. Foi demonstrada especialização em nível de hospedeiro dentro desse clado, por meio de inoculações cruzadas de isolados de diversos locais. Dessa

maneira, Baker *et al.* (2003) levataram a hipótese de que populações locais de *C. fimbriata* são hospedeiro-especializadas; algumas dessas populações são espécies emergentes e alguns genótipos hospedeiro-especializados têm sido movimentados para novas áreas pela ação do homem. Engelbrecht & Harrington (2005), ao parearem isolados de grupos de compatibilidade opostos para encontrar interesterilidade entre eles, constataram que dois grupos interestéreis corresponderam, respectivamente, a linhagens de batata doce e *Platanus* sp. A linhagem do patógeno de cacau apresentou dois grupos interestéreis correspondendo a duas sublinhagens genéticas. Além disso, foram estudadas características morfológicas das linhagens hospedeiro-especializadas; e com base nos resultados, esses autores descreveram uma nova espécie, denominada de *C. cacaofunesta,* e elevaram o patógeno oriundo de *Platanus* sp. (*C. fimbriata* f sp. *platani*) para *C. platani*. O patógeno de batata-doce foi mantido dentro de *C. fimbriata,* de onde foi originalmente descrito. Com base nesses estudos, foi sugerido que a evolução e divergência dessas espécies podem ter sido conduzidas pela especialização por hospedeiro, já que apenas pequenas diferenças morfológicas entre as espécies foram observadas. Em outros estudos com membros do clado da América Latina de *C. fimbriata* (Engelbrecht *et al.*, 2004, 2007b; van Wyk *et al.*, 2006; Ocasio-Morales *et al.*, 2007) e espécies de *Ceratocystis* relacionadas (Roux *et al.*, 2001; Barnes *et al.*, 2005) foi possível distinguir populações possivelmente naturais de introduzidas. O patógeno pode ser introduzido em novas áreas pela movimentação de material vegetal infectado e ferramentas (Harrington, 2000; Baker *et al.*, 2003; CABI, 2005; Ocasio-Morales *et al.*, 2007; Engelbrecht *et al.*, 2007a). Populações introduzidas tem menor diversidade quando comparadas a populações possivelmente nativas, e muitas vezes somente um único genótipo é identificado nas populacões introduzidas (McDonald, 1997).

Os mecanismos potenciais de disseminação da doença no campo e seus efeitos sobre os componentes de desenvolvimento de epidemias estão intimamente ligados à estrutura genética de populações (McDonald & Linde, 2002). Os processos de disseminação de doenças têm efeitos principais nos componentes de distribuição espacial e temporal do desenvolvimento de epidemias (Campbell & Madden, 1990). Nas doenças causadas por *C. fimbriata* alguns mecanismos foram estudados. Por exemplo, em árvores de *Platanus* sp. foi

verificada a dispersão do patógeno via contato radicular (Accordi, 1986, 1989) e ferimentos na raiz (Vigouroux *et al.*, 1999; Vigouroux & Olivier, 2004). Rossetto & Ribeiro (1990) também comprovaram a infecção em *M. indica* pelo inóculo do solo. Para a cultura do eucalipto, Ferreira (2004) examinou árvores no campo e observou a infecção via inóculo no solo. Outro mecanismo comum verificado na literatura é a dispersão do patógeno por disseminadores secundários, como cupins e coleobrocas *Scolytidae* em *Populus* (Hinds, 1972), *Prunus* (Moller *et al.*, 1969) e *M. indica* (Batista, 1960; Viégas, 1960). Sugere-se que coleobrocas que se alimentam de fungos adquirem inóculo de *C. fimbriata* e o dispersam para plantas suscetíveis, principalmente por serragem contaminada. Além disso, verificou-se que esporos de *C. fimbriata* podem ser carregados sobre os corpos de besouros *Ambrosia* e sobreviver pela passagem através do intestino do inseto (Iton, 1960; Crone, 1963). Essas coleobrocas, pertencentes aos gêneros *Xyleborus* e *Hypocryphalus,* são atraídas pelas plantas doentes, que produzem forte aroma e muitas galerias no tronco e galhos dessas plantas (Goitia & Rosales, 2001; Wingfield & Robison, 2004). Também foi observada a infecção de *Ipomoea* em ferimentos feitos por insetos e roedores (Clark & Moyer, 1988).

Ferreira *et al.* (2006) inspecionaram um grande número de plantas clonais de eucalipto afetadas pela murcha de *C. fimbriata* no Brasil e verificaram que somente duas apresentaram perfurações de inseto da família *Platybodidae* nas lesões de *C. fimbriata*; também não foram constatadas perfurações de insetos nas lesões novas e longitudinais, das plantas vizinhas. Desse modo, foi interpretado que a associação de insetos somente em lesões mais velhas é uma forma secundária ou não precursora da doença. Todavia, esses insetos, ao saírem das galerias do lenho infectado, podem transmitir endoconídios e clamidósporos do patógeno para outras plantas lenhosas com xilema alterado por fator abiótico ou biótico, como acontece com outras doenças por *C. fimbriata* em outras culturas (Sinclair *et al.*, 1987; Wingfield *et al*., 1993; Baker *et al*., 2003).

Além das coleobrocas, cupins podem agir como agentes secundários de disseminação da doença. Ferimentos são pontos de entrada para *C. fimbriata* e também podem ser disseminados por ferramentas utilizadas nos tratos culturais (Walter, 1946; Walter *et al.*, 1952; Teviotdale & Harper, 1991). Ademais, materiais propagativos podem constituir método efetivo de dispersão do patógeno. Em *Populus*, foi constatada a presença de *C. fimbriata* em estacas

assintomáticas (Vujanovic *et al.*, 1999). A presença de *C. fimbriata* em mudas assintomáticas de eucalipto (Ferreira, 2004) também sugeriu a dispersão do patógeno pelo homem para várias regiões eucaliptocultoras.

Até o momento, poucos são os estudos referentes à variabilidade genética de *C. fimbriata* e ao padrão espaço-temporal da doença no campo, objetos deste trabalho. Este estudo foi dividido em três artigos. No primeiro, o principal objetivo foi avaliar a diversidade genética de populações de *C. fimbriata* no Brasil, com interesse em identificar populações introduzidas e nativas, usando 15 marcadores microssatélites polimórficos, verificar a interfertilidade entre representantes de algumas populações brasileiras com populações de *C. cacaofunesta* e *C. platani* e confirmar se as populações brasileiras de *C. fimbriata* pertencem a uma única espécie biológica e são capazes de recombinação genética. O segundo objetivou distinguir populações naturais de introduzidas e comparar os genótipos das diferentes populações de *C. fimbriata*. No terceiro artigo, objetivou-se fornecer informações sobre a epidemiologia da murcha-de-ceratocystis, bem como caracterizar o padrão espacial em áreas de coleta de brotos e em plantios convencionais e determinar a taxa de progresso da doença para o patossistema *Eucalyptus*-*Ceratocystis fimbriata.*

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ARTIGO 1

Genetic variation and interfertility among Brazilian populations of the *Ceratocystis fimbriata* sensu strict

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Abstract

Ceratocystis fimbriata is an insect-associated wilt pathogen that causes serious losses on many important crops in Brazil, including *Eucalyptus* spp. (eucalyptus), *Mangifera indica* (mango), *Ficus carica* (fig), *Colocasia esculenta* (inhame), and *Gmelina arborea*. It was suspected that some populations of *C. fimbriata* in Brazil are host-specialized, cryptic species, but mating studies showed that isolates of *C. fimbriata* from these hosts and sweet potato are interfertile, and progeny from those crosses showed normal segregation for microsatellite markers. We used 15 highly-polymorphic microsatellite markers to compare genetic diversity among 13 populations of *C. fimbriata* collected from six states in Brazil. The gene diversity values of most eucalyptus and mango populations from Minas Gerais, Bahia, Rio de Janeiro, and São Paulo were similar to putatively-natural populations of *C. platani* and *C. cacaofunesta*, two other species in the *C. fimbriata complex* that are homothallic. Index of association values indicated substantial asexual reproduction or selfing in populations on mango and eucalyptus. Most of these eucalyptus and mango populations were not highly differentiated from each other, there was evidence of gene flow among them, and the populations and genotypes appeared to be related to each other by UPGMA analyses. In contrast, the *Gmelina* population from Pará and the fig and inhame populations from São Paulo had relatively low levels of diversity and were highly differentiated from each other and all other studied populations, suggesting that they were from different origins and had gone through genetic bottlenecks. One of the eucalyptus populations in Bahia consisted of a single genotype and may have been introduced to the site in infected cuttings from another Bahia location. Similarly, a mango population from Mato

Grosso do Sul consisted of a single genotype, which was identical to one of the genotypes found on mango in São Paulo. Aside from introductions by humans, mating studies and genetic analyses suggest that limited dispersal distance and a high degree of selfing or asexual reproduction lead to local populations of *C. fimbriata* that have limited diversity but are highly differentiated from other populations.

Introduction

Ceratocystis fimbriata Ellis & Halsted causes lethal, wilt-type diseases in woody hosts or rot of corms in many economically important hosts in Brazil, but these important hosts are not native to Brazil (CABI, 2005). The first report of Ceratocystis wilt in Brazil was in the state of São Paulo on *Crotalaria juncea* (Costa & Krug, 1935). Later, the disease was reported on *Mangifera indica* (mango) in the states of Pernambuco and São Paulo (Viégas, 1960). The pathogen also causes substantial mortality to mango in eastern Rio de Janeiro (Baker *et al*., 2003; Silveira *et al*., 2006). The fungus was first observed on *Eucalyptus* spp. (eucalyptus) in the south of Bahia (Ferreira *et al*., 1999), but it is also common in the nearby state of Minas Gerais (Alfenas *et al*., 2004), as well as in Maranhão and Mato Grosso do Sul (AC Alfenas, unpublished). Substantial mortality of *Ficus carica* (edible fig) is known in São Paulo, the only major fig growing area in Brazil (Valarini & Tokeshi, 1980). *C. fimbriata* and relatives cause a corm rot of *Colocasia esculenta* (taro or inhame) and related Araceae, and *C. fimbriata sensu stricto* is a common cause of black rot in corms of inhame in the state of São Paulo (Harrington *et al*., 2005; Thorpe *et al*., 2005). The pulpwood species *Gmelina arborea* is seriously affected in plantations at Project Jari in the Amazonian state of Pará (Muchovej *et al*., 1978; Fearnside, 1988). Ceratocystis wilt of *Theobroma cacao* (cacao), caused by the related *C. cacaofunesta*, is thought to be native to the upper Amazon Basin (Rondônia) and was introduced to Bahia on propagation material (Engelbrecht & Harrington, 2005; Engelbrecht *et al*., 2007b). Aside from *C. cacaofunesta*, it is not known if there are other host-specialized, cryptic species within the *C. fimbriata* complex in Brazil.

C. fimbriata is primarily a xylem pathogen (Harrington, 2000; Ferreira *et al*., 2006). On trees (mango, eucalyptus, fig, *Gmelina*, etc.), infection typically occurs through fresh wounds, often those made by contaminated cutting tools (Giraldo, 1957; Rossetto & Ribeiro, 1990; Viégas, 1960). However, the fungus is soilborne, and root infections are also common (Ribeiro *et al*., 1986; Rossetto & Ribeiro, 1990; Laia *et al*., 2000; CABI, 2005). *Ceratocystis* species are strongly associated with insects (Harrington, 2009), though specific vectors of *C. fimbriata sensu stricto* are not known. Trees with Ceratocystis wilt are frequently attacked by ambrosia beetles (Coleoptera: Curculionidae: Scolytinae and Platypodinae), and the fungus is readily dispersed by wind and rain as aleurioconidia (a special, thick-walled conidium) in the insect frass that is expelled from the tree by the adult beetles (Iton, 1960; Iton and Conway, 1961; Baker *et al*., 2003; Engelbrecht *et al.*, 2007b; Ocasio-Morales *et al*., 2007). Infection may take place if the infested frass lands on a wound of a susceptible host or through root infection via soilborne aleurioconidia. Besides dispersal through aleurioconidia, sticky ascospore masses formed atop long-necked perithecia may be dispersed by insects such as flies and nitidulid beetles (Harrington, 2009). All members of the *C. fimbriata* complex are homothallic through unidirectional mating type switching (Harrington & McNew, 1997; Witthuhn *et al.*, 2000), so even sexual reproduction is usually through selfing, which tends to limit genetic diversity in *Ceratocystis* populations (Harrington *et al*., 1998). Limited dispersal of ascospores by insects or aleurioconidia in insect frass, along with a preponderance of selfing and asexual reproduction, are thought to result in isolated populations of the *C. fimbriata* complex that have relatively low genetic diversity and are highly differentiated from other populations (Roux *et al.*, 2001; Engelbrecht *et al.*, 2004; Engelbrecht *et al.*, 2007b).

There is substantial genetic variation within the *C. fimbriata* complex (Barnes *et al.*, 2001; Baker *et al.*, 2003; Marin *et al.*, 2003; Steimel *et al.*, 2004; Johnson *et al.*, 2005; Thorpe *et al.*, 2005; Engelbrechet & Harrington, 2005), and much of this variation is among clades and lineages associated with geographic regions or specific hosts. Three geographic clades are believed to be centered in North America, Asia, and Latin America, respectively (Harrington, 2000; Johnson *et al.*, 2005). All isolates from Brazil have been placed in the Latin American clade of the *C. fimbriata* complex, along with the *Ipomoea batatas* (sweet potato) form of the

pathogen, on which the species was first described (Halsted, 1890; Engelbrecht & Harrington, 2005). Within the Latin American clade, inoculation studies and intersterility tests have shown that the cause of Ceratocystis wilt of cacao and Ceratocystis wilt of *Platanus* spp. are distinct species, known as *C. cacaofunesta* and *C. platani*, respectively (Baker *et al.*, 2003; Engelbrecht & Harrington, 2005; Engelbrecht *et al.*, 2007b). Brazilian isolates of *C. fimbriata sensu stricto* have shown variation in aggressiveness to cultivated hosts, but there has been little evidence of host specialization, except that *Gmelina* isolates were the most aggressive to seedlings of *Gmelina arborea*, and some eucalyptus isolates were particularly aggressive to eucalyptus hybrids (Baker *et al.*, 2003; Zauza *et al.*, 2004; Thorpe, 2004; Thorpe *et al.*, 2005).

Previous studies with members of the Latin American clade of *C. fimbriata* (Engelbrecht *et al.*, 2004, 2007b; van Wyk *et al.*, 2006; Ocasio-Morales *et al.*, 2007) and related *Ceratocystis* species (Roux *et al.*, 2001; Barnes *et al.*, 2005) were able to distinguish putatively natural populations of the pathogen from introduced populations. These pathogens can be readily introduced to new areas on solid wood packing material, contaminated tools, and infected propagative material (Harrington, 2000; Baker *et al.*, 2003; CABI, 2005; Ocasio-Morales *et al.*, 2007; Engelbrecht *et al.*, 2007a). Introduced populations have shown signs of having gone through a severe genetic bottleneck, that is, there has been substantially less diversity in the putatively introduced populations compared to the putatively native populations, often with only a single genotype identified in introduced populations (McDonald, 1997).

The objective of this study was to evaluate population diversity of *C. fimbriata* in Brazil with the hopes of identifying what might be considered introduced and native populations using highly polymorphic microsatellite markers. Interfertility among representatives of some of the Brazilian populations and with *C. cacaofunesta* and *C. platani* was also determined to confirm that the Brazilian populations of *C. fimbriata* are of a single biological species and capable of genetic recombination.

Materials and Methods

Fungal isolates

Isolates were collected from diseased trees in the field or, in the case of inhame, from corms with symptoms of black rot in grocery stores or markets. The fungus was baited from diseased wood or rotted corm tissue by placing pieces of discolored tissue between two discs of carrot root (Moller & DeVay, 1968). Ascospores masses from perithecia forming on the carrot discs were transferred to agar media for purification and then storage. Only one isolate per tree or corm was stored and used in genetic analyses. At each collection site, an attempt was made to collect samples from trees as far apart as possible. Pure cultures were stored at Iowa State University on malt agar media at - 80ºC on agar slants (isolates beginning with the letter C). The cultures stored at the Universidade Federal de Viçosa (cultures with isolate numbers beginning with other letters) were stored at room temperature (Castellani, 1939).

Fungal populations

We analyzed 13 Brazilian populations (Table 1) of *C. fimbriata* from five hosts in six states: Pará, Bahia, Minas Gerais, Rio de Janeiro, São Paulo, and Mato Grosso do Sul (Fig. 1). Each population was from a single host species in an area usually less than 500 km^2. In the case of eucalyptus, each population was from a single plantation of a single eucalyptus clone. We compared gene diversity and other measures of the Brazilian populations of *C. fimbriata* with previously published data using the same microsatellite markers applied to a nearly-clonal, worldwide collection of *C. fimbriata* from sweet potato (Steimel *et al.*, 2004), natural and introduced populations of *C. cacaofunesta* (Engelbrecht *et al.*, 2007b), and natural and introduced populations of *C. platani* (Engelbrecht *et al.*, 2004).

Eucalyptus populations

Plantations of *Eucalyptus* species and hybrids were sampled in south Bahia and Minas Gerais, which are in the high-rainfall, Atlantic Rainforest forest type and the relatively dry Cerrado forest type, respectively. The EucBA1 plantation was north of Eunápolis and was heavily diseased when sampled in 2003. Plantation

EucBA1 was heavily damaged in a windstorm in 2000, and in 2001 it was used as a source material for the production of rooted cuttings that were planted elsewhere in south Bahia. The site was a farm of unknown crops immediately prior to planting eucalyptus. The EucBA2b plantation was a hybrid of *E. grandis* x *E. urophylla* in its third rotation, with the first rotation started in 1989, but the vegetation previous to eucalyptus is not known. It was sampled in 2007. The EucBA2a population was sampled in 2007 from a second rotation of a different *E. grandis* x *E. urophylla* clone. Previous to the eucalyptus plantations, the EucBA2a site was in pasture.

Three eucalyptus plantations at three locations were sampled in Minas Gerais, and all sites were likely native Cerrado forest type prior to eucalyptus cultivation. The EucMG1 population was an *E. grandis* x *E. urophylla* hybrid, but the vegetation before this planting is not known. The EucMG2 and EucMG3 populations were planted with the same eucalyptus clone, which differed from the clone used in EucMG1. Both EucMG2 and EucMG3 were in an agroforestry system, where agronomic plants were interplanted with eucalyptus. The EucMG2 plantation was sampled in 2007, and previous to the eucalyptus agroforestry system, the site was natural Cerrado vegetation. The EucMG3 population was also sampled in 2007. It had been first planted with eucalyptus seedlings 21 years earlier, but a neighboring eucalyptus plantation was planted to mango 33 years earlier.

Mango populations

Isolates from mango were collected from dead or dying trees in experimental plantings, small farms, private gardens, or along streets. The ManRJ1 population was sampled in 2003 from trees along streets or private gardens in southwestern Rio de Janeiro. The isolates from population ManRJ2 were collected from 1999 to 2003 in northeastern Rio de Janeiro from small farms near the town of São Fidélis. Three isolates of the ManSP1 population were collected from experimental mango plantings at the Instituto Agronômico de Campinas in 2000; two isolates were sampled from a commercial plantation in 2001; and three isolates was received from Instituto Biológico de São Paulo (M. Barreto Figueiredo), but the exact location of the diseased trees was unknown (Baker *et al.*, 2003, Thorpe, 2004). Isolates for the ManMS population were collected in 2008 from mature, diseased street trees in the

city of Aquidauana, Mato Grosso do Sul, where the disease was just recently recognized.

Populations on other hosts

The GmePA population was collected in 1996 from a small number of trees of *Gmelina arborea* that survived in project Jari in the state of Pará, near the mouth of the Amazon River (Muchovej *et al.*, 1978; Fearnside, 1988). The twenty isolates of the fig population FicSP2 were sampled in 2001 and 2002 from commercial plantations in the vicinity of Valinhos, São Paulo (Valarini & Tokeshi, 1980), the primary area of commercial fig production in Brazil. At least two of the plantations were on sites that were previously in pasture. The inhame population ColSP3 consisted of isolates obtained in 2002 from corms of *Colocasia esculenta* with black rot symptoms purchased directly from farmers in distribution markets or a small grocery store near the major inhame growing region of Piedade, São Paulo (Harrington *et al.*, 2005).

DNA extraction

Two methods were used to obtain DNA from cultures for use as template in polymerase chain reactions (PCR). Isolates were grown in 25 ml of liquid medium (2% malt extract and 0.2% yeast extract) at room temperature for 2 weeks, and DNA extraction followed the method of DeScenzo and Harrington (1994) or a CTAB-based protocol (Murray & Thompson, 1980).

Microsatellite markers

We analyzed 15 microsatellite loci (CfAAG8, CfAAG9, CfCAA9, CfCAA10, CfCAA15, CfCAA38, CfCAA80, CfCAT1, CfCAT3K, CfCAT1200, CfCAG5, CfCAG15, CfCAG900, CfGACA60, and CfGACA650) developed from the total genomic DNA of a strain of *Ceratocysits cacaofunesta* (Steimel et al., 2004) and used in population studies on *C. cacaofunesta* (Engelbrecht et al., 2007b) and *C. platani* (Engelbrecht *et al.*, 2004; Ocasio-Morales *et al.*, 2007). Of the 16 loci

used in earlier studies, we were unable to consistently resolve alleles for locus CfCAT9X, and this marker was not included in the analysis.

For each primer pair specific to the flanking regions of the 15 simple sequence repeat regions, one of the primers was fluorescently labeled. PCR amplifications of all microsatellite loci were performed using a 96-well thermal cycler (PTC-100, MJ Research Inc., Watertown, Massachusetts). Cycling conditions were a hot start at 85ºC for 2 m and an initial denaturing step of 95ºC for 95 s, followed by 35 cycles of 58ºC for 60 s, 72ºC for 72 s, and 94ºC for 30 s, followed at 58ºC for 1 m, with a final extension at 72ºC for 30 m. Each reaction (20 µl) contained 2 µl of 10X reaction buffer (Promega Inc., Madison, WI), 200 µM of each dNTP, 4 mM $MgCl_2$, 5.0 pmol of each primer, 0.5 U of *Taq* DNA polymerase (Promega Inc., Madison, WI), and 1-2 µl of template DNA (50-300 ng/µl).

The PCR products were electrophoresed using a four-capillary ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems Inc.). Band sizes of the products were determined using marker standards and ABI GeneScan Analysis Software v3.1.2 and Genotyper 2.0 software (Applied Biosystems Inc., Foster City, CA). Each product length (within 1 bp) was considered a different allele. For most loci, alleles differed by increments of 3 bp. Earlier studies (Engelbrecht *et al*., 2004; Steimel *et al*., 2004; Engelbrecht *et al*., 2007b) used polyacrylamide gel electrophoresis to determine product size, and there were some differences in the band sizes (±2 bp) between alleles using the capillary system and the polyacrylamide system (Ocasio-Morales *et al.*, 2007). Alleles sizes of isolates from *C. platani* (southeastern USA), *C. cacaofunesta,* the sweet potato isolates, and some eucalyptus isolates of *C. fimbriata* that were determined by the polyacrylamide system in earlier work were converted to those of the capillary system for analyses.

Analyses

Nei's gene diversity for each population was calculated without and with clone-corrected data using PopGen 1.32 software (Yeh & Boyle, 1997). Clone-corrected datasets were a subset of the population left after removing isolates that were genetically identical, that is, a genotype within a population was counted only once. Weir and Cockerham's theta (θ), which is comparable to Wright's F_{ST} (Weir

& Cockerham, 1984), was calculated among pairs of populations using the program Multilocus (Mac version 1.21, Department of Biology, Imperial College at Silwood Park, UK). Gene flow between pairs of populations (N_m) was estimated based on the average coefficient of gene differentiation (G_{ST}) across all loci using PopGen 1.32.

Nei's genetic distance between populations and UPGMA (unweighted pair group method with arithmetic mean) dendrograms were constructed using PopGen 1.32. Bootstrap values for branches of the population trees were calculated from 100 replicates using SEQBOOT, GENDIST, NEIGHBOR and CONSENSE in PHYLIP version 3.6 (Felsenstein 1989, 1993). Relationships among genotypes were also examined using genetic distance (Nei's) matrices, UPGMA trees, and 1000 bootstrap replications generated with PAUP* (Swofford, 1998).

Partition of total variance using analysis of molecular variance (AMOVA) (Excoffier, 1992) on Euclidean distances was performed on mango and eucalyptus populations using ARLEQUIN 2.0 (Excoffier *et al.*, 2005). The significance of the variance components associated with different levels of genetic structure (geographic regions and populations) was tested using nonparametric permutations procedures (Slatkin, 1985; Barton & Slatkin 1986).

To test for random mating within mango and eucalyptus populations, linkage disequilibrium was analyzed using the index of association (I_A) statistic in Multilocus. The populations were clone-corrected before analysis. We used randomization procedures (1000 replications, without replacement) to test the significance of the departure of the observed I_A value from zero (randomly mating population).

Mating experiments

Tester strains from representative isolates from the five Brazilian hosts (*Gmelina*, eucalyptus, mango, fig, and inhame) and representative testers of a sweet potato isolate of *C. fimbriata*, *C. cacaofunesta*, and *C. platani* (Engelbrecht & Harrington, 2005) were used in mating experiments. Each pairing was attempted at least twice.

Isolates of *C. fimbriata* are of two mating types, MAT-1 and MAT-2, but MAT-2 isolates are capable of selfing through uni-directional mating type switching (Harrington & McNew, 1997). Self-sterile, single ascospore progeny (MAT-1) of isolates were recovered from selfings of MAT-2 field isolates. For MAT-2 testers, spontaneous, self-sterile mutants were recovered from sectors of otherwise self-fertile isolates. These MAT-2 sectors generally lacked protoperithecia and, thus, could not form perithecia through selfing and were only used as males in mating tests. To confirm that they still had the *MAT1-2* gene, genomic DNA of each sector was extracted using the methods of DeScenzo & Harrington (1994), and PCR was performed to amplify a portion of the *MAT1-2* gene following the protocol of Engelbrecht & Harrington (2005) using primers CFM2-1 (5'-GCTACATTTTGTATCGCAAAGAC-3') and CFM2-2 (5'-TAGTGGGGATATGTCAACATG-3').

The MAT-1 testers (serving as females) were grown on MYEA for 7 days at room temperature and then spermatized by MAT-2 testers. The conidial suspension of the MAT-2 tester was prepared by flooding a 7-day-old MYEA plate with 10 ml of sterilized deionized water (SDW), scraping the mycelium with a sterile spatula, and filtering the suspension through four layers of sterile cheesecloth. One ml of the inoculum was dispersed over the MAT-1 colony.

Cultures were observed for 4 weeks for the presence of perithecia and ascospore masses. Ascospore masses were examined microscopically (400X) to see if there were abundant, normal-appearing ascospores (indication of an interfertile cross) or if there were few or no ascospores, or if the ascospores were misshapen, which is typical of an interspecific cross or hybrid (Harrington & McNew, 1998; Harrington *et al*., 2002). Also, ascospore masses from one or more perithecia of a cross were streaked onto fresh MYEA to observe whether the ascospores were viable and if the progeny had a uniform mycelial morphology, indicating an induced selfing had occurred, or if progeny showed the mycelial phenotypes of the two parents, indicating a successful cross (Harrington & McNew, 1998).

Single-ascospore progeny were recovered from selected pairings by dispersing an ascospore mass into Isopar M (a light, sulfur oil, Exxon Corp.) in a deep-glass slide and streaking onto MYEA (Harrington & McNew, 1997). Recovered single-ascospore progeny were tested for meiotic segregation of

microsatellite markers. Genomic DNA from each of the progeny was extracted using the Prepman Ultra kit (Applied Biosystems, Foster City, CA), and PCR amplification was performed using fluorescently-labeled and unlabeled primers flanking each of three microsatellite loci (CAA80, CAT1200, and CAA38 or CAA9) described in Steimel *et al.* (2004). The sizes of PCR products (alleles) were determined as described earlier, and comparisons were made between progeny and their respective parents. Segregation of the three microsatellite markers among the progeny (deviation from 1:1:6, male-parental:female-parental:non-parental genotype) was analyzed using chi-square (SAS statistical software, SAS Institute, Cary, NC).

Results

Of the 15 microsatellite loci, 14 were polymorphic among isolates of the 13 Brazilian populations of *C. fimbriata* (Table 2). When previous data from the sweet potato form of *C. fimbriata* and populations of *C. cacaofunesta* and *C. platani* were included, all loci were polymorphic, each locus had between 2 and 27 alleles, and there were 106 microsatellite genotypes identified from among the 278 isolates tested (Table 2). Only one microsatellite genotype was found in more than one population; the five mango isolates from population ManMS in Mato Grosso do Sul had an identical genotype, and this genotype was also found in one of the mango isolates from the São Paulo population (ManSP1).

Nei's gene diversity (*H*) was calculated for each of the 13 Brazilian populations of *C. fimbriata* (Table 1). A second calculation of gene diversity was made using only unique genotypes from each population (clone-corrected for population), and these values were compared to those of a worldwide collection of *C. fimbriata* isolates from sweet potato, populations of *C. cacaofunesta*, and three populations of *C. platani*. Relatively high levels of diversity were found in populations EucBA1, EucBA2b, EucMG1, EucMG2, EucMG3, ManRJ1, and ManSP1, as well as in the Rondônia population of *C. cacaofunesta* population and in the eastern North American population of *C. platani*, which are putatively natural populations (Table 1). A genetic diversity value of 0.0 (all isolates from the population had the identical genotype) was found in populations EucBA2a, ManMS, and GmePA. Relatively low values of diversity were found in populations ManRJ2,

FicSP2, and ColSP3, as well as the putatively introduced populations of the sweet potato population (IpoWW), the Ecuadorian, Costa Rican, Colombian and Bahian populations of *C. cacaofunesta*, and the California and European populations of *C. platani* (Table 1).

Weir and Cockerham's Theta (θ) was used to estimate the degree of differentiation between populations. Theta varies from 0.0 (no differentiation among populations) to 1.0 (populations completely differentiated). Most of the eucalyptus and mango populations were similar to each other, with θ values ranging from 0.082 to 0.629, with the exception of the ManMS and ManRJ2 populations (Table 3). The ManMS population was represented by a single genotype, which was least differentiated from the ManSP1 population (θ = 0.356). The ManRJ2 population was differentiated from all other populations (θ = 0.754 and above). Aside from the comparisons among mango and eucalyptus populations, all other θ values were above 0.553, indicating substantial differentiation among the populations.

The estimate of gene flow (N_m) was also used to make pairwise comparisons of populations (Table 3). The value of N_m ranges from 0.0 (no gene flow) to infinity (complete gene flow between populations). The analysis revealed that the comparisons between eucalyptus and mango populations, except for the comparisons of populations ManRJ2 and ManMS and populations EucBA2a and ManSP1, respectively, had relatively high values of probable gene flow, ranging from 0.537 to 3.256. The ManMS and ManSP1populations had a relatively high value for gene flow (N_m=0.962). With the exception of comparisons between mango and eucalyptus populations and the pairwise comparisons ManSP1/ColSP2, EucMG1/CacBRA, ManSP1/CacBRa, EucMG1/MaRJ1, and ManSP1/PlaUS, all other comparisons had N_m values below 0.367, suggesting relatively little gene flow between populations (Table 3).

The UPGMA tree constructed using allele frequencies of populations showed that the 13 Brazilian populations, the sweet potato population, two populations of *C. cacaofunesta*, and the eastern USA population of *C. platani* were relatively distinct, with few populations connected by short branch lengths or with bootstrap support (Fig. 2). The branch connecting populations ManSP1 and ManMS had weak

bootstrap support (66%), as did the branch connecting all the eucalyptus populations (46%) (Fig. 2).

The UPGMA trees constructed using genotypes separated the isolates into 9 groups, PlaUS, CacBR, CacEC, FicSP2, ManRJ2, ColSP3, GmelPA, IpoWW and all the eucalyptus and mango populations except ManRJ2 (Fig. 3). There was moderate support for branches grouping the genotypes from populations ColSP3 (82%), CacEC (51%), and FicSP2 (51%). The genotypes from ManRJ2 grouped separately from other mango genotypes. Aside from the ManRJ2 population, the mango genotypes and all the eucalyptus genotypes appeared to be related to each other (Fig. 3), and the eucalyptus isolates and mango isolates tended to be in two clusters, one dominated by mango genotypes (except the ManRJ2 genotypes) and another dominated by eucalyptus genotypes (Fig. 4). However, there was no bootstrap support for the branches separating the mango and eucalyptus clusters.

Because most of the eucalyptus and mango populations had relatively high levels of diversity and appeared to form natural populations, we grouped them into geographic regions and utilized AMOVA to determine the amount of variation due to regions, among populations within regions, and within populations (Table 4). The single-genotype populations ManMS and EucBA2a were considered atypical and were excluded from the analysis. The results showed that most (48%) of the variation was due to variation among regions, 41% was attributable to the diversity within populations, and only 11% of the variation was due to variation among populations within regions.

The index of association (I_A) was used to test for random mating (using clone-corrected data) in the ManRJ1, ManRJ2, ManSP1, EucBA1/EucBA2b, and EucMG1/EucMG2/EucMG3 regions (Table 5). The value of I_A is expected to be near zero in a randomly mating population, and 1000 randomized replicates were performed to assess whether the value obtained was significantly different from zero. Considering all populations together, the I_A value obtained showed evidence for non-random mating or asexual reproduction (I_A = 2.7, $P < 0.001$). The populations in the five regions each showed significant departures from the values expected for random mating, with the three EucMG populations showing the lowest value of I_A and the São Paulo mango population showing the highest value I_A.

Mating studies

Testers from isolates of each of the five Brazilian host groups successfully crossed with the majority of the other Brazilian testers of opposite mating type (Table 6). In most of these crosses, there were many more than 25 perithecia with normal ascospore masses per plate. In successful crosses, perithecia usually developed within a week and produced thick, creamy ascospore masses at the tips of perithecial necks.

Analyses of single-ascospore progeny from thick, creamy ascospore masses demonstrated that the ascospore masses were not from an induced selfing. Single-ascospore progeny produced colonies of the mycelial morphology of the male and female parents in a roughly 1:1 ratio. Ten sets of progeny from ten crosses were analyzed for three microsatellite markers (Table 7). Eight to 24 progeny were tested for each set of progeny. All sets showed normal segregation (not deviating from 1:1:6 by the chi-square test) for male-parental:female-parental:non-parental types (Table 7), confirming that there had been a successful cross and meiotic segregation of three unlinked loci.

Some of the female testers performed poorly in crosses, perhaps through loss of femaleness (poor protoperithecia or perithecia development). Four female/MAT-1 testers (C925, C1590, C1783, and C1858) usually produced only a few perithecia per plate when paired with what appeared to be compatible MAT-2 testers (Table 6). Three of the four *Gmelina* female/MAT-1 testers (C918, C920, and C925) formed few or no perithecia with ascospores when spermatized with MAT-2 testers from eucalyptus, mango, or inhame. However, when MAT-2 *Gmelina* testers were used as males, they successfully crossed with all of the female/MAT-1 testers from Brazil.

The Brazilian isolates did not successfully cross with testers of *C. platani* or *C. cacaofunesta*, except fewer than 25 perithecia with ascospores formed between the mango female/MAT-1 tester C1657 and the male/MAT-2 *C. platani* tester (Table 6). In contrast, when the MAT-2 sweet potato tester of *C. fimbriata* was used as a male, there was production of abundant perithecia with numerous ascospores by many of the Brazilian female/MAT-1 testers (Table 6). The crossings between the male sweet potato isolate and female/MAT-1 *Eucalyptus* tester from C1347 and female/MAT-1 *Gmelina* tester from C924 were confirmed by the presence of

recombinant progeny as determined by microsatellite markers (Table 7). In contrast, when the female/MAT-1 sweet potato tester was spermatized with male/MAT-2 testers of Brazilian isolates, few perithecia were produced, and the ascospore masses from these perithecia were watery, not creamy, and microscopic examination showed there to be misshapen ascospores and aborted asci in the perithecial centrum.

Discussion

Ceratocystis fimbriata populations on mango in São Paulo and Rio de Janeiro and on eucalyptus in Minas Gerais and Bahia have gene diversity (*H*) values ranging from 0.1889 to 0.3813, comparable to the values (using the same microsatellite markers) of what have been considered natural populations of other homothallic species from the *C. fimbriata* complex: H = 0.1979 for an Upper Amazon population of *C. cacaofunesta* (Engelbrecht *et al*., 2007b) and H = 0.2178 for the eastern USA population of *C. platani* (Engelbrecht *et al*., 2004). Using other neutral markers, similar values have been found for putatively natural populations of *C. albifundus* in South Africa (Roux *et al*., 2001; Barnes *et al*., 2005). These values of gene diversity are relatively low, but expected, for homothallic *Ceratocystis* species (Harrington *et al*., 1998). Selfing and asexual reproduction, as well as limited dispersal by insects or in insect frass should lead to isolated populations that are highly differentiated from each other and with relatively little gene flow between them (Harrington *et al*., 1998; Harrington, 2000; Baker *et al.*, 2003). Aside from the eucalyptus populations in Minas Gerais and Bahia and the mango populations from Sao Paulo and southwestern Rio de Janeiro, which appeared to be closely related, most Brazilian populations of *C. fimbriata* were highly differentiated from each other and showed evidence of limited gene flow. Some of the Brazilian populations were of a single genotype or of only a few closely-related genotypes, and these appear to be the result of introductions by humans on plant propagative material.

Although some of the Brazilian populations were highly differentiated from each other and had low values of gene flow (N_m), they remain a single biological species that is interfertile with the sweet potato strain of *C. fimbriata*, on which the species is based (Halsted, 1890; Engelbrecht & Harrington, 2005). In general,

crosses between different species of *Ceratocystis* produce no interaction when paired, or they form only a few perithecia with few ascospores that are misshapen, germinate poorly, and produce colonies of aberrant morphology (Engelbrecht & Harrington, 2005; Harrington & McNew, 1997; Harrington *et al*., 2001; Johnson *et al*., 2005). In our crosses, attempts were made to recover single-ascospore progeny and determine if the progeny segregated for colony morphology, which would indicate that the ascospore mass was the result of a crossing of two parents, or if the single ascospore progeny were uniform in appearance, an indication that the ascospore mass was the result of a selfing. Selected progeny were further studied for segregation of three microsatellite markers, and a 6:1:1 segregation ratio confirmed meiotic segregation. Fully interfertile crosses were between representatives of the Brazilian populations and also with *C. fimbriata ss*, but with one exception, the Brazilian testers were not interfertile with *C. platani* or *C. cacaofunesta*. Thus, the Brazilian populations should be considered a single biological species*, C. fimbriata sensu stricto*, and distinct from *C. cacaofunesta* and *C. platani*, which are host-specialized to the native American plant genera *Theobroma* and *Platanus*, respectively (Baker *et al*., 2003; Engelbrecht & Harrington, 2005).

The Brazilian populations most closely related to each other based on genetic distance analyses of populations and genotypes, as well as based on values of theta and estimates of gene flow, were the eucalyptus and mango populations from Minas Gerais, Bahia, São Paulo, and southwestern Rio de Janeiro. The analysis of molecular variance showed that most of the genetic variation among these populations was due to variation among regions and variation within populations, and little of the variation was due to variation among populations within regions. Thus, even in this limited part of Brazil, there are regional differences among populations.

High index of association values found in eucalyptus and mango populations suggest that much of the reproduction is asexual or through selfing, as has been found in *C. cacaofunesta* (Engelbrecht *et al*., 2007b), *C. platani* (Engelbrecht *et al*., 2004), and *C. albifundus* (Barnes *et al*., 2005). Most of the eucalyptus plantations sampled that showed genetic diversity were formerly Cerrado vegetation or were previously farmland, and it is presumed that the eucalyptus trees from which the fungus was collected were infected from soilborne inoculum (Laia *et al.*, 2000). The

soilborne inoculum was likely in the form of aleurioconidia that was originally formed in the wood of infected natural or cultivated trees, perhaps expelled from the trees by the tunneling of ambrosia beetles or other wood-boring insects (Carvalho, 1938; Viégas, 1960; Iton, 1960, 1961; Rossetto *et al*., 1997; Goitia & Rosales, 2001; Ocasio-Morales *et al*., 2007).

Surprisingly, few native hosts of *C. fimbriata ss* have been identified in Brazil, and then only on cultivated native hosts, such as *Hevea brasiliensis* (CABI, 2005). However, the high incidence of the disease and relatively high genetic diversity of the pathogen in eucalyptus plantations of Minas Gerais on sites that were recently natural Cerrado vegetation (Machado et al., 2004) suggest that *C. fimbriata* is native to this relatively dry forest type. At least one of the diverse Bahia populations (EucBA1) was on a site that was previously a farm, but the natural vegetation history of the Bahia populations and the sites of the mango isolates is not known. However, these regions of Bahia, Rio de Janeiro, and São Paulo would have been Mata Atlântica rainforest previous to agriculture or urban development. Thus, the region of the greatest population diversity of *C. fimbriata* in Brazil spans the very different Cerrado and Mata Atlântica forest types.

One of the mango populations, near the town of São Fidélis in northeastern Rio de Janeiro, differed substantially from the other mango and eucalyptus populations in UPGMA trees and in values of theta and N_m. The level of gene diversity for the São Fidélis population was somewhat lower than for most of the other mango and eucalyptus populations, and it is possible that the São Fidélis population is not natural and the fungus was brought into the area with the recent commercial plantings of mango. The isolates collected from the São Fidélis area were from scattered small farms, so more diversity had been expected for this population. However, much of this region was in pasture before cattle grazing, and the region may not have had much woody vegetation prior to establishment of these small farms. Aside from mango, cultivated annona or sugar-apple (*Annona squamosa*) is also a host of *C. fimbriata* in the São Fidélis area (Baker *et al*., 2003; Silveira *et al*., 2006), so the host range of this population may differ from the other eucalyptus and mango populations.

Only five closely-related genotypes were found among the 12 inhame isolates from the small commercial growing area in São Paulo. The clone-corrected

value for gene diversity (H = 0.1600) for this population was somewhat less that the values found for most of the mango and eucalyptus populations. Earlier genetic studies have suggested that strains of *C. fimbriata* and other *Ceratocystis* species have been readily moved on corms of inhame and related Araceae (Thorpe *et al.*, 2005), and it was earlier speculated that much of the *C. fimbriata* on inhame in Brazil had originated from São Paulo (Harrington *et al*., 2005). Limited diversity in the inhame population that we sampled suggests that the genotypes of *C. fimbriata* found there may have been introduced from some other region on corms, which are the primary means of propagation. Alternatively, selection for aggressiveness (Pariaud *et al.*, 2009) to inhame may have created a genetic bottleneck in an otherwise diverse local population of *C. fimbriata* in this part of São Paulo.

Other sampled populations in Brazil had little or no genetic diversity and were presumably the result of introductions of the fungus on plant propagative material, as has been seen in other populations of the *C. fimbriata* complex (Harrington, 2000; Engelbrecht & Harrington, 2005; Johnson *et al*., 2005; Thorpe *et al*. 2005; Engelbrecht *et al*., 2007b, 2007b; Ocasio-Morales *et al*., 2007). Eucalyptus is usually planted in Brazil as rooted cuttings, and high-yielding and/or high quality clones are mass-propagated from field-collected cuttings emanating from fresh stumps or from special mini-hedges in nurseries (Alfenas *et al*., 2004). Infected, six-month-old rooted cuttings were found in an eucalyptus nursery in Bahia (Baker *et al*., 2003), and the fungus can be introduced to new sites in such symptomless rooted cuttings. This is believed to be the case with plantation EucBA2a, where all six of the sampled isolates had the identical genotype, which was genetically similar to the other genotypes found on eucalyptus at site EucBA2b. Plantation EucBA2a was in its second cycle of eucalyptus, and before eucalyptus, the site was pasture for cattle grazing, and there was probably no soilborne inoculum of *C. fimbriata* before the fungus was introduced in rooted cuttings of eucalyptus. Recent reports of *C. fimbriata* on eucalyptus in Africa and Uruguay (Barnes *et al*., 2003; Roux *et al*., 2004; van Wyk *et al*., 2006) suggest that the fungus has been introduced to other countries on infected cuttings of eucalyptus. Likewise, *C. fimbriata* was probably introduced on mango nursery stock to Mato Grosso do Sul, where Ceratocystis wilt on mango was recently recognized. The mango population on street trees in Mato Grosso do Sul was of a single genotype, and this genotype was also isolated from a

mango tree in São Paulo, where there are many nurseries producing mango seedlings, Ceratocystis wilt has been recognized for more than 70 years (Costa & Krug, 1935; Viégas, 1960), and the mango pathogen may be native.

Until recently, mango has been reported as a host of *C. fimbriata* only in Brazil (Viégas, 1960). Recently, serious mortality of mango in Oman and Pakistan has been reported, and anecdotal evidence suggests that the strains in Oman and Pakistan were introduced from Brazil on mango seedlings or grafted material (Al Adawi *et al*., 2006; Fateh *et al*., 2006). The DNA sequences of mango isolates from Oman and Pakistan have indicated that the *C. fimbriata* there is closely related to other South American isolates of *C. fimbriata* (van Wyk et al. 2007). Although detailed comparisons with Brazilian populations of *C. fimbriata ss* were not made, it was concluded by van Wyk et al. (2007) that the mango pathogen in Oman and Pakistan should be distinguished from *C. fimbriata* as a new species, *C. manginecans*, on the basis of the presence of barrel-shaped conidia. Isolates of the sweet potato strain of *C. fimbriata* do not produce barrel-shaped conidia (Engelbrecht & Harrington, 2005), but we have found the presence of barrel-shaped conidia to be a variable character in the Latin American clade of *C. fimbriata*, including isolates from Brazil, which we consider *C. fimbriata ss*. Thus, the history of Ceratocystis wilt on mango and DNA sequences suggest that *C. manginecans* is likely based on one or two strains of *C. fimbriata* from Brazil, perhaps from São Paulo, and until further study, *C. manginecans* should be considered a synonym of *C. fimbriata*.

Two other Brazilian populations of *C. fimbriata* had lower gene diversity values than expected. *Gmelina* is known as a host of *C. fimbriata* only in Pará, at Project Jari, near the mouth of the Amazon. In contrast, isolates from the other Brazilian hosts we studied were from regions previously in the Mata Atlântica or Cerrado forest types, far south of the Amazon. It was assumed that the fungus was native to the lower Amazon because *Gmelina* plants were observed within two years of planting of seedlings in recently-cleared, natural rainforest (Muchovej *et al.*, 1978; Fearnside, 1988). We only sampled five isolates from five *Gmelina* trees in a single plantation, and all were of a single, unique genotype, suggesting that they were not from natural, soilborne inoculum. More isolates need to be sampled from

more plantations to more accurately reflect the genetic diversity of the population there.

The fig population from São Paulo was collected from a small area where most of the commercial figs in Brazil are grown. Isolates were collected from various plantations, yet only two genotypes were identified among the 20 isolates studied. Two of the plantations were on sites previously in pasture for cattle, so it is likely that the fungus was introduced on vegetatively-propagated figs in at least these two plantations. The disease in these and the other fig plantations appeared in discrete, circular foci of dead and dying trees, suggesting that one plant had been infected initially and the fungus spread to adjacent plants through root systems.

Isolates of *Ceratocystis fimbriata* collected from exotic hosts in Brazil have shown substantial variation in rDNA sequences and microsatellite markers, and they have varied in aggressiveness to exotic hosts (Baker *et al*., 2003; Thorpe 2004; Thorpe *et al*., 2005). These isolates from Brazil do not appear to be highly host specialized, except that *Gmelina* isolates from Pará (population GemPA) are particularly aggressive on *Gmelina* seedlings, and two isolates from eucalyptus (populations EucBA2) were especially aggressive on eucalyptus (Baker *et al*., 2003; Thorpe, 2004). Zauza *et al.* (2004) tested 18 commercial clones of the hybrid *Eucalyptus grandis* × *E. urophylla* using the same two eucalyptus isolates, and there was significant clone × isolate interaction in the amount of discoloration found in the inoculated hosts. Baker et al. (2003) found that mango and eucalyptus isolates were pathogenic to both mango and eucalyptus, but there was substantial variation in aggressiveness. Thorpe *et al.* (2005) reported that inhame isolates from Brazil caused significantly greater discoloration than the control inoculations in inhame, whereas mango and fig isolates did not. Baker *et al.* (2003) reported that *C. fimbriata ss* from sweet potato was not pathogenic to mango or inhame, and Brazilian isolates from mango, eucalyptus, and *Gmelina* were not pathogenic to sweet potato or *Platanus occidentalis*.

These inoculation studies and the population genetic analyses presented here suggest that geographically isolated populations of *C. fimbriata ss* tend to be particularly aggressive on different exotic hosts, or at least there appears to have been selection for genotypes with particular aggressiveness (Pariaud *et al*., 2009). In Brazil, most of our isolations have been from dead and dying cultivated plants, and

thus our samples are biased towards the portion of the population aggressive to these exotic hosts. These aggressive phenotypes may be further selected as they are spread and maintained on vegetatively propagated material of these same exotic hosts. In the most extreme example of such a genetic bottleneck, it is likely that isolates from sweet potato around the world originated from a single strain (Steimel *et al*., 2004) that was likely selected by humans on propagative material (storage roots), perhaps taken from northern South America, where the cultivated sweet potato may have originated (Engelbrecht & Harrington, 2005). Thus, the species *C. fimbriata ss* is based on a single, highly-selected strain of the fungus on sweet potato, taken from an unstudied population, probably a population of wound-colonizers (Johnson *et al*., 2005; Roux *et al*., 2007) that may have originally varied in aggressiveness to a wide variety of hosts.

Recognizing that populations of *C. fimbriata* tend to be geographically isolated and highly differentiated by limited spore dispersal, care must be taken to distinguish between populations and species. Mechanisms of speciation and the species concept will continue to be controversial issues in the mycological community (Taylor *et al*., 2000; Kohn, 2005), but a functional species definition is needed for important plant pathogens like *C. fimbriata*. Harrington & Rizzo (1999) proposed that a fungus species is "the smallest aggregation of populations with a common lineage that share unique, diagnostic phenotypic characters" (Harrington & Rizzo, 1999). Within the Latin American clade of *C. fimbriata*, *C. cacaofunesta* and *C. platani* can be distinguished by an ecologically significant phenotype (host specialization to native hosts), phylogenetic analyses, and intersterility (Engelbrecht & Harrington, 2005). The microsatellite markers we used here are too sensitive to mutation to be useful for phylogenetic analyses (Dettman *et al*., 2004), as can be seen in the distinction of the two geographically-isolated populations of *C. cacaofunesta* (Engelbrecht *et al*., 2007b), which are phenotypically indistinguishable and monophyletic based on DNA sequence analyses (Baker *et al*., 2003; Engelbrecht & Harrington, 2005; Engelbrecht *et al*., 2007b). Our DNA sequence analyses of multiple genes have failed to distinguish isolates of the Brazilian populations from each other or the sweet potato strain of *C. fimbriata* (Harrington, unpublished), and the Brazilian populations studied were fully interfertile with themselves and the male tester strain from sweet potato. More cryptic species will likely be delimited in

the *C. fimbriata* complex, and perhaps more species will be recognized in Brazil. However, the evidence to date suggests that much of the variation in Brazil is found among geographically-isolated populations that vary in aggressiveness to certain exotic hosts. The level of aggressiveness to some of these cultivated hosts and the ease of spread of these strains to new regions in propagative or woody material is becoming an increasing concern.

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Table 1 Number of genotypes and Nei's gene diversity (*H*) of populations of *Ceratocystis fimbriata, C. cacaofunesta,* and *C. platani* based on 15 microsatellite loci.

				No. of isolates		Nei's gene diversity (*H*)	
Species	Population	Host	Local	No. of isolates	No of genotypes	all isolates	Clone-corrected[a]
C. fimbriata	EucBA1	*Eucalyptus* spp	Eunápolis, Bahia	26	14	0.2128	0.2476
	EucBA2a	*Eucalyptus* spp	Caravelas, Bahia a	6	1	0.0000	0.0000
	EucBA2b	*Eucalyptus* spp	Caravelas, Bahia b	6	4	0.2296	0.2583
	EucMG1	*Eucalyptus* spp	Curvelo, Minas Gerais	18	14	0.2903	0.3083
	EucMG2	*Eucalyptus* spp	Paracatu, Minas Gerais	6	6	0.2778	0.2778
	EucMG3	*Eucalyptus* spp	João Pinheiro, Minas Gerais	6	5	0.2407	0.2502
	ManRJ1	*Mangifera indica*	Campus, Rio de Janeiro	14	10	0.3306	0.3813
	ManSP1	*Mangifera indica*	São Paulo	8	5	0.3187	0.3627
	ManMS	*Mangifera indica*	Aquidauana, Mato Grosso do Sul	5	1	0.0000	0.0000
	ManRJ2	*Mangifera indica*	São Fidélis, Rio de Janeiro	19	6	0.1108	0.1889
	FicSP2	*Ficus carica*	Valinhos, São Paulo	20	2	0.0170	0.0333
	ColSP3	*Colocasia esculenta*	Piedade, São Paulo	12	5	0.1019	0.1600
	GmelPA	*Gmelina arborea*	Jari, Pará	5	1	0.0000	0.0000
	All Brazilian populations			151	74	0.6253	0.5526
	IpoWW	*Ipomoea batatas*	World wide	15	2	0.0083	0.0333
C. cacaofunesta[b]		*Theobroma cacao*	Rondônia, Brazil	3	3	ND[c]	0.1979
			Bahia, Brazil	44	4	ND	0.0308
			Columbia	4	4	ND	0.0938
			Costa Rica	17	4	ND	0.0386
		Theobroma cacao	Ecuador[b]	9	5	ND	0.1020
C. platani[d]	PlaUS	*Platanus occidentalis*	Eastern United States[c]	33	11	ND	0.2178
			Europe	27	3	ND	0.0191
			California	7	4	ND	0.0625

[a]Clone correction removed isolates that had genotypes identical to other isolates from the same site.
[b] Data from Engelbrecht *et al*. (2007b) based on 16 loci.
[c] Not determined.
[d] Data from Engelbrecht *et al*. (2004) based on 16 loci.

Table 2 Microsatellite alleles found for 15 loci among isolates of 17 populations of *Ceratocystis fimbriata*, *C. cacaofunesta,* and *C. platani.*

Populations	Loci														
	AAG8	AAG9	CAA9	CAA10	CAA15	CAA38	CAA80	CAT1	CAT1200	CAG5	CAG15	CAG900	GACA60	GACA650	CAT3K
EucBA1	C[a]	C	B, C	A, B, C	B, C	A, H, L, M	C, D, G	D	B, C, D	C	A, B	A	A, B, C	C, E	A
EucBA2a	C	C	B	B	B	M	D	D	C	C	A	A	B	C	A
EucBA2b	B, C	C	A, B, I	A, B	A, B	E, I, M, N	D, F	D, E	C	C	A	A	B, C	C	A
EucMG1	C, D	B, C	A, B, G, J	A, B, C	B, C	F, M, N, P	A, F, H, I	C, D	B, C	C	A, C, D, E, F, H, J	A	B	C	A
EucMG2	C	C	A, B	B, C	A, B, C	M, N, O	A, D, F, H	D, E	C	C	E,F, I	A	C	C, D	A
EucMG3	C	C	B	A, C	B, C	M, N	A, F, G	D, E	C	B, C	A, E,F, I	A	C	C, D	A
ManRJ1	A, C, D	A, C	B	A, B, C, Null	B, C	A, B, M	D, G, H, K	B, D	B, C	A, C, F	A, I, K, M	A	B, C	C, E	A, F
ManSP1	A, C	C	B, E	A, B, C	B, C	C, I, J, M	E, F,H, J	C, D	A, B, C	C	B, E, I, L	A	B	C,E	C
ManMS	A	C	E	A	C	J	J	C	B	C	I	A	B	E	C
ManRJ2	E,F	A	H	A	D	D	D	A, D	A,D,E	D, E	G, I	A	B, E	D	B
FicSP2	C	D	D, E	B	D	G	H	B	F	G	M	A	B	A	F
ColSP3	C	D, H	F	A	B, C	K	A, E	A, D	A	E	F, H	A	B, D	B, C	C
GmePA	A	B	B	D	C	E	D	G	E	C	B	A	B	C	D
IpoWW	A, E	C	E	C	B	R	A	C	C	C	N	A	B	E	B
PlaUS	A	E	V, X, Y, Z, W, AA	E	H, E	C, Q	N, O, P	C	I, J	E	P, Q, R, S, T, U, V	A	B	F, G, H, I, J, K	B, D, F
CacBR	E, H, I	E	L, N, Q, R, S, T, U	A, B	B, C, F	L, N, S, T	B, E, M	C, H, I	C	H	E, F, G	A	B, F	A, D, F	D, E
CacEC	G	F, G	K, M, O, R	B	G	C	L	B	G, H	E	B, I, O	B	B	A	F
No. of alleles	9	8	27	5	8	20	16	9	10	8	22	2	6	11	6
Size range (bp)	171-232	393-416	156-406	124-136	286-343	132-271	265-331	246-269	370-406	298-338	174-396	189-192	184-219	209-327	309-330

[a] Different letters in the same column represent different alleles for that locus based on different sizes (base pairs) of the PCR product.

Table 3 Estimate of gene flow (N_m, above diagonal) and estimate of differentiation (theta, θ, below diagonal) among 17 populations of *Ceratocystis fimbriata*, *C. cacaofunesta* and *C. platani* using 15 microsatellite loci.

Populations	EucBA1[a]	EucBA2a	EucBA2b	EucMG1	EucMG2	EucMG3	ManRJ1	ManSP1	ManMS	ManRJ2	FicSP2	ColSP3	GmePA	Ipo	CacBR	CacEC	PlaUS
EucBA1		0.589	1.226	1.142	1.390	1.026	1.374	0.949	0.191	0.219	0.178	0.275	0.203	0.198	0.310	0.236	0.310
EucBA2a	0.467		0.756	0.641	0.537	0.566	0.408	0.000	0.070	0.012	0.069	0.000	0.007	0.111	0.076	0.133	0.403
EucBA2b	0.405	0.483		0.756	1.986	1.984	0.846	0.732	0.179	0.246	0.174	0.286	0.186	0.217	0.321	0.226	0.307
EucMG1	0.313	0.298	0.264		2.330	2.484	1.739	1.352	0.275	0.285	0.259	0.359	0.284	0.326	0.431	0.295	0.403
EucMG2	0.388	0.531	0.202	0.217		3.256	1.173	0.937	0.225	0.281	0.218	0.362	0.248	0.268	0.367	0.262	0.347
EucMG3	0.459	0.581	0.202	0.194	0.082		0.997	0.782	0.186	0.244	0.175	0.324	0.193	0.235	0.343	0.223	0.312
ManRJ1	0.406	0.504	0.466	0.324	0.384	0.422		1.958	0.412	0.325	0.319	0.391	0.339	0.320	0.398	0.368	0.450
ManSP1	0.507	0.629	0.500	0.377	0.432	0.482	0.267		0.962	0.317	0.290	0.426	0.319	0.391	0.429	0.337	0.489
ManMS	0.737	1.000	0.800	0.667	0.759	0.794	0.572	0.356		0.070	0.010	0.074	0.000	0.008	0.119	0.072	0.161
ManRJ2	0.804	0.896	0.817	0.768	0.804	0.820	0.754	0.780	0.891		0.081	0.154	0.074	0.077	0.209	0.143	0.238
FicSP2	0.828	0.981	0.908	0.792	0.888	0.908	0.775	0.836	0.983	0.923		0.080	0.010	0.015	0.117	0.117	0.148
ColSP3	0.750	0.905	0.775	0.698	0.736	0.753	0.679	0.692	0.892	0.856	0.934		0.063	0.064	0.195	0.137	0.215
GmePA	0.724	1.000	0.794	0.658	0.740	0.787	0.623	0.666	1.000	0.885	0.983	0.907		0.005	0.105	0.074	0.146
IpoWW	0.792	0.989	0.872	0.722	0.847	0.863	0.746	0.761	0.987	0.917	0.984	0.941	0.991		0.138	0.076	0.165
CacBR	0.778	0.823	0.758	0.730	0.769	0.769	0.759	0.751	0.811	0.808	0.852	0.811	0.830	0.816		0.183	0.317
CacEC	0.778	0.901	0.776	0.733	0.781	0.805	0.691	0.727	0.899	0.867	0.914	0.869	0.897	0.936	0.766		0.253
PlaUS	0.756	0.805	0.802	0.712	0.748	0.758	0.697	0.685	0.766	0.787	0.842	0.791	0.784	0.810	0.762	0.827	

[a] Shaded values are those greater than 0.5 for N_m, and less than 0.5 for θ, indicating relatively low gene flow and high differentiation between populations, respectively.

Table 4 Analysis of molecular variance (AMOVA) of *Ceratocystis fimbriata* populations on eucalyptus and mango in five regions in Minas Gerais, Bahia, Rio de Janeiro, and São Paulo in Brazil based on 15 microsatellite loci.

Source of variation	df	Sum of squared deviations	Variance components	Proportion of variance components (%)	p[a]
Among regions[b]	4	216.940	2.270	48.14	< 0.001
Among populations within regions	3	19.778	0.528	11.20	< 0.001
Within populations	95	182.204	1.917	40.66	0.003
Total	102	418.922	4.716		

[a]The *p* value is for the null hypothesis that there is no significant variation at that level based on 1023 permutations.
[b] The populations were distributed in five regions: (1) ManRJ1; (2) ManRJ2; (3) ManSP1; (4) EucBA1, EucBA2b; (5) EucMG1,EucMG2, EucMG3.

Table 5 Index of association of *Ceratocystis fimbriata* populations on eucalyptus and mango in five regions in Bahia, Minas Gerais, Rio de Janeiro, and São Paulo.

Populations	Hosts	Locations	Index of Association (I_A)	Probability[a]
ManRJ1	mango	Southwestern Rio de Janeiro	1.037	< 0.001
ManRJ2	mango	Northeastern Rio de Janeiro	0.745	= 0.044
ManSP1	mango	São Paulo	1.913	< 0.001
EucBA1, EucBA2b	eucalyptus	Eunápolis and Caravelas, Bahia	0.658	= 0.010
EucMG1, EucMG2, EucMG3	eucalyptus	Curvelo, João Pinheiro, and Paracatu, Minas Gerais	0.583	= 0.002
All Populations			2.680	< 0.001

[a] Probability that the index of association differs from a purely sexually outcrossing population

Table 6 Mating experiments between isolates representing different populations of *Ceratocystis fimbriata, C. platani* (from *Platanus*)*, C. cacaofunesta* (from *Theobroma*) using MAT-2, self-sterile sector (sec) strains as males and MAT-1 self-sterile (ss) strains as females.

		MAT-2, Male[a]								
	MAT-1,	*Gmelina*		*Eucalyptus*		*Mangifera*	*Colocasia*	*Ipomoea*	*Platanus*	*Theobroma*
Host	Female[a]	920sec (GmePA)	925sec (GmePA)	1347sec (EucBA2)	1440sec (EucBA2)	1657sec (ManSP1)	1907sec (ColSP2)	1418sec (IpoWW)	1317sec (PlaUS)	1587sec (CacBR)
Gmelina	918ss (GmePA)	C[b]	C	-	C (1)	-	-	-	H (2)	-
	920ss (GmePA)	C	C	-	-	-	-	-	-	-
	924ss (GmePA)	C	C	C	C	C[c]	C	C[c]	H	-
	925ss (GmePA)	C	C	C (Few)	C (2)[c]	C (2)	-	C (1)	H (Few)	-
Eucalyptus	1347ss (EucBA2)	C[z]	C	C	C	C (Few)	-	C[c]	H (Few)	-
	1440ss (EucBA2)	C	C[c]	C	C	C	C (Few)	C (1)	H (Few)	-
Mangifera	1590ss (ManRJ2)	C (Few)	C	C	C (Few)	C (Few)	C (Few)	C	H	-
	C1657ss (ManSP1)	C	C[c]	C[c]	C	C	C	C	C (Few)	-
Ficus	1783ss (FicSP3)	C (Few)	C	C	C (Few)	C (1)	C (Few)	C (1)	-	-
	1858ss (FicSP3)	C (Few)[c]	C	C	C	-	C (Few)	-	H (Few)	-
Colocasia	1907ss (ColSP2)	C	C	C	C[c]	C	C	C	H	-
	1926ss (ColSP2)	C	C	C	C	C (Few)	C	C (Few)	H	-
Ipomoea	1418ss	H	H	H	H	H	H	C	H	-

Platanus	(IpoWW) 1317ss	H	-	H	H	H	H	H	C	-
Theobroma	(PlaUS) 1587ss (CacBR)	H	H	-	H (Few)	H	H	H	H	-

[a]sec = strains from MAT-2, self-sterile sectors recovered from self-fertile isolates; ss = MAT-1, female-competent strains with protoperithecia.
[b]C = successful cross with many perithecia per plate (greater than 25) and normal looking ascospore masses and the mycelial morphology of each parent found among the progeny; - = no perithecia produced; in parentheses: Few = less than 25 perithecia produced, or a specific number indicating the actual number of perithecia with ascospore masses; H = hybrid cross with either watery ascospore masses of few spores and no germination of ascospores when plated on MYEA, typical of a cross between two different species.
[c]Progeny compared to parents for segregation of microsatellite markers (see Table 7).

Table 7. Segregation of alleles of three microsatellite loci among single ascospore progeny from crosses between strains of *Ceratocystis fimbriata* from Brazil or a sweet potato strain (C1418) from North Carolina, USA.

Parents[a]						
Male Parent	Female Parent	Total Progeny	Male Parental Type	Female Parental Type	Non-parental Type	Deviation from 1:1:6 (*P*)[b]
C1347sec	C1657ss	12	0	0	12	0.1801
C920sec	C1347ss	20	3	5	12	0.5455
C1440sec	C925ss	8	3	3	2	0.1353
C925sec	C1657ss	10	1	2	7	0.8967
C920sec	C1858ss	8	1	2	5	0.8089
C1418sec	C1347ss	10	0	4	6	0.2397
C1440sec	C1907ss	20	5	4	11	0.4076
C1657sec	C924ss	19	2	2	15	0.9591
C925sec	C1440ss	17	6	4	7	0.1290
C1418sec	C924ss	24	1	5	18	0.4724
Total		148	22	31	95	0.0953

[a]sec = self-sterile MAT-2 strains; ss = MAT-1 strains.

[b]Probability of deviation from 1:1:6 segregation of male parental:female parental:non-parental genotypes based on chi-square tests.

Figure Legend

Figure 1 Geographic distribution of the 13 studied *Ceratocystis fimbriata* populations in Brazil. The first three letters of each population name indicate the host (*Gmelina arborea*, *Eucalyptus* spp*., Mangifera indica*, *Colocasia esculenta*, and *Ficus carica*), the last two letters indicate the state of origin, and if more than one population was sampled in that state, they were numbered.

Figure 2 Dendrogram of *Ceratocystis fimbriata*, *C. cacaofunesta,* and *C. platani* populations generated by UPGMA (unweighted pair group method, arithmetic mean) based on alleles frequencies of 15 microsatellite loci. Bootstrap values are shown alongside the branches. The number of isolates sampled from each population is in parenthesis.

Figure 3 Dendrogram of *Ceratocystis fimbriata, C. cacaofunesta* and *C. platani* genotypes generated by UPGMA (unweighted pair group method, arithmetic mean) based on Nei's genetic distance. Bootstrap values greater than 50 are shown alongside the branches. If two or more isolates shared a genotype, only on isolate number is listed but the number of isolates with that genotype is in parentheses.

Figure 4 Dendrogram generated by UPGMA (unweighted pair group method, arithmetic mean) based on Nei's genetic distance among *Ceratocystis fimbriata* populations on *Eucalyptus* spp. and *Mangifera indica* in Brazil. Bootstrap values greater than 50 are shown alongside the branches. Isolates numbers following a 'C' are stored at Iowa State University, and all other isolates are stored at the Universidade Federal de Viçosa.

GmePA
PARÁ
BAHIA
EucBA1
EucBA2a
EucBA2b
EucMG2
EucMG3
EucMG1
ManMS
MINAS
GERAIS
FicSP2
ManSP1
ManRJ2

ARTIGO 2

Movement of genotypes of *Ceratocystis fimbriata* among Eucalyptus plantations in Brazil

Abstract

One of the most important diseases on *Eucalyptus* species in Brazil is Ceratocystis wilt, which is caused by the fungus *Ceratocystis fimbriata*. The disease was first recognized in 1999 in the State of Bahia, but it is now known to occur in the states of Espírito Santo, Mato Grosso do Sul, Minas Gerais, São Paulo, and recently in Maranhão and Pará. Although the pathogen can be soilborne and is thought to be native to some forest sites in Minas Gerais and Bahia, some of the rapid increase in the incidence of the disease may be due to movement of select genotypes of the pathogen in infected, rooted cuttings from nurseries to plantations. We used six highly-polymorphic microsatellite markers to determine the genotype of 177 isolates of *C. fimbriata* from individual trees in 20 plantations in the states of Minas Gerais, Bahia, and São Paulo. The gene and genotypic diversity values of eucalyptus populations (plantations) from Minas Gerais and some populations in Bahia were similar to those expected for natural, soilborne populations. However, some of the other populations showed little or no genetic diversity. Populations of high genetic diversity data were found in plantations that were formerly farmland or forest, suggesting that *C. fimbriata* was established in the soil before eucalyptus was planted. In contrast, one or only a few genotypes were found in some plantations on previous pastureland (with no woody hosts for *C. fimbriata*), and these same genotypes were found in nurseries or plantations that were the source for rooted cuttings. São Paulo populations of *C. fimbriata* were closely related to some of the Bahia populations, and many of the genotypes found in São Paulo were also found in Bahia, often on the same eucalyptus clones. Thus, although eucalyptus strains of *C. fimbriata* appear to be native to some areas of Minas Gerais and Bahia, the pathogen apparently can be introduced to new areas in infected eucalyptus cuttings from diseased mother plants taken from plantations or hedges in nurseries.

Additional keywords: eucalyptus, genetic diversity, population genetics, introduced populations, natural populations, Ceratocystis wilt

Introduction

The Brazilian forestry sector is responsible for 4% of the national GNP (gross national product) in Brazil and 2 million jobs. Eucalyptus plantations in Brazil are among the most productive forests in the world, covering 4 million hectares and typically producing more than 40 m^3/ha/yr of wood (ABRAF, 2008; SBS, 2001). Eucalyptus is currently the most planted forest species worldwide, meeting the high demand of fiber for pulp and paper production, charcoal, furniture, utility poles, fence poles, fuel wood, and building materials. Two of the most important diseases on eucalyptus in Brazil are Ralstonia wilt and Ceratocystis wilt, caused by the bacterium *Ralstonia solanacearum* and the fungus *Ceratocystis fimbriata* Ellis & Halsted, respectively. Both pathogens can be disseminated in infected rooted cuttings of eucalyptus (Giraldo, 1957; Rossetto & Ribeiro, 1990; Engelbrecht et al., 2007a, 2007b; Harrington, 2000; Alfenas et al., 2006).

In addition to eucalyptus, *C. fimbriata* causes lethal, wilt-type diseases in many economically important hosts in Brazil, such as *Mangifera indica*, *Gmelina arborea*, *Hevea brasiliensis*, and others (CABI, 2005). The fungus was first observed on *Eucalyptus* spp. (eucalyptus) in the south of Bahia (Ferreira et al., 1999), but it is also common in the nearby state of Minas Gerais (Alfenas et al., 2004), and the disease is also known in São Paulo, Mato Grosso do Sul, Maranhão, and Pará (AC Alfenas, unpublished). The disease in southern Bahia may reduce volumetric growth by 58% in *E. grandis* x *E. urophylla* hybrids (Ferreira et al., 2007) and reduce pulp yields by 14%. Considering the mean annual increment of 52 m^3/ha in the region and wood value of R\$45/m^3, the losses can be as high as R\$9,495/ha in a single rotation. Losses for pulpwood production may be R\$17,645/ ha (92.0 t cellulose/ha valued at R\$1,400/t). Additionally, pulpwood from diseased trees may increase the need for alkali digestion by 45% (Alfenas & Ferreira, 2008).

Eucalyptus plants may be infected by *C. fimbriata* through their roots by soilborne inoculum in the form of aleurioconidia, or they may be infected through fresh wounds by inoculum in the form of spores on insects or airborne/rainsplashed

insect frass (Laia et al, 2000, CABI, 2005). After penetration, the fungus colonizes the xylem, including radial parenchyma cells, eventually darkening infected tissues and causing wilting and death of the whole plant or individual branches (Alfenas et al., 2004, Ferreira et al., 2006). The typical symptoms of radial darkening of infected wood are observed in transverse sections along the infected stem, from the roots to often the top branches. Crown die-back and stem cankers may be observed (Laia et al., 2000, Alfenas et al. 2004).

Recent studies showed that there are natural and introduced populations of *C. fimbriata* in Brazil (Ferreira et al., submitted). *C. fimbriata* and relatives can be readily introduced to new areas on contaminated tools and infected propagative material (Harrington, 2000; Baker *et al.*, 2003; CABI, 2005), and introduced populations typically show little or no genetic variation because of genetic bottlenecks (Ocasio-Morales *et al.*, 2007; Engelbrecht *et al.*, 2007b; Harrington 2009; Ferreira et al., submitted). The objective of this study was to distinguish natural, genetically diverse, soilborne populations of *C. fimbriata* in Minas Gerais, Bahia, and São Paulo from genetically limited populations due to introduction of only a few genotypes on infected cuttings. Genotypes of the fungus were identified based on six highly polymorphic microsatellite markers, and populations were compared to see if they shared common genotypes.

Materials and Methods

Fungal isolates

Isolates were collected from diseased trees in the field or, in the case of inhame, from corms with symptoms of black rot in grocery stores or markets. The fungus was baited from diseased wood or rotted corm tissue by placing pieces of discolored tissue between two discs of carrot root (Moller & DeVay, 1968). Ascospores masses from perithecia forming on the carrot discs were transferred to agar media for purification and then storage. Only one isolate per tree or corm was stored and used in genetic analyses. Pure cultures were stored at Iowa State University on malt agar media at - 80 C on agar slants (isolates beginning with the letter C). The cultures stored at the Universidade Federal de Viçosa (cultures with

isolate numbers beginning with other letters) were stored at room temperature (Castellani, 1939).

Fungal populations

We analyzed 20 Brazilian populations (Table 1) of *C. fimbriata* from *Eucalyptus* spp. and hybrids in three states: Bahia, Minas Gerais, and São Paulo. Each population was from a single plantation of a single clone of *Eucalyptus*. An attempt was made to collect samples from trees scattered throughout the plantation.

Isolates were collected from dead or dying trees by baiting from diseased wood by placing pieces of discolored tissue between two discs of carrot root (Moller & DeVay, 1968). Ascospores masses from perithecia forming on the carrot discs were transferred to agar media for purification and then storage. Only one isolate per tree was stored and used in genetic analyses. Pure cultures were stored at Iowa State University on malt agar media at - 80 C on agar slants (isolates beginning with the letter C). The cultures stored at the Universidade Federal de Viçosa (cultures with isolate numbers beginning with other letters) were stored at room temperature (Castellani, 1939).

Bahia populations. Plantations of *Eucalyptus* species and hybrids were sampled near Teixeiras de Freitas, Eunápolis, Caravelas, and Itabela in south Bahia from five eucalyptus clones. This region is in a tropical, high-rainfall area that would naturally be in the Atlantic Rainforest forest type. The BA1sA plantation, north of Eunápolis, was a farm of unknown crops immediately prior to planting eucalyptus. Plantation BA1sA (letter 's' designates this plantation as a source of cuttings for rooting in the nursery) was heavily damaged in a windstorm in 2000, and in 2001 it was used as a source material for the production of rooted cuttings that were planted elsewhere in south Bahia, including the BA2A and BA3A plantations. Plantation BA1sA was heavily diseased when sampled in 2003, but only a small percentage of eucalyptus trees showed symptoms in plantations BA2A and BA3A (Table 1).

Stump sprouts from plantation BA4sB were used as source material for cuttings that were rooted in a nearby nursery, BA5nB ('n' signifies that the population was in the nursery). Site BA4sB was in its second rotation of eucalyptus, and the site was in pasture before eucalyptus. However, the plantation was adjacent

to a forest of natural vegetation. A population in Sao Paulo (SP1B) was planted with cuttings of clone B rooted in nursery BA5nB.

Plantation BA6D, sampled in 2007, was a hybrid of *E. grandis* x *E. urophylla* in its third rotation, started in 1989, but the vegetation previous to eucalyptus is not known. The BA7E population was sampled in 2007 from a second rotation of a different *E. grandis* x *E. urophylla* clone. Previous to eucalyptus, the BA7E site was in pasture. The BA8H plantation, sampled in 2006, was a hybrid of *E. grandis* x *E. urophylla* in its first rotation, started in 2003, and the vegetation previous to eucalyptus was pasture that had substantial woody regeneration. The BA9E population was sampled in 2006 from a first rotation of a different *E. grandis* x *E. urophylla* clone. Previous to eucalyptus, the BA9E site was in pasture with substantial woody regeneration (Table 1).

São Paulo populations. Three eucalyptus plantations at three locations were sampled near the towns of Lençóis Paulista, and Angatuba, respectively. The SP1B plantation was in its fourth eucalyptus rotation, most recently planted with cuttings of clone B from BA4sB and rooted at nursery BA5nB (Table 1). The vegetation at site SP1B before eucalyptus planting was pasture with some citrus trees. The SP2I and SP3J populations were from two different *E. grandis* x *E. urophylla* hybrids, but the vegetation before these plantings is not known (Table 1).

Minas Gerais populations. Plantations of three different Eucalyptus species or hybrids were sampled in near the towns of Curvelo, Paraopeba, João Pinheiro, Paracatu, and Buritizeiro. The Minas Gerais plantations were in natural Cerrado forest vegetation type before eucalyptus cultivation, though some of the plantations had a mixture of agroforestry or *Pinus* cultivation along with natural Cerrado forest immediately before eucalyptus cultivation (Table 1).

Stump sprouts from MG1sC were used as a source of cuttings for rooting in a nursery, though it is not clear if any of these cuttings were used to plant clone C in the other plantations. Plantations MG3F and MG4F were in an agroforestry system, where agronomic plants were interplanted with eucalyptus. The MG4F plantation had been first planted with eucalyptus seedlings 21 years before, and a neighboring plantation was planted to mango 33 years before. The MG5G and MS6G plantations were of an *E. grandis* x *E. urophylla* hybrid planted after clearing Cerrado forests. The MG7C and MG8C plantations were first planted with eucalyptus seedlings 21

years earlier, but a neighboring eucalyptus plantation was planted to mango 33 years earlier (Table 1).

DNA extraction

Two methods were used to obtain DNA from cultures for use as template in polymerase chain reactions (PCR). Isolates were grown in 25 ml of liquid medium (2% malt extract and 0.2% yeast extract) at room temperature for two weeks, and DNA extraction followed the method of DeScenzo & Harrington (1994) or a CTAB-based protocol (Murray & Thompson, 1980).

Microsatellite markers

We analyzed 6 highly polymorphic, PCR-based microsatellite loci (CfCAA9, CfCAA10, CfCAA15, CfCAA38, CfCAA80, and CfCAG15) developed from *Ceratocystis cacaofunesta* (Steimel et al., 2004). The primer pairs were selected from 15 markers used in an earlier study of Brazilian isolates of *C. fimbriata* and were found to be among the most polymorphic of the markers (Ferreira et al., submitted). For each primer pair, one of the primers was fluorescently labeled. PCR amplifications of all microsatellite loci were performed using a 96-well thermal cycler (PTC-100, MJ Research Inc., Watertown, Massachusetts) following the earlier described conditions (Ferreira et al., submitted). The PCR products were electrophoresed using a four-capillary ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems Inc.). Band sizes of the products were determined using marker standards and ABI GeneScan Analysis Software v3.1.2 and Genotyper 2.0 software (Applied Biosystems Inc., Foster City, CA). Each product length (within 1 bp) was considered a different allele. For most loci, alleles differed by increments of 3 bp.

Analysis

Nei's gene diversity for each population was calculated with and without clone-corrected data using PopGen 1.32 software (Yeh & Boyle, 1997). Clone-

corrected datasets were a subset of the population left after removing isolates that were genetically identical, that is, a genotype within a population was counted only once. Comparisons of Nei's gene diversity were made for five eucalyptus populations for which there were data available for the six highly polymorphic microsatellite loci in contrast to the 15 microsatellite loci used in an earlier study (Ferreira et al., submitted). Multilocus genotypic diversity was estimated with the Stoddart and Taylor's G index (Stoddart & Taylor, 1988). Bootstrapping was conducted using SAS (SAS Institute, Cary, NC, 2003) modified to calculate indices of diversity and evenness. Bootstrapping was conducted using 2,000 replicates at a confidence interval of 95% using the accelerated bootstrap procedure (BCa method) (Grünwald et al., 2003).

Partition of total variance using analysis of molecular variance (AMOVA) on Euclidean distances was performed eucalyptus populations from Minas Gerais using ARLEQUIN 2.0 (Excoffier *et al.*, 2005). The significance of the variance components associated with different levels of genetic structure (geographic regions and populations) was tested using nonparametric permutations procedures (Barton & Slatkin 1986).

A dendrogram comparing the relatedness of populations was generated based on Nei's genetic distance between populations and UPGMA (unweighted pair group method with arithmetic mean) using PopGen 1.32 (Yeh & Boyle, 1997). Bootstrap values for the population trees were calculated from 100 bootstrap replicates of 100 UPGMA trees using SEQBOOT, GENDIST, NEIGHBOR and CONSENSE in PHYLIP version 3.6 (Felsenstein, 1993). Relationships among genotypes were also examined using genetic distance matrices with Dice (Nei & Li) coefficient and UPGMA trees generated using GENES (Cruz, 2008).

Results

All six microsatellite loci were polymorphic among eucalyptus isolates from the 20 Brazilian plantations. A total of 79 genotypes were identified among 177 isolates tested using the six loci, although more genotypes would have been detected if more microsatellite loci had been used. In comparison with data from 62 isolates from six plantations that were also included in the earlier study with 15

microsatellite loci (Ferreira et al., submitted), the six polymorphic loci distinguished 40 genotypes, while 43 genotypes were distinguished using all 15 loci (Table 2). Because the six loci were among the most polymorphic of the original 15 loci, the gene diversity values were about twice as high when the six loci were used than when all 15 loci were used (Table 2).

Each population was a collection of isolates from an individual plantation, except BA5nB, which was a collection of isolates from infected rooted cuttings in a nursery. The UPGMA tree constructed using allele frequencies of populations showed that some of the populations were closely related to each other (Fig. 1). The branch connecting populations BA1sA, BA2A, and BA3A were all from the same eucalyptus clone, and the branch connecting those three populations had strong bootstrap support (89%). The other Bahia populations and the three São Paulo populations were connected by short branch lengths and weak bootstrap support (Fig. 1). The populations collected from a plantation (BA4sB) that was a source of cuttings and a nursery (BA5nB) where the cuttings were rooted were in this second Bahia/São Paulo group of populations. The populations from SP1B and BA6D had intermediate bootstrap support (75%), and populations from BA8H and SP3J had low bootstrap support (52%).

The branch connecting populations MG7C and MG8C had moderate bootstrap support (Fig. 1). These plantations were planted at about the same time with the same eucalyptus clone, and the plantations were near each other. The MG1sC and MG2C plantations were of the same clone as the MG7C and MG8C plantations, and MG1sC was a source of cuttings for other plantings, but the populations of *C. fimbriata* from MG1sC and MG2C were not closely related to the MG7C and MG8C populations. There was some support for grouping the MG1sC, MG2C, MG4F, and M5G populations, but populations MG3F and MG6G were distinct (Fig. 1).

Genotypic diversity (G) and Nei's gene diversity (*H*) were calculated for each of the 20 Brazilian populations of *C. fimbriata* as well as for the combined populations of each State (Table 1). The Bahia populations had lower gene diversity (*H* ranged from 0.0 to 0.4352) and genotypic diversity (G ranged from 1.32 to 6.36) than the Minas Gerais populations (Table 1). There were only 39 genotypes among the 103 isolates from the nine Bahia populations (G = 12.5), and the overall gene

diversity value of the combined Bahia populations was H = 0.5526. Plantations BA2A and BA3A were planted with rooted cuttings taken from BA1sA, and their *C. fimbriata* populations had lower gene diversity (H = 0.0180 and 0.2857, respectively), than the source population (H = 0.3836). Most of the genotypes found in plantation BA1sA appeared to be closely related to each other based on the UPGMA analysis (Fig. 2). One of the five genotypes identified in plantations BA2A and BA3A were also found in the source of the rooted cuttings, plantation BA1sA (Table 3), and the four genotypes (from population BA2B and BA3B were closely related to the BA1sA genotypes). The most common genotype found in BA1sA (genotype CBCICA) was also the most common genotype found in BA2A and BA3A, but this genotype was not found in any other plantation (Table 3).

Likewise, the nursery population from rooted-cuttings of clone B (BA5nB) had lower gene diversity than the population sampled from the source of the cuttings of the same eucalyptus clone, plantation BA4sB (H = 0.2292 and H = 0. 3356, respectively). Population BA4sB was made up of many unrelated genotypes according to the UPGMA analysis (Fig. 2), but 11 of the 27 isolates sampled were of the same genotype, BCBJDA (Table 3). The three genotypes found in the nursery BA5nB were also found in the source plantation BA4sB (Table 3).

Bahia plantation BA6D was in the second rotation of eucalyptus following pasture, and its *C. fimbriata* population had a gene diversity value of H = 0.0 (all isolates of the same genotype, BBBJDA). Twenty isolates were identified with genotype BBBJDA in Bahia and São Paulo, including two of the five isolates sampled from nursery BA5nB (Table 3). Besides the source plantation BA4sB and the nursery BA5nB, genotype BBBJDA was identified from three other Bahia plantations and three other São Paulo plantations (Table 3).

The three São Paulo plantations (SP1B, SP2I, SP3J) had relatively low values of gene diversity (H values of 0.1389, 0.3958, and 0.2407, respectively), and only 7 unique genotypes (G = 4.34) were found among the 18 São Paulo isolates (Table 1). Four of the seven genotypes found in São Paulo were also found in Bahia, and 9 of the 18 isolates from São Paulo had genotypes identified in nursery BA5nB (Table 3). For all three São Paulo populations combined, the gene diversity value was lower (H = 0.3323) than for the combined Bahia populations (H = 0.5526) and the combined Minas Gerais populations (H = 0.6732) (Table 1).

Almost all of the genotypes found in Bahia and São Paulo were identical to (Table 3) or related to (Fig. 2) genotypes found in populations BA1sA or BA4sB. The most common genotypes, CBCICA and BBBJDA, were each found 20 times (Table 3 and Fig. 2). Genotype CBCICA was the dominant genotype in source plantation BA1sA, and genotype BBBJDA was the dominant genotype in the nursery population BA5nB. Genotypes from the source plantation BA1sA were also found in six other plantations. Genotypes from the source plantation BA4sB and the nursery population BA5nB were found in nine other plantations (Table 3). Overall, 17 of the 79 genotypes identified in this study were found in more than one population, and 11 of these genotypes were found in a source of eucalyptus cuttings, that is, populations BA1sA, BA4sb, BA5nB, or MG1sC (Table 3).

Relatively high levels of gene diversity (*H* ranged from 0.4352 to 0.6145) and genotypic diversity (G ranged from 2.88 to 12.00) were found in populations from Minas Gerais (Table 1). Combining the eight Minas Gerais populations together, there were 40 genotypes out of the 56 isolates sampled, with a gene diversity value of $H = 0.6732$ and a genotypic diversity value of G = 32.25. A wide diversity of genotypes were found in Minas Gerais (Fig. 2). Only eight of the 40 genotypes found in Minas Gerais were found in more than one plantation, and no genotype in Minas Gerais was found in more than three plantations (Table 3). Two genotypes were found in a source of rooted cuttings for clone C (MG1sC) and in plantation MG2C. Two genotypes were found in both Bahia and Minas Gerais populations, and another genotype was found in both São Paulo and Minas Gerais populations (Table 3).

We were able to identify the previous vegetation type of 16 of the 20 eucalyptus plantations (Table 1). The populations with the highest gene diversity values (average $H = 0.4991$) and genotypic diversity values (average G = 5.14) and were from the eight plantations that followed natural Cerrado vegetation, all of which were in Minas Gerais (Table 4). One of the Bahia plantations (BA1sA) was planted on a former farm site, and it had an average gene diversity of $H = 0.3836$ and genotypic diversity of G = 6.36. Five plantations (four in Bahia and one in São Paulo) were on former pastureland, presumably without hosts for *C. fimbriata*. These five populations had relatively low average gene diversity values (average $H = 0.1556$) and genotypic diversity (average G = 2.40) (Table 4). Two Bahia

plantations (BA8H and BA9H) were on former pasture sites that had substantial woody regeneration before clearing and planting of eucalyptus. The *C. fimbriata* populations sampled from these sites had an average gene diversity value of H = 0.3611 and an average genotypic diversity value of G = 3.91, higher than for the pasture plantations without woody regeneration (Table 4).

Because the eight populations from Minas Gerais had relatively high levels of diversity and appeared to form natural populations, we grouped them into six geographic regions and utilized AMOVA to determine the amount of variation due to regions, among populations within regions, and within populations (Table 5). The results showed that 13% of the variation was due to variation among regions, 84% was attributable to the diversity within populations, and only 2.5% of the variation was due to variation among populations within regions.

Discussion

There was substantial variation in levels of genetic diversity among the sampled populations of *C. fimbriata* from eucalyptus plantations. Some *C. fimbriata* populations had relatively high levels of genetic diversity, suggesting that the eucalyptus trees were infected by natural, soilborne inoculum that was established before planting eucalyptus. The most diverse populations were found on sites that were formerly Cerrado forests, all of which were in Minas Gerais, while the least diverse populations tended to be from eucalyptus plantations planted on former pastureland. Generally, genotypes of the pathogen found in populations of limited genetic diversity were also found in plantations that were used as a source of cuttings or in a nursery where the cuttings were rooted. These data suggest that *C. fimbriata* populations with limited genetic diversity arose primarily through introduction of relatively few strains in infected cuttings. Thus, both natural soilborne inoculum and infected cuttings appear to be sources of infection in eucalyptus plantations in Brazil.

The amount of genetic diversity found in the Minas Gerais populations suggest that *C. fimbriata* is native to this region and probably occurs in natural Cerrado forests, as was concluded in an earlier study (Ferreira et al., submitted). The populations of *C. fimbriata* sampled in Minas Gerais had much higher levels of

genotypic diversity and gene diversity than the sampled populations in Bahia and São Paulo. The levels of gene diversity found in the Minas Gerais populations are similar to those of other putatively natural populations of homothallic *Ceratocystis* species (Roux et al, 2000, Engelbrecht et al, 2004, Engelbrecht et al., 2007a, 2007b, Ferreira et al., submitted,). Most of the variation found in the Minas Gerais populations was found within the populations, though the Minas Gerais populations were generally quite distinct based on UPGMA analysis of allele frequencies. Still, some movement of genotypes from plantations that were the source of cuttings may have been moved to other plantations. Two genotypes found in plantation MG2C were also found in the source of cuttings for that clone, MG1sC. Also, two adjacent plantations in Minas Gerais (MG7C and MG8C) were planted with the same clone, and these plantations were related to each other, suggesting that some of the eucalyptus trees may have been infected at the source of the cuttings or in the nursery. However, these adjacent plantations may have been planted on sites with similar populations of *C. fimbriata* in the soil. Movement of genotypes among populations seems to be have a minor influence on the population structure of *C. fimbriata* on eucalyptus in Minas Gerais. All of the Minas Gerais plantations followed clearing of native Cerrado forest type, and it is likely that the *C. fimbriata* populations were naturally established in the soil before planting of eucalyptus.

Root infection of mango (*M. indica*) and other hosts by *C. fimbriata* has been noted (CABI 2005; Rossetto & Ribeiro, 1990), and the fungus can be readily isolated from soil using carrot discs as bait (Laia et al, 2000). Soilborne inoculum of *C. fimbriata* is likely in the form of aleurioconidia, which may enter the soil from the activities of insects boring into the wood of diseased trees, expelling frass from galleries as they tunnel through discolored xylem colonized by *C. fimbriata* (Viégas, 1960; Iton, 1960, 1961; Rossetto et al., 1997; Goitia & Rosales, 2001; CABI 2005; Ocasio-Morales et al., 2007). Although it is hypothesized that trees native to the Cerrado are hosts of *C. fimbriata*, no such native host has been identified, and further investigations into the presence of the fungus associated with ambrosia beetles or other wood-boring insects is needed in the Cerrado vegetation type.

Most of the *C. fim*briata populations on eucalyptus in Bahia and São Paulo did not show characteristics of natural populations. Many Bahia and São Paulo populations had lower levels of genotypic and gene diversity than expected, and

some of the populations seem to be related to each other. The São Paulo populations showed particularly low levels of diversity.

Some of the Bahia and São Paulo sites were planted on former pastureland, which would not have woody hosts for *C. fimbriata*, and thus little soilborne inoculum. Sites that had pastureland with woody regeneration before planting eucalyptus had *C. fimbriata* populations of higher diversity than plantations on pastureland with no woody regeneration. One of the sources of cuttings was plantation BA1sA, which was formerly a small farm, and it had a *C. fimbriata* population with diversity values intermediate between populations on former pastureland and former Cerrado forest. The genotypes from BA1sA were closely related to each other, and they, too, may have been introduced to the farm in propagative material of fruit trees or other hosts for *C. fimbriata* (Ferreira et al., submitted). Two plantings of eucalyptus cuttings from BA1sA on former pastureland had *C. fimbriata* populations of low diversity, and most of the isolates in these two plantations had genotypes that were also found in BA1sA, suggesting that the genotypes were brought into the plantations in infected cuttings. Another source of cuttings, plantation BA4sB, planted on former pastureland, had a *C. fimbriata* population with a moderate level of diversity, but this plantation was adjacent to native Mata Atlântica forest, which may have been a source of soilborne inoculum. This source plantation for cuttings had higher genetic diversity than the nursery where the cuttings were rooted, and even lower diversity was seen in the SP1B planting of clone B. Thus, the SP1B population may have gone through several genetic bottlenecks, from native Mata Atlântica forest to nearby source plantation BA4sB to nursery BA5nB to plantation SP1B. Genotypes identical to or closely related to those found in BA4sB and BA5nB were found throughout Bahia and São Paulo.

The two most commonly encountered genotypes in the study were CBCICA and BBBJDA, which were found in BA1sA and BA4sB, respectively, which were sources of cuttings that were brought to nurseries for rooting and distribution. Genotype BBBJDA was found also in cuttings of clone B that were being rooted at nursery BA5nB. Although the CBCICA genotype was only recovered from eucalyptus clone A, genotype BBBJDA was found in five different eucalyptus clones, perhaps spread to other eucalyptus clones on contaminated tools (Giraldo,

1957; Rossetto & Ribeiro, 1990; CABI 2005). Once infected cutting are brought to a nursery, the pathogen could be easily spread to other eucalyptus clones on scissors and other equipment. Also, plantations where cuttings are collected may have substantial tree-to-tree spread on tools used to harvest the cuttings. In each of the populations from plantations that were earlier a source of cuttings (BA1sA, BA4sB, and MG1sC), a third to a half of the isolates sampled were of a single genotype. Of all the plantations sampled, a plantation used as a source of cuttings, MG1sC, had the highest incidence of disease, 50%, perhaps due to repeatedly entering the plantation for collection of cuttings and spreading of the pathogen on cutting tools.

Most of the São Paulo genotypes were identical or closely related to the Bahia genotypes found in BA4sB or BA5nB, and these appear to be the result of introductions by humans on contaminated tools, or more likely, on plant propagative material (CABI 2005; Thorpe et al., 2005). Ferreira (2004) observed asymptomatic cuttings of eucalyptus that were infected with *C. fimbriata* and suggested the dissemination of the pathogen to new areas in contaminated cuttings. Genotypes of *C. fimbriata* have been recently found in *Eucalyptus* plantations in Uruguay and Africa (add the other reference from for Uruguay- Barnes et al.?; Roux et al., 2000), and the ITS sequences of those isolates are similar to those of eucalyptus isolates from Bahia (Harrington, unpublished).

The pathogen was first observed on *Eucalyptus* spp. (eucalyptus) in the south of Bahia (Ferreira *et al*., 1999) on only a few clones, especially on clone B, and then the disease was later recognized in Minas Gerais, Maranhão, São Paulo, and Mato Grosso do Sul (AC Alfenas, unpublished). Populations of *C. fimbriata* on eucalyptus in Bahia and Minas Gerais appear to be natural (Ferreira et al., submitted), but populations of *C. fimbriata* on eucalyptus in São Paulo and elsewhere may have arisen through human activity. Care should be taken not to move the fungus to new areas on contaminated equipment and in infected, symptomless cuttings.

The disease on eucalyptus may be harder to manage in Minas Gerais than elsewhere because there appears to be abundant natural inoculum in the Cerrado forest type, at least in some regions of Minas Gerais. But in all locations it is important that the source of eucalyptus cuttings for rooting be collected from plants free of *C. fimbriata*. Besides using uninfected planting stock, cleaning of tools should be practiced after working with infected plants (Harrington, 2000).

Resistance to particular genotypes or populations of *C. fimbriata* (Zauza, et al., 2004; Alfenas & Ferreira, 2008) may be important management tools where the fungus is already established in the soil.

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Table 1 – Description of the plantations, disease incidence, and genetic diversity of populations of *Ceratocystis fimbriata* based on six microsatellite loci.

Populations	City.State	Vegetation before eucalyptus	Number of Rotations in Eucalyptus	Age of Plantation at time of sampling (months)	Clone of eucalyptus	Disease Incidence (%)	No. of isolates sampled	No of Genotypes	Stoddart & Taylor's genotypic diversity (G)	Nei's gene diversity (*H*)	
										All isolates	Clone corrected[a]
BA1s[b]A[d]	Eunápolis., Bahia	Small farm	First	84	43	14.8	31	14	6.36 (5.15-7.87)[f]	0.3836	0.4932
BA2A	Eunápolis., Bahia	Pasture	First	17	43	2.3	7	2	1.32 (1.23-1.48)	0.018	0.1667
BA3A	Eunápolis., Bahia	Pasture	First	19	43	3.8	7	4	2.58 (2.21-3.01)	0.2857	0.3333
BA4sB	Teixeiras de Freitas, Bahia	Pasture	Second	Unkown	1172	ND[e]	27	13	5.10 (4.00-6.67)	0.3356	0.428
BA5n[c]B	Teixeiras de Freitas, Bahia	Nursery	Nursery	Nursery	1172	ND	4	3	2.67 (2.39-2.88)	0.2292	0.2593
BA6D	Caravelas, Bahia	Pasture	Second	12	1501	13.3	6	1	1	0	0
BA7E	Caravelas, Bahia	Unknown	Third	10	6011	1.3	6	4	3.60 (3.33-3.89)	0.4352	0.4583
BA8H	Eunápolis, Bahia	Pasture with woody regeneration	Second	40	1028	39.8	8	6	4.56 (3.92-5.26)	0.3958	0.4074
BA9H	Itabela, Bahia	Pasture with woody regeneration	Second	40	1028	48.9	7	4	3.26 (2.94-3.63)	0.3265	0.3333
	All Bahia populations						103	39	12.5 (10.00-16.66)	0.5526	0.5593
SP1B	Angatuba, São Paulo	Pasture/Citrus	Fourth	48	1172	ND	6	3	2.00 (1.74-2.32)	0.1389	0.2222

SP2I	Lençóis Paulistas	*Unkown	Unkown	Unkown	H13	ND	6	4	3.60 (3.86-3.90)	0.3958	0.4167
SP3J	Lençóis Paulistas	*Unkown	Unkown	Unkown	TC30	ND	6	4	3.00 (2.58-3.43)	0.2407	0.25
	All São Paulo populations						18	7	4.34 (3.70-5.26)	0.3323	0.3338
MG1sC	Curvelo, Minas Gerais	Cerrado Forest	Unkown	Unkown	1288	50.1	6	4	3.00 (2.58-3.43)	0.4537	0.5208
MG2C	Curvelo, Minas Gerais	Cerrado Forest	Unkown	Unkown	1288	15.6	12	12	12.00 (11.90-12.10)	0.6145	0.6145
MG3F	Paracatu Minas Gerais	Agroforestry/Cerrado	Fourth	120	8B	ND	6	5	4.50 (4.03-4.83)	0.5741	0.6
MG4F	João Pinheiro, Minas Gerais	Agroforestry/Cerrado	First	75	8B	ND	7	5	3.77 (3.20-4.32)	0.4352	0.4267
MG5G	Paraopeba, Minas Gerais	Cerrado	First	24	VM3	8	6	5	4.50 (4.02-4.85)	0.4762	0.5067
MG6G	João Pinheiro, Minas Gerais	Cerrado	First	29	VM3	ND	6	5	4.50 (4.02-4.85)	0.4537	0.4933
MG7C	Buritizeiro, Minas Gerais	Pinus/Cerrado	First	37	1172	ND	6	6	5.99 (5.98-6.00)	0.5093	0.5093
MG8C	Buritizeiro, Minas Gerais	Pinus/Cerrado	First	35	1172	ND	7	3	2.88 (2.67-2.99)	0.4762	0.4815
	All Minas Gerais populations						56	40	32.25 (31.25-33.33)	0.6732	0.6793

All populations	177	79	25.25 (20.83-33.33)	0.6302	0.6709

[a]Clone correction removed isolates that had genotypes identical to other isolates from the same site.
[b] Source of cuttings for clonal propagation of *Eucalyptus* spp.
[c] Root cuttings from BA4sB.
[d] Clone of *Eucalyptus* spp.
[e] Not determined.
[f] Confidence interval (95%) based on bootstrapping.

Table 2. Comparison of genotypic diversity and Nei's gene diversity in five populations of *Ceratocystis fimbriata* from eucalyptus plantations using six highly polymorphic microsatellite loci vs. 15 microsatellite loci

Populations	No of isolates sampled	Six most polymorphic loci			All (15) microsatellite loci		
			Average Gene Diversity (H)			Average Gene Diversity (H)	
		No. of genotypes	All	Clone corrected	No. of genotypes	All	Clone corrected
BA1sA	26	12	0.3792	0.4699	14	0.2128	0.2476
BA7E	6	4	0.4352	0.4583	4	0.2296	0.2400
MG1sC	6	4	0.4537	0.5208	4	0.2185	0.2333
MG2C	12	12	0.6145	0.6145	12	0.3004	0.3004
MG5G	6	5	0.5741	0.6000	6	0.2778	0.2778
MG6G	6	5	0.4352	0.4267	5	0.2407	0.2347
All	62	40	0.6486	0.6714	43	0.3216	0.3616

Table 3. Genotypes of *Ceratocystis fimbriata* from eucalyptus found in more than one population.

Genotypes	Number of Isolates	Populations (number of isolates)
CBCICA	20	BA1sA (10), BA2A (6), BA3A (4)
BBCIDA	2	BA1sA (1), BA2A (1)
BCCFDA	4	BA1sA (1), BA9H (3)
BCBJEA	2	BA1sA (1), SP1B (1)
BBBJDA	20	BA4sB (2), BA5nB (2), BA6D (6), BA8H (1), BA9H (2), SP1B (4), SP2I (2), SP3J (1)
BCBJDA	12	BA4sB (11), BA1sA (1)
BABKEA	3	BA4sB (1), BA5nB (1), MG2C (1)
BBBGDA	3	BA5nB (1), SP1B (1), SP2I (1)
ABCKEA	2	BA4sB (1), MG6G (1)
BABGDA	2	BA8H (1), BA9H (1)
ABBGDA	9	BA7E (2), BA8H (3), SP2I (1), SP3J (3)
BCCJEF	2	SP2I (1), MG2C (1)
ABBJEE	2	MG1sC (1), MG2C (1)
BCBKGF	4	MG1sC (3), MG2C (1)
BCBKAI	2	MG4F (1), MG6G (1)
ACCLEI	2	MG3F (1), MG7C (1)
ECCEIJ	2	MG7C (1), MG8C (1)

Table 4. Average genotypic diversity and gene diversity based on six microsatellite loci of *Ceratocystis fimbriata* populations found in eucalyptus plantations planted on four different previous vegetation types.

Vegetation before planting eucalyptus	Plantations	Average Stoddart & Taylor's genotypic diversity (G) of the populations	Average population gene diversity (H)	
			All isolates	Clone correct
Pasture	**BA2A, BA3A, BA4sB, BA6D, SP1B**	2.40	0.1556	0.2300
Pasture with woody regeneration	**BA8H, BA9H**	3.91	0.3611	0.3703
Small Farm	**BA1sA**	6.36	0.3836	0.4932
Cerrado Forest	**MG1sC, MG2C, MG3F, MG4F, MG5G, MG6G, MG7C, MG8C**	5.14	0.4991	0.5191

Table 5 Analysis of molecular variance (AMOVA) of *Ceratocystis fimbriata* populations on eucalyptus in six regions in Minas Gerais, Brazil based on six microsatellite loci.

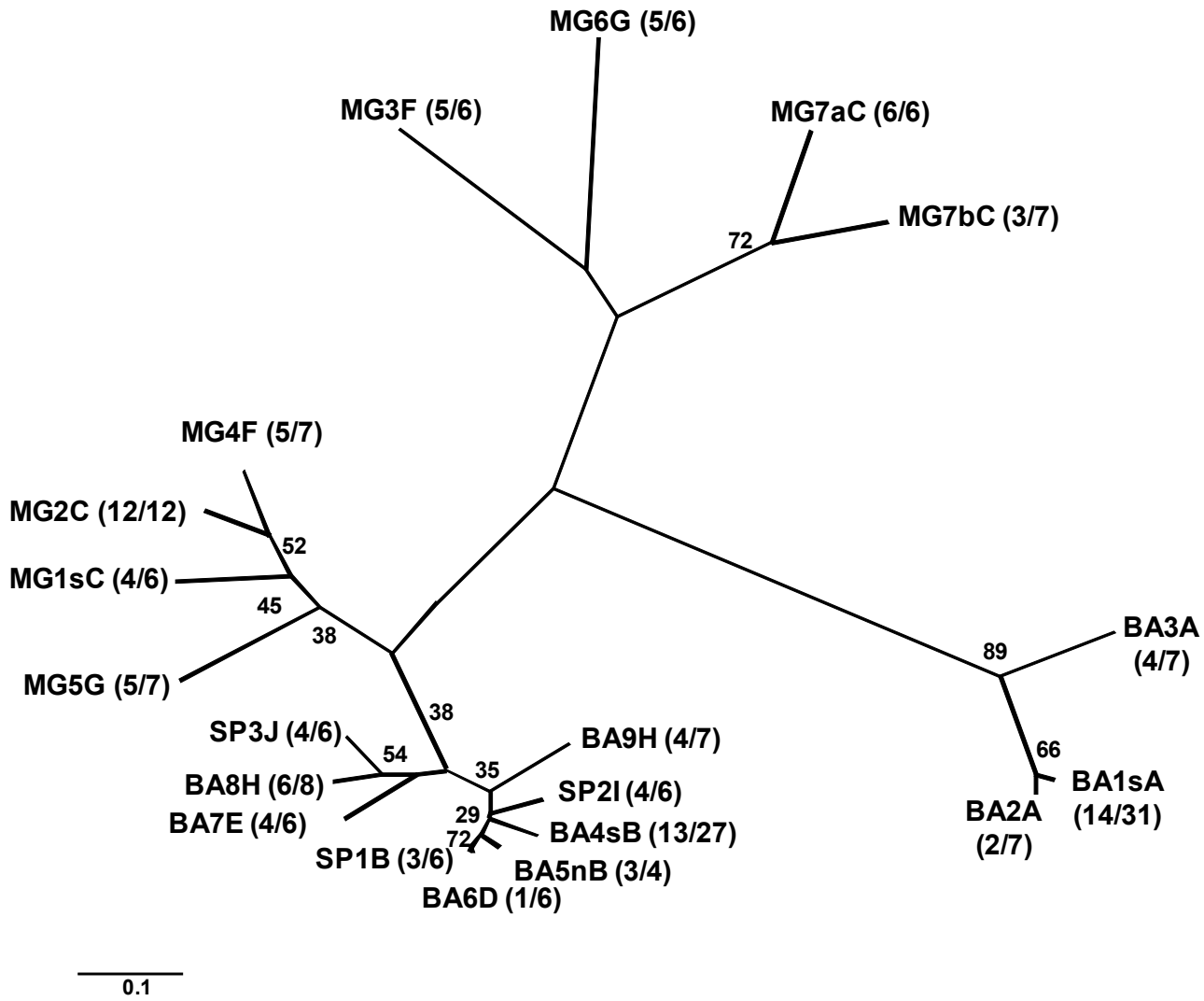
Source of variation	df	Sum of squared deviations	Variance components	Proportion of variance components (%)	p[a]
Among regions[b]	5	22.995	0.277	13.17	< 0.001
Among populations within regions	2	4.321	0.054	2.54	0.004
Within populations	48	85.167	1.774	84.29	0.005
Total	55	112.482	2.105		

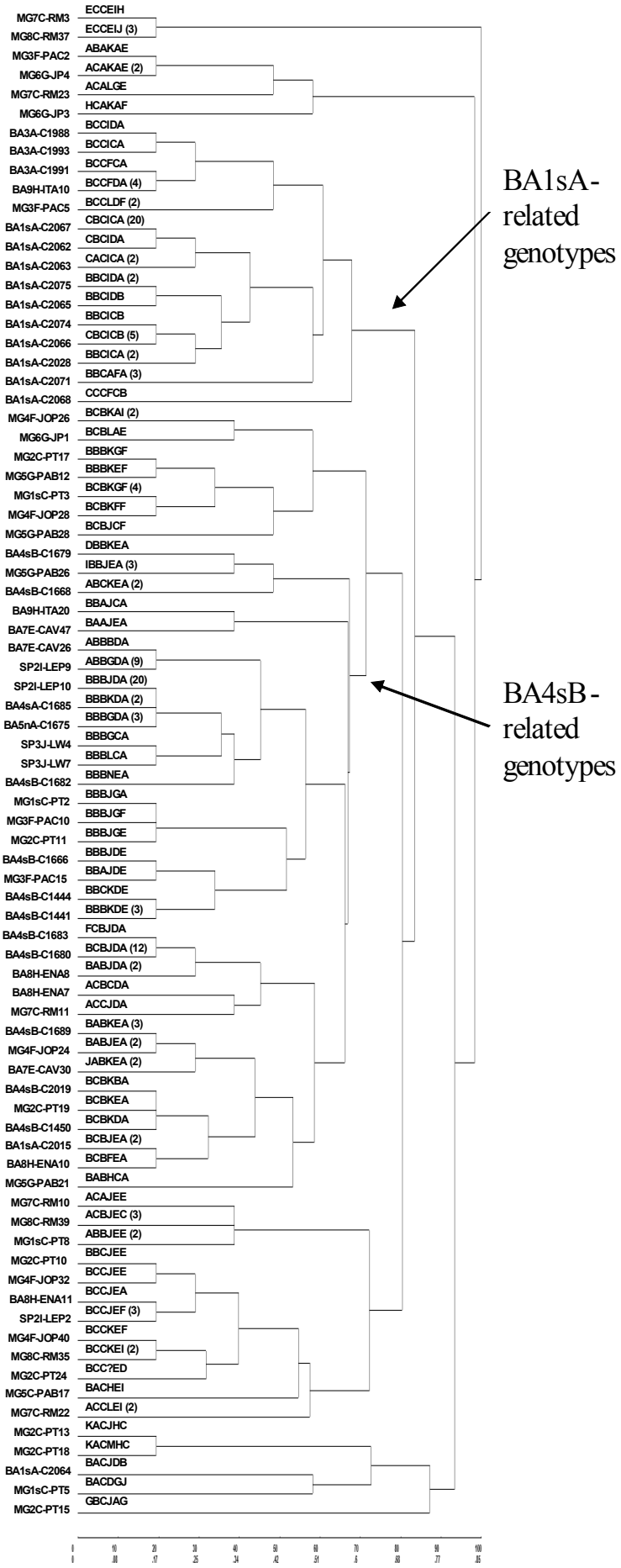
[a]The *p* value is for the null hypothesis that there is no significant variation at that level based on 1023 permutations.
[b] The populations were distributed in six regions in Minas Gerais: (1) MG1sC, MG2C; (2) MG3F; (3) MG4F; (4) MG5G; (5) MG6G; (6) MG7C, MG8C.

Figure Legend

Figure 1 Dendrogram of 20 *Ceratocystis fimbriata* eucalyptus populations generated by UPGMA (unweighted pair group method, arithmetic mean) based on allele frequencies of six microsatellite loci. Bootstrap values are shown alongside the branches. Populations are designated by state (two-letter abbreviation), location number, and clone of eucalyptus (capital letter). A nursery population is designated with a "n." Two populations that were used as a source for rooted cuttings for that clone are designated with a "s." The number of genotypes and isolates from each population are in parenthesis.

Figure 2 Dendrogram of 79 genotypes of *Ceratocystis fimbriata* from Minas Gerais, Bahia, and São Paulo obtained from the complement of the similarity coefficient (DICE 1945) generated by UPGMA (unweighted pair group method, arithmetic mean). Each genotype is given above the branch based on the respective alleles (each letter represents a unique allele) of six microsatellite loci. The isolate number ('C' number if stored at Iowa State University) of a representative strain of the genotype at each population follows the population number. If more than one isolate from a population had that genotype, the number of isolates follows in parentheses. Genotypes found in more than one population are shown in Table 3.


MG6G (5/6)
MG3F (5/6)
MG7aC (6/6)
MG7bC (3/7)
72
MG4F (5/7)
MG2C (12/12)
52
MG1sC (4/6)
45
38
MG5G (5/7)
38
89
BA3A
(4/7)
SP3J (4/6)
54
BA9H (4/7)
66
BA8H (6/8)
35
SP2I (4/6)
BA1sA
(14/31)
BA7E (4/6)
29
BA4sB (13/27)
BA2A
(2/7)
72
SP1B (3/6)
BA5nB (3/4)
BA6D (1/6)
0.1


MG7C-RM3 ECCEIH
MG8C-RM37 ECCEIJ (3)
MG3F-PAC2 ABAKAE
MG6G-JP4 ACAKAE (2)
MG7C-RM23 ACALGE
MG6G-JP3 HCAKAF
BA3A-C1988 BCCIDA
BA3A-C1993 BCCICA
BA3A-C1991 BCCFCA
BA9H-ITA10 BCCFDA (4)
MG3F-PAC5 BCCLDF (2)
BA1sA-C2067 CBCICA (20)
BA1sA-C2062 CBCIDA
BA1sA-C2063 CACICA (2)
BA1sA-C2075 BBCIDA (2)
BA1sA-C2065 BBCIDB
BA1sA-C2074 BBCICB
BA1sA-C2066 CBCICB (5)
BA1sA-C2028 BBCICA (2)
BA1sA-C2071 BBCAFA (3)
BA1sA-C2068 CCCFCB
MG4F-JOP26 BCBKAI (2)
MG6G-JP1 BCBLAE
MG2C-PT17 BBBKGF
MG5G-PAB12 BBBKEF
MG1sC-PT3 BCBKGF (4)
MG4F-JOP28 BCBKFF
MG5G-PAB28 BCBJCF
BA4sB-C1679 DBBKEA
MG5G-PAB26 IBBJEA (3)
BA4sB-C1668 ABCKEA (2)
BA9H-ITA20 BBAJCA
BA7E-CAV47 BAAJEA
BA7E-CAV26 ABBBDA
SP2I-LEP9 ABBGDA (9)
SP2I-LEP10 BBBJDA (20)
BA4sA-C1685 BBBKDA (2)
BA5nA-C1675 BBBGDA (3)
SP3J-LW4 BBBGCA
SP3J-LW7 BBBLCA
BA4sB-C1682 BBBNEA
MG1sC-PT2 BBBJGA
MG3F-PAC10 BBBJGF
MG2C-PT11 BBBJGE
BA4sB-C1666 BBBJDE
MG3F-PAC15 BBAJDE
BA4sB-C1444 BBCKDE
BA4sB-C1441 BBBKDE (3)
BA4sB-C1683 FCBJDA
BA4sB-C1680 BCBJDA (12)
BA8H-ENA8 BABJDA (2)
BA8H-ENA7 ACBCDA
MG7C-RM11 ACCJDA
BA4sB-C1689 BABKEA (3)
MG4F-JOP24 BABJEA (2)
BA7E-CAV30 JABKEA (2)
BA4sB-C2019 BCBKBA
MG2C-PT19 BCBKEA
BA4sB-C1450 BCBKDA
BA1sA-C2015 BCBJEA (2)
BA8H-ENA10 BCBFEA
MG5G-PAB21 BABHCA
MG7C-RM10 ACAJEE
MG8C-RM39 ACBJEC (3)
MG1sC-PT8 ABBJEE (2)
MG2C-PT10 BBCJEE
MG4F-JOP32 BCCJEE
BA8H-ENA11 BCCJEA
SP2I-LEP2 BCCJEF (3)
MG4F-JOP40 BCCKEF
MG8C-RM35 BCCKEI (2)
MG2C-PT24 BCC?ED
MG5C-PAB17 BACHEI
MG7C-RM22 ACCLEI (2)
MG2C-PT13 KACJHC
MG2C-PT18 KACMHC
BA1sA-C2064 BACJDB
MG1sC-PT5 BACDGJ
MG2C-PT15 GBCJAG
BA1sA-C1sA-related genotypes
BA4sB-C1sB-related genotypes

ARTIGO 3

Padrão espaço-temporal da murcha-de-ceratocystis em plantios clonais de eucalipto

Resumo

Os mecanismos potenciais de dispersão de doenças no campo e seus efeitos sobre os componentes de desenvolvimento de epidemias estão intimamente ligados à estrutura genética de populações; os processos de disseminação de doenças têm efeitos principais nos componentes de distribuição espacial e temporal do desenvolvimento de epidemias. Embora a murcha-de-ceratocystis tenha grande impacto na produção de madeira, inexistem estudos epidemiológicos da doença em condições naturais de infecção. Este estudo teve por objetivos estudar o progresso da doença em plantios clonais de eucalipto, caracterizar o padrão espacial em áreas de coleta de brotos e analisar a dinâmica espacial da doença ao longo do tempo, bem como avaliar o uso de ferramentas no incremento na incidência da murcha-de-ceratocystis no campo. A partir do mapeamento das árvores assintomáticas e sintomáticas, avaliou-se o padrão espacial de 35 parcelas em talhões clonais de eucalipto, e quatro destas parcelas corresponderam a áreas de coleta de brotações para enraizamento. Além disso, avaliou-se também o progresso temporal de um plantio clonal de eucalipto na região de Bocaiúva-MG. Foram feitos três tipos de análises: 'ordinary runs', autocorrelação e distribuições de frequências. Das 35 parcelas analisadas, 25 apresentaram padrão espacial agregado. Na área de coleta de brotações da Bahia, observou-se padrão espacial aleatório. Em contrapartida, todas as parcelas (BA2D, BA3D, BA4D e BA5D) com mudas provenientes da área de produção de brotações (BA1sD) mostraram forte agregação dentro da linha de plantio pela análise de autocorrelação espacial, bem como pelas análises de 'ordinary runs'e ajuste da distribuição betabinomial. Nas áreas de coleta de brotações de Minas Gerais, observou-se alta incidência da doença (variando de 22,6 a 50,5%) e forte agregação na linha e entrelinha de plantio. Quanto ao padrão temporal da doença, o modelo monomolecular linearizado foi o que melhor se ajustou com menor QMR (0,0074), maior coeficiente de determinação (0,71) e ausência de

tendências indesejáveis no gráfico de resíduos. O valor k (taxa de progresso) da murcha-de-ceratocystis foi de 0,0004/dia. A predominância do padrão espacial agregado de árvores doentes em algumas áreas indica que mudas infectadas podem ter influenciado o padrão da doença. Entretanto, para a maioria das áreas, sobretudo as localizadas no Estado de Minas Gerais, a hipótese mais provável é que as infecções ocorreram por inóculo primário presente no solo. Desse modo, com este estudo é possível concluir que a maior parte das áreas apresentaram padrão agregado de distribuição da doença no campo, e a baixa taxa de progresso da doença (comum para doenças poliéticas) sugere que o patógeno está presente no solo em algumas áreas. O padrão espacial pode ter sido influenciado pela utilização de mudas contaminadas das áreas de coleta de brotos para as áreas de plantio convencional; essas as áreas apresentaram maior incidência, provavelmente em decorrência da transmissão da doença pelas ferramentas utilizadas durante a coleta de brotos para enraizamento.

Introdução

A maioria dos estudos epidemiológicos com espécies florestais é incipiente, pois em geral pouco se sabe sobre as condições do ambiente favoráveis para o desenvolvimento da doença. Para que se conheça um patossistema e se obtenham informações suficientes para traçar estratégias de controle, os primeiros estudos a serem realizados devem ser direcionados para a determinação da influência da temperatura e umidade favoráveis à infecção. Desse modo, a maior parte desses estudos no Brasil envolve apenas experimentos em casas de vegatação ou em viveiros florestais. No campo, os estudos estão direcionados a correlacionar variáveis climáticas com severidade e incidência das doenças. Isso está relacionado à dificuldade de avaliar o desenvolvimento da doença por longos períodos. Contudo, quando uma espécie arbórea é plantada em larga escala e a doença compromete a produção, há também o surgimento do interesse em entender melhor o patossistema para traçar estratégias de controle, como ocorre atualmente com a eucaliptocultura brasileira. Atualmente, o Brasil possui quatro milhões de hectares plantados com a cultura do eucalipto (ABRAF, 2009; SBS, 2001). Com a extensão da eucaliptocultura no Brasil, houve aumento

na incidência de doenças causadas por patógenos acidentalmente introduzidos ou endêmicos. No campo, as doenças comuns são o cancro (*Crysoporthe cubensis*), ferrugem (*Puccinia psidii*), queima de folha e desfolha (*Cylindrocladium* spp., *Rhizoctonia* spp.); recentemente, consideradas emergentes, destacam-se a desfolha de *E. globulus* causada por *Teratosphaeria* sp. e as murchas vasculares causadas por *Ralstonia solanacearum* e *Ceratocystis fimbriata* (Alfenas & Ferreira, 2008).

A murcha-de-ceratocystis causada por *Ceratocystis fimbriata*, foco deste trabalho, é atualmente uma das doenças mais importantes na cultura do eucalipto. Foi observada primeiramente em eucalipto no Brasil em 1997, no Estado da Bahia (Ferreira *et al.*, 1999). É encontrada hoje em dia nos Estados do Espírito Santo, Mato Grosso do Sul, Minas Gerais, São Paulo (Alfenas *et al.*, 2004), Maranhão e Pará (A.C. Alfenas, 2009, informação pessoal). A sintomatologia da murcha-de-ceratocystis é caracterizada pela emissão de brotações epicórmicas ao longo do tronco, pela murcha e pelo secamento de ramos laterais ou apicais "die-back" ou morte da planta. Em cortes perpendiculares ao eixo principal da árvore, é possível visualizar a descoloração no sentido radial do lenho (Alfenas *et al.*, 2004). Alguns estudos mostraram que, em consequência da doença, pode haver redução de 58% no incremento volumétrico de madeira de um clone híbrido de *E. grandis x E. urophylla* (Ferreira *et al.*, 2007) e de 13,7% no rendimento depurado de celulose (Alfenas & Ferreira, 2008).

Embora a murcha-de-ceratocystis tenha grande impacto na produção de madeira, inexistem estudos epidemiológicos da doença em condições naturais de infecção. Dentre os estudos necessários, a determinação da dinâmica temporal e espacial é importante para a compreensão da epidemia em condições naturais. Os estudos da dinâmica espacial de doenças têm sido usados para detectar e fornecer informações sobre a quantidade de inóculo inicial (Campbell & Madden, 1990) no campo, os mecanismos de dispersão do patógeno, os efeitos das práticas culturais e biológicas (Vincelli & Lorbeer, 1987) e dos fatores ambientais sobre a infecção e disseminação dos patógenos e das doenças (Swallow, 1985; McRobertis *et al*., 1996; Hudges & Madden, 1997a, 1998). Já os estudos da dinâmica temporal são amplamente utilizados para determinar sobretudo a intensidade inicial e a taxa de progresso da doença, com o objetivo final de efetuar o manejo de epidemias (Bergamin Filho, 2005). Desse modo, em virtude

do desconhecimento sobre a dinâmica espaço-temporal da murcha-de-ceratocystis, este estudo teve por objetivos estudar o progresso da doença em plantios clonais de eucalipto, caracterizar o padrão espacial em áreas de coleta de brotos e analisar a dinâmica espacial da doença ao longo do tempo.

Material e Métodos

Coleta de dados

A incidência da doença foi visualmente monitorada em 35 parcelas de eucalipto localizadas nos Estados de Minas Gerais e Bahia, entre os anos de 2003 e 2008. O número de árvores avaliadas variou de 480 a 2.912. A incidência da doença foi avaliada pela inspeção visual (murcha parcial, total e morte) de todas as árvores em cada parcela. A localização de cada árvore sintomática e assintomática foi mapeada. Todas as parcelas avaliadas continham diferentes clones híbridos de *Eucalyptus* suscetíveis em diferentes idades. Foram avaliadas quatro parcelas (MG1sA, MG2sA, MG3sA e BA1sD), onde foi feita a coleta de brotações para enraizamento (plantios com intervenção pelo uso de tesouras de poda), e 31 parcelas com plantios convencionais sem intervenção (Tabela 1).

Padrão espacial da murcha-de-ceratocystis

A partir do mapeamento das árvores assintomáticas e sintomáticas, foram determinados os arranjos espaciais das plantas doentes em cada área experimental, por meio de três tipos de análises: "ordianry runs", autocorrelação espacial e distribuições de frequência. Para verificar se houve mudança no padrão espacial ao longo do tempo, nas parcelas BA6E e BA7E, Eunápolis-BA, e BA9F, Teixeiras de Freitas-BA (Tabela 1), foi feita mais de uma avaliação. Nas parcelas BA6E e BA7E foram realizadas duas avaliações em um intervalo de nove meses entre elas, e na parcela BA9F foram realizadas conco avaliações em intervalos que variaram de 11 a 12 meses. Nas demais parcelas, foi realizada apenas uma avaliação.

'Ordinary runs': Com a análise de 'ordinary runs' (Campbell & Madden, 1990) foi investigada a existência de agregação entre plantas doentes imediatamente adjacentes dentro das linhas, considerando-se linhas combinadas e entre linhas.

Valores de *Z* menores que -1,64 (α>0,05) indicaram rejeição à hipótese de nulidade (padrão aleatório), em favor da hipótese alternativa (padrão agregado).

Autocorrelação espacial: O padrão espacial de plantas doentes entre árvores foi examinado pela análise de autocorrelação espacial, utilizando-se o software LCOR2 (Gottwald et al., 1992). A localização espacial [x,y] de cada árvore e a posição das plantas foram utilizadas como dados de entrada para a análise de autocorrelação espacial em cada área e período de avaliação. Foram calculados os números de SL+ contíguos e descontíguos à posição lag [0,0], que forma um grupo discreto.

Distribuição binomial e betabinomial: Para ajuste de distribuição binomial e betabinomial, 45 parcelas foram divididos em *quadrats*. O número de árvores/*quadrat* e o número total de *quadrat* foram determinados pelo método de Greig-Smith (1952). A distribuição binomial foi analisada e comparada com a distribuição betabinomial. Quando houve um padrão aleatório de árvores doentes, a distribuição binomial foi a mais apropriada para representação da frequência de indivíduos doentes por unidade amostral. Nesse caso, todas as árvores mostraram uma probabilidade igual e constante de estarem doentes. Os estimadores ρ e θ foram calculados pelo método dos momentos, utilizando o software BBD (Madden & Hudges, 1994).

Progresso da murcha-de-ceratocystis

Estudou-se o progresso de murcha-de-ceratocystis em dois plantios clonais de eucalipto nas regiões de Bocaiúva-MG e de Teixeiras de Freitas-BA. Na região de Bocaiúva, avaliou-se um plantio com a idade de inicial de 24 meses. Para verificar o progresso da doença, foram avaliadas cinco parcelas de 100 plantas/parcela, totalizando 500 plantas em 18 avaliações, e o intervalo entre as avaliações variou de 15 a 178 dias. A idade do plantio após a última avaliação foi de 60 meses. Na região de Teixeiras de Freitas-BA, foram feitas cinco avaliações em cinco parcelas de 100 plantas/parcela, totalizando 500 plantas. Adicionalmente, comparou-se a precipitação média (mm) histórica com a precipitação no ano de 2007. A incidência foi determinada pela porcentagem de plantas com sintomas da doença em relação ao total de plantas avaliadas em cada parcela.

Curvas de progresso e de taxa absoluta de progresso da murcha-de-ceratocystis foram plotadas, utilizando-se os valores de incidência no tempo, em cada parcela. As taxas absolutas de progresso da doença foram calculadas conforme Campbell & Madden (1990). Os dados de intensidade das doenças em proporção (y), originais ou as formas linearizadas dos modelos exponencial [y = ln (y)], monomolecular [y = ln[1/(1-y)]], logístico [y = ln[y/(1-y)]] e de Gompertz [y = -ln[-ln(y)]] (Campbell & Madden, 1990), foram ajustados a modelos de regressão linear simples, tendo o tempo em meses como variável independente. Os melhores ajustes foram selecionados com base no maior coeficiente de determinação da regressão (R^2) para reciprocidade entre valores observados e previstos de incidência da doença, no menor quadrado médio do resíduo (QMR) e na ausência de tendências indesejáveis no gráfico de dispersão de resíduos. Utilizando os melhores ajustes, foi estimada a taxa de progresso da doença (k), determinada pelo parâmetro b da equação de regressão.

Resultados

Padrão espacial da murcha-de-ceratocystis em áreas de coleta de brotos para estaquia e plantios convencionais

Ordinary runs. O primeiro nível de hierarquia espacial examinado foi o padrão de plantas doentes imediatamente adjacentes, pela análise de "ordinary runs". As três parcelas (MG1sA, MG2sA e MG3sA) de coleta de brotações localizadas em Curvelo-MG (Figura 1) apresentaram padrão agregado de doença pela análise de "ordinary runs" ($Z < -1,64$; $P=0,05$). Das parcelas de plantio convencional, correspondentes à região de Curvelo-MG, apenas MG1aA e MG4B apresentaram padrão aleatório ($Z= -1,64$; $P=0,05$). Nas áreas de coleta de brotações, a incidência foi superior às áreas de plantios convencionais em até 40,8% (Tabela 2). Contariamente às parcelas localizadas em Curvelo-MG, a parcela de coleta de brotações BA1sD localizada em Eunápolis-BA (Figura 2) apresentou padrão aleatório de doença; as parcelas de plantio convencional (BA2D, BA3D, BA4D e BA5D) mostraram padrão agregado de doença ($Z < -1,64$; $P=0,05$). Todas as demais parcelas analisadas nas regiões de João Pinheiro-MG, Teixeiras de Freitas-BA e Eunápolis-BA apresentaram padrão agregado de doença ($Z < -1,64$; $P=0,05$), à exceção das parcelas BA8fF, BA8gF, BA8hF,

BA8iF e BA8jF, cujo padrão foi aleatório (Tabela 2). Na região de João Pinheiro-MG, houve padrão agregado da doença no campo; o número de *lags* contínuos variou de 3 a 47 unidades dentro da linha e de 1 a 6 unidades entre as linhas de plantio. Além disso, observou-se alta incidência da doença, com variação de 45,8 a 51,2% (Tabela 2).

Autocorrelação espacial. Com as análises de autocorrelação, encontraram-se os mesmos resultados obtidos pela análise de 'ordinary runs' (Tabela 2). No tocante à orientação, nas parcelas localizadas em Curvelo-MG houve predominância de agregação de plantas doentes dentro das linhas, embora também tenha sido detectada a agregação entre linhas e diagonal ao agregado principal com menor intensidade em quatro situações (Tabela 2). A dependência espacial contígua variou de 2 a 20 unidades ou *lags* dentro da linha e de 10 a 32 *lags* entrelinhas de plantio (Tabela 2). Nas parcelas de Eunápolis-BA, em relação à orientação, constatou-se também predominância de agregação dentro da linha de plantio. A dependência espacial contígua variou de 2 a 19 *lags* dentro da linha de plantio, de 1 a 3 entre linhas e de 1 a 2 na diagonal (Tabela 2). Quanto às demais parcelas analisadas nas regiões de João Pinheiro-MG, Teixeiras de Freitas-BA e Eunápolis-BA, obtiveram-se os mesmos resultados da análise de 'ordinary runs' (Tabela 2).

Distribuição binomial e betabinomial. O padrão de plantas doentes dentro de quadriláteros, pela análise da adequação à distribuição betabinomial, foi o terceiro nível de hierarquia espacial examinado. O tamanho ideal de *quadrats* foi 4x4 árvores pelo método de Greig-Smith (1952) para todas a parcelas, à exceção de BA6E e BA7E, cujo tamanho ideal foi de 2x2 árvores. Houve predominância do padrão agregado de plantas doentes, indicado pelos valores do teste qui-quadrado para os testes de homogeneidade de variâncias e adequação aos modelos beta-binomial e binomial. Adicionalmente, o resultado final na decisão-padrão foi o mesmo levando-se em consideração análises de 'ordinary runs', autocorrelação e ajuste de distribuições betabinomial e binomial (Tabela 3).

Padrão espacial ao longo do tempo. Ao analisar as parcelas BA6A, constatou-se que não houve mudança no padrão espacial da doença no intervalo entre as duas avaliações. Tanto pelas análises de 'ordinary runs' quanto por autocorrelação espacial e ajuste de distribuições, o padrão permaneceu agregado. O mesmo foi observado para a parcela BA9F, ou seja, não houve mudança no padrão espacial

da doença durante o período de avaliação. Contudo, houve aumento na incidência da doença de 12,9% entre a primeira e a última avaliação (Tabela 3).

Padrão temporal da murcha-de-ceratocystis

Para os dados de Bocaiúva-MG, entre os modelos utilizados, o monomolecular foi o que melhor se ajustou, com menor QMR (0,0074), maior coeficiente de determinação (0,71) e ausência de tendências indesejáveis no gráfico de resíduos (Tabela 5). O valor k (taxa de progresso) da murcha-de-ceratocystis foi de 0,0004/dia (Figura 3 e Tabela 3). Entretanto para os dados de Teixeiras de Freitas-BA, a análise de regressão não foi significativa entre os valores de incidência e o tempo analisado em nível de 5% de significância. Durante o período de avaliação houve redução acentuada da precipitação média em 2007, quando comparado a uma média histórica dos anos de 1996 a 2006. Concomitante com a redução na precipitação, houve incremento na incidência em 2,5% em 2007 e 10,4% em 2008 (Figura 4A). Na região de Teixeiras de Freitas (sul da Bahia), em fevereiro de 2007, o índice pluviométrico foi superior ao da média histórica da região, e a partir de março houve redução significativa da intensidade de chuvas (Figura 4B).

Discussão

O padrão espacial de doenças de plantas é influenciado pela interação de vários fatores, incluindo o ambiente, o homem e os vetores, dependendo do patossistema (Madden *et al.*, 1982). Embora descrições estatísticas não propiciem uma explicação dos mecanismos responsáveis pelos padrões espaciais (Campbell & Madden, 1990), padrões agregados de distribuição de plantas doentes sugerem que houve disseminação planta a planta ou que o inóculo estava espacialmente agregado, enquanto padrões aleatórios indicam que o patógeno não foi disperso ao longo da linha ou que, se disperso ao longo da linha, não induziu sintomas de maneira agregada (Campbell *et al.*, 1984). Quanto à disseminação, padrões agregados são mais caracteristicamente associados com fontes de inóculo próximas, ou mesmo, dentro de populações do hospedeiro, ao passo que padrões aleatórios geralmente resultam do inóculo, chegando a uma população de plantas de uma fonte distante ou de material de plantio infectado (Burdon, 1987). Na

cultura do eucalipto, o padrão espacial de doença no campo pode estar relacionado com o sistema de propagação clonal. Durante o processo de propagação clonal, mudas podem ser mantidas nas mesmas caixas desde o início do enraizamento até a expedição para plantio no campo. Assim, um lote de mudas infectadas pode ser distribuído em linhas durante o plantio, gerando um padrão agregado de doença no campo. Nesse caso, como observado neste trabalho, o maior número de parcelas com padrão agregado de distribuição dentro da linha de plantio pode estar relacionado com a disseminação da doença através de mudas contaminadas. Entretanto, quando a variabilidade genética de *C. fimbriata* é alta, como visto em muitas das áreas analisadas, pode significar que existem populações naturais do patógeno no solo (Ferreira *et al*., 2009, submetido).

Quando se comparam áreas de produção de brotos com áreas de plantio convencional sem intervenção de um mesmo clone, há diferenças entre as parcelas analisadas. Na área de coleta de brotações da Bahia, observou-se um padrão espacial aleatório. Em contrapartida, todas as parcelas (BA2D, BA3D, BA4D e BA5D) com mudas provenientes dessa área de produção de brotações (BA1sD) mostraram forte agregação dentro da linha de plantio pela análise de autocorrelação espacial, bem como pelas análises de 'ordinary runs' e ajuste da distribuição betabinomial. Em estudos prévios, foi comprovada alta variabilidade do fungo na área de coleta de brotações, menor nas áreas de plantio e presença dos mesmos genótipos do patógeno oriundos da área de coleta (Ferreira *et al*., 2009, submetido). Estes resultados permitem inferir que mudas infectadas com patógeno podem ter sido disseminadas das áreas de produção de brotações para as outras áreas. Nas áreas de coleta de brotações de Minas Gerais, observou-se alta incidência da doença (variando de 22,6 a 50,5%) em todas as áreas de coleta de broto e forte agregação na linha e entrelinha de plantio, sendo que uma das áreas apresentou 20 *lags* contíguos. Em termos biológicos, isso significa que uma árvore infectada tem alta capacidade para infectar 20 plantas dentro da linha de plantio. Entretanto, neste estudo, provavelmente, a forte agregação dentro da linha está mais relacionada à direção mais usada pelos trabalhadores que fazem coletas de brotações nessas áreas. Provavelmente, a alta incidência da doença nas áreas de coleta de brotos está relacionada com a disseminação pelas ferramentas de poda utilizadas na coleta das brotações. Em árvores (mangueira, eucalipto, figo, gmelina, etc), as infecções tipicamente ocorrem através de ferimentos recentes,

muitas vezes feitos por ferramentas contaminadas (Walter, 1946; Walter *et al*., 1952; Giraldo, 1957; Viégas, 1960; Rossetto & Ribeiro, 1990; Teviotdale & Harper, 1991). Por exemplo, em *Populus*, foi constatada a presença de *C. fimbriata* em estacas assintomáticas (Vujanovic *et al.*, 1999). A presença de *C. fimbriata* em mudas assintomáticas de eucalipto (Ferreira, 2004) também sugeriu a dispersão do patógeno pelo homem para várias outras regiões eucaliptocultoras.

A predominância do padrão espacial agregado de árvores doentes indica que mudas infectadas podem ter influenciado o padrão da doença. Contudo, para a maioria das áreas, sobretudo as localizadas no Estado de Minas Gerais, a hipótese mais provável é que as infecções ocorreram principalmente pelo inóculo primário presente no solo. Rossetto & Ribeiro (1990) comprovaram a infecção em *M. indica* pelo inóculo do solo. Adicionalmente, alguns autores discutem a dispersão do patógeno via contato radicular (Accordi, 1986, 1989) e ferimentos na raiz (Vigouroux *et al*., 1999; Vigouroux & Olivier, 2004). Outro mecanismo comum verificado na literatura é a dispersão do patógeno por disseminadores secundários, como cupins e coleobrocas do gênero *Scolytidae* em *Populus* (Hinds, 1972), *Prunus* (Moller *et al.*, 1969) e *M. indica* (Batista, 1960; Viégas, 1960). Sugere-se que coleobrocas adquirem inóculo de *C. fimbriata* e contribuem para a dispersão para plantas suscetíveis. Além disso, verificou-se que esporos de *C. fimbriata* podem ser carregados sobre os corpos de besouros *Ambrosia* e sobreviver pela passagem através do intestino do inseto (Iton, 1960; Crone, 1963). Essas coleobrocas pertencentes aos gêneros *Xyleborus* e *Hypocryphalus,* são atraídas pelas plantas doentes que produzem forte aroma devido à colonização do fungo (Goitia & Rosales, 2001; Wingfield & Robison, 2004). Foi observada a infecção de *Ipomoea* em ferimentos feitos por insetos e roedores (Clark & Moyer, 1988). Ferreira *et al.* (2006) inspecionaram plantas clonais de eucalipto afetadas pela murcha de *C. fimbriata* no Brasil e verificaram que somente duas apresentaram perfurações de inseto da família Platybodidae nas lesões de *Ceratocystis;* também não foram constatadas perfurações de insetos nas lesões novas, longitudinais, das plantas vizinhas. Desse modo, foi interpretado que a associação de insetos somente em lesões mais velhas é uma forma secundária ou não precursora da doença. Todavia, esses insetos, ao saírem das galerias do lenho infectado, podem transmitir endoconídios e clamidósporos do patógeno para outras plantas lenhosas com xilema alterado por fator abiótico ou biótico, como

acontece com outras doenças causadas por *C. fimbriata* em outras culturas (Sinclair *et al*., 1987; Wingfield *et al*., 1993; Baker *et al*., 2003). Além das coleobrocas, cupins podem agir como agentes secundários de disseminação da doença (Ferreira, 2005). Assim, embora os insetos possam ter algum papel na disseminação da doença, pouco contribuem para o seu aumento em condições de campo, como verificado na curva de progresso da doença.

As murchas vasculares são monocíclicas, ou seja, o inóculo é constante durante a estação de crescimento do hospedeiro ou, poliéticas, em que o inóculo presente no final de uma estação cujas condições são favoráveis será inóculo inicial (y_0) para a próxima estação favorável ao patógeno (Bergamin Filho *et al*.; 1995; Vale *et al.*, 2004; Agrios, 2005). Geralmente, o modelo que melhor se ajusta a esse tipo de doença é o monomolecular (Bergamin Filho *et al*., 1995). Neste estudo foi observada uma menor taxa (k=0,0004/dia) de progresso da doença, que é comparável à de outros patossistemas envolvendo murchas vasculares (Bergamin Filho *et al*., 1995). Vale *et al*. (2004) citam o exemplo de *C. fimbriata* associado à seca da mangueira, que na década de 1960 dizimou os plantios comerciais do cultivar Bourbon em Jardinópolis-SP.

Foi possível observar, neste estudo, a influência das condições climáticas sobre o incremento da incidência de murcha-de-ceratocystis no sul da Bahia. Em 2007, houve a seca de ponteiros generalizada no sul da Bahia, provavelmente decorrente de fatores abióticos. Análises das condições climáticas mostraram que o índice pluviométrico foi inferior aos registados nos anos anteriores (Alfenas *et al*., 2007). Nesse caso, é possível inferir que o déficit hídrico pode ter sido responsável pelo aumento da incidência da murcha-de-ceratocystis. Ferreira (2006) também observou o aparecimento da murcha-de-ceratocystis após um período de déficit hídrico no sul da Bahia no ano de 1997.

O plantio de materiais resistentes é a única forma de controle da murcha-de-ceratocystis utilizada atualmente (Alfenas et al., 2004). Entretanto, a compreensão dos processos epidemiológicos envolvidos podem auxiliar na tomada de decisão para adoção de medidas de controle da doença, sobretudo quando a doença já se encontra instalada na área de plantio. Assim, com este estudo é possível concluir que a maior parte das áreas apresenta padrão agregado de distribuição da doença no campo; baixa taxa de progresso da doença (comum para doenças poliéticas) sugere que o patógeno está presente no solo em algumas

áreas. Pode-se também concluir que o padrão espacial pode ter sido influenciado pela utilização de mudas contaminadas das áreas de coleta de brotos para as áreas de plantio convencional. As áreas de coleta de brotos apresentaram maior incidência da doença, provavelmente em decorrência da transmissão pelas ferramentas utilizadas durante a coleta de brotos.

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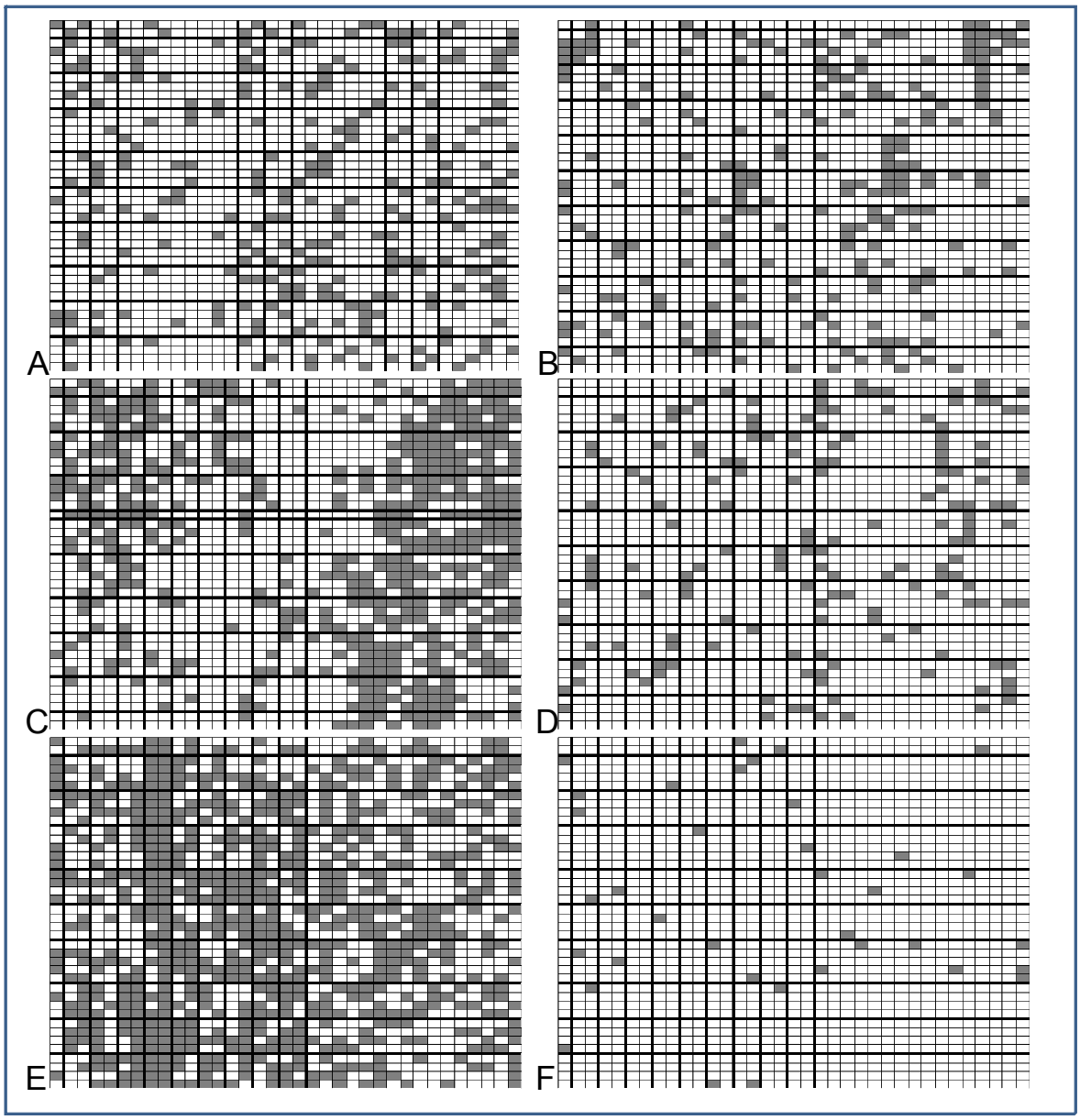

Figure 1 – Padrão espacial da murcha-de-ceratocystis em áreas de coleta de brotações e áreas de plantio convencional sem intervenção em Curvelo-MG, Brasil. A, C e E; Parcelas de coleta de brotações para enraizamento e B, D, e F; Parcelas de plantios sem intervenção.

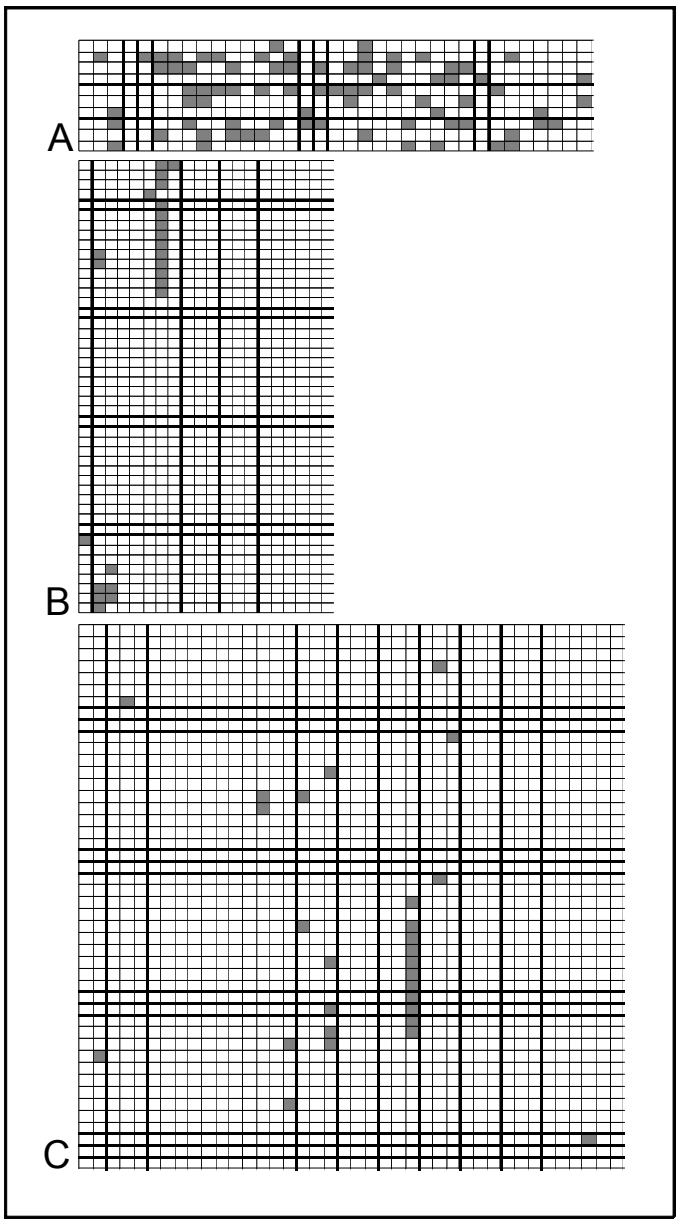

Figure 2 – Padrão espacial da murcha-de-ceratocystis em áreas de coleta de brotações e áreas de plantio convencional sem intervenção em Eunápolis-BA, Brazil. A; Parcela BA1sA, área de coleta de brotações, B e C; Parcelas BA4D e BA2D, áreas de plantio, cujas mudas foram originadas da parcela BA1sA.

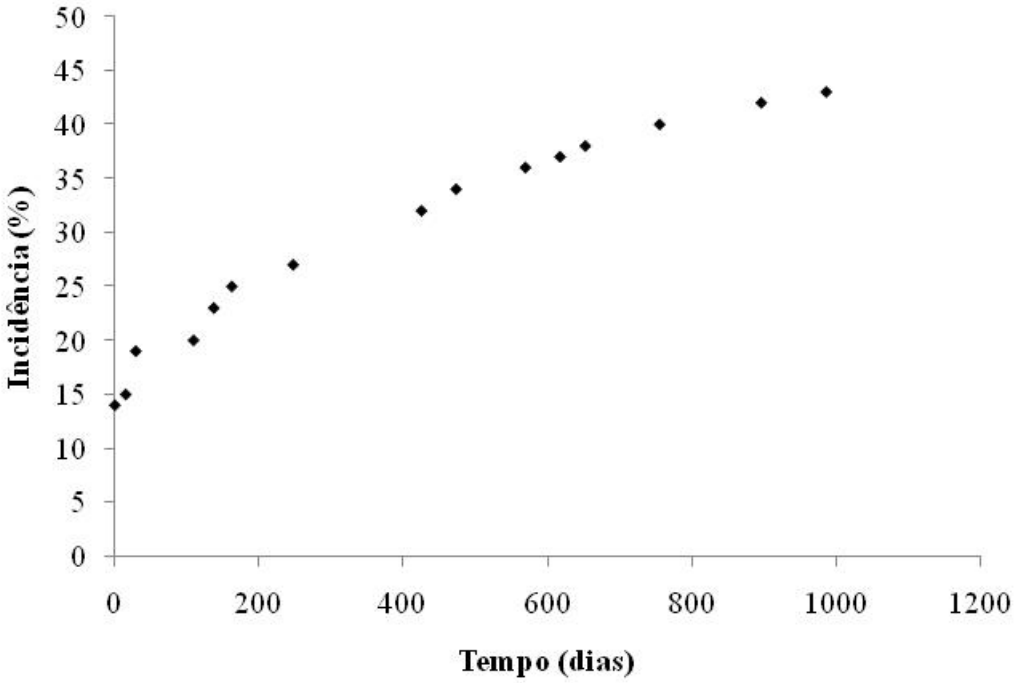

Figura 3 – Incremento médio da incidência da murcha-de-ceratocystis ao longo do tempo na região de Bocaiúva-MG.

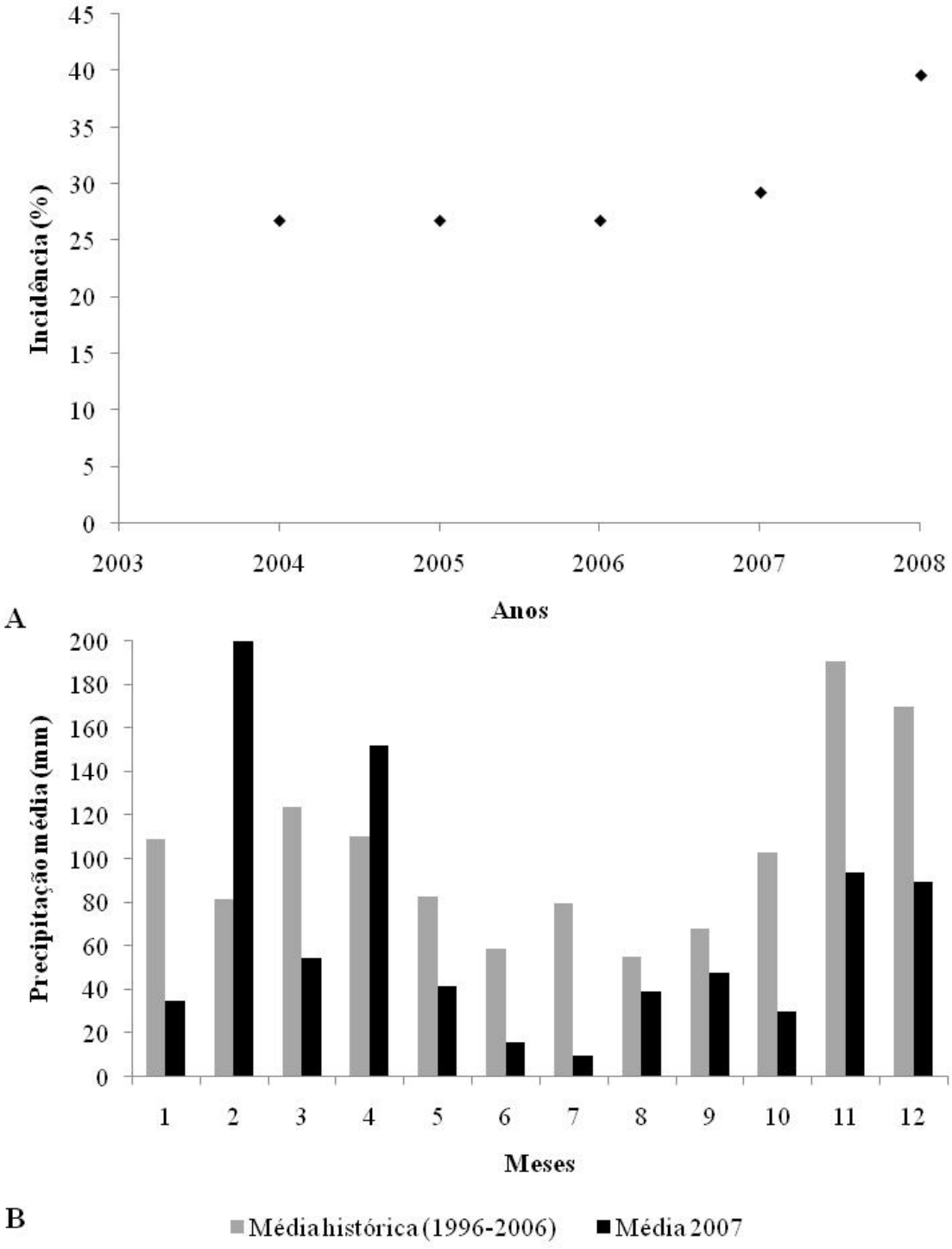

Figura 4 – A) Incremento médio da incidência da murcha-de-ceratocystis ao longo do tempo n região de Teixieras de Freitas –BA entre os anos de 2003 a 2008. B) Precipitqação média mensal histórica (1996-2006) e média mensal do ano de 2007.

Tabela 1 – Descrição das parcelas avaliadas quanto á incidência, padrão espacial e temporal da murcha-de-ceratocystis.

Parcelas	Cidade/Estado	Idade do plantio	Ciclos da cultua	Clone	Espaçamento	Número de árvores	Vegetação antes do eucalipto
MG[a]1[b]s[c]A[d]	Curvelo/MG	ND[a]	ND	1288	3x2	1600	Cerrado
MG1aA		ND	ND	1265	3x2	1600	Cerrado
MG2sA		ND	ND	1265	3x2	1600	Cerrado
MG2aA		ND	ND	1288	3x2	1600	Cerrado
MG3sA		ND	ND	1288	3x2	1600	Cerrado
MG3aA		ND	ND	1288	3x2	1600	Cerrado
MG4B		ND	ND	1288	3x2	1600	Cerrado
MG5B		ND	ND	1288	3x2	1600	Cerrado
MG6aC	João Pinheiro/MG	33	1	VM3	6x3x2	500	Cerrado
MG6bC		33	1	VM3	6x3x2	500	Cerrado
MG6cC		33	1	VM3	6x3x2	500	Cerrado
MG6dC		33	1	VM3	6x3x2	500	Cerrado
BA1sD	Eunápolis/BA	84	1	43	5x2.4	390	Pequena propriedade
BA2D		17	1	43	5x2.4	2912	Patagem
BA3D		19	1	43	5x2.4	1250	Pastagem
BA4D		18	1	43	5x2.4	1000	Pastagem
BA5D		18	1	43	5x2.4	1000	Pastagem
BA6E		40	2	1028	5x2.4	936	Pastagem em regeneração
BA7E		40	2	1028	5x2.4	676	Pastagem em regeneração
BA8aF	Teixeiras de Freitas/BA	48	1	1172	3x3	500	Pastagem
BA8bF		48	1	1172	3x3	500	Pastagem
BA8cF		48	1	1172	3x3	500	Pastagem
BA8dF		48	1	1172	3x3	500	Pastagem
BA8eF		48	1	1172	3x3	500	Pastagem
BA8fF		48	1	1172	3x3	500	Pastagem
BA8gF		48	1	1172	3x3	500	Pastagem
BA8hF		48	1	1172	3x3	500	Pastagem

BA8iF	48	1	1172	3x3	500	Pastagem
BA8jF	48	1	1172	3x3	500	Pastagem
BA9F	23	2	1172	3x3	120	Pastagem
BA10aF	12	1	1172	3x3	2100	Pastagem
BA10bF	12	1	1172	3x3	2000	Pastagem
BA11F	13	1	1172	3x3	2000	Pastagem
BA12G	12	2	1501	3x3	1050	Pastagem
BA12H	10	3	6011	3x3	1188	ND

[a] As duas primeiras letras maiúsculas equivalem ao Estado onde as parcelas foram avaliadas.
[b] O número equivale as subáreas onde as parcelas foram alocadas.
[c] A letra minúscula equivale as subparcelas alocadas em áreas próximas, sendo que 'n' equivale a áreas de coleta de brotações.
[d] A última letra maiúscula equivale ao clone de eucalipto avaliado.
[e] ND, Não determinado.

Tabela 2 – Análises de incidência, 'ordinary runs' e autocorrelação em cinco áreas de coleta de brotações e oito plantios convencionais de eucalipto sem intervenção atacadas por *Ceratocystis fimbriata* nos estados de Minas Gerais e Bahia, Brasil

	'ordinary runs[a]				Aucorrelação espacial					
					Lags contíguos (+SL)			***Lags descontíguos (+SL)***		
Parcelas	No de linhas testadas	Dentro das linhas	No de linhas testadas	Entrelinhas	Dentro das linhas	Entrelinhas	Diagonal	Dentro das linhas	Entrelinhas	**Padrão espacial**
MG1aA	26	0.000	25	0.000	0	0	0	37*[b]	23*	Aleatório
MG1sA	40	0.025	40	0.000	12*	6*	3**	31**	9**	Agregado
MG2aA	40	0.150	39	0.025	2**	0	0	26**	0	Agregado
MG2sA	40	0.225	40	0.275	20*	7**	7**	26**	32**	Agregado
MG3aA	39	0.128	40	0.050	5**	1**	1**	17**	31**	Agregado
MG3sA	40	0.350	40	0.025	3**	0	1**	26*	15*	Agregado
MG4B	31	0.032	36	0.000	0	0	0	16**[c]	10**	Aleatório
MG5B	37	0.108	40	0.000	5**	1**	1**	32*	32**	Agregado
MG6aC	10	0.120	49	0.000	47**	3**	5*	0	0	Agregado
MG6bC	10	0.040	49	0.000	3*	1**	0	41*	3*	Agregado
MG6cC	10	0.160	43	0.000	11*	7*	6*	41*	0	Agregado
MG6dC	10	0.140	50	0.000	5*	1**	1**	33*	0	Agregado
BA1sD	35	0.000	10	0.000	0	0	0	0	8**	Aleatório
BA2D	7	0.429	28	0.107	11**	3**	2**	16**	8*	Agregado
BA3D	4	0.750	13	0.154	8**	0	0	12**	7**	Agregado
BA4D	6	0.500	23	0.000	12**	1**	1**	39*	4*	Agregado
BA5D	5	0.400	10	0.000	5**	1**	1**	0	0	Agregado
BA6E*	52	0.154	18	0.111	5**	2**	0	0	31**	Agregado
BA6E	52	0.192	18	0.000	4**	2**	0	0	31**	Agregado
BA7E	52	0.096	13	0.077	0	0	0	0	0	Aleatório
BA8aF	25	0.053	19	0.000	2*	0	0	4*	14*	Agregado

BA8bF	25	0.050	20	0.000	1**	0	1**	21*	0	Agregado
BA8cF	24	0.053	19	0.000	1**	0	0	6*	2*	Agregado
BA8dF	25	0.100	19	0.000	1**	0	0	13*	10**	Agregado
BA8eF	25	0.000	20	0.000	0	1**	0	7*	0	Agregado
BA8fF	25	0.000	20	0.000	0	0	0	19*	3**	Aleatório
BA8gF	25	0.000	20	0.000	0	0	0	16*	0	Aleatório
BA8hF	25	0.000	20	0.000	0	0	0	20*	2*	Aleatório
BA8iF	25	0.320	20	0.400	0	0	0	14*	14*	Aleatório
BA8jF	25	0.320	20	0.650	0	0	0	17*	6**	Aleatório
BA9F**	10	0.100	55	0.000	2**	1**	0	45*	0	Agregado
BA9F	10	0.100	55	0.000	2**	1**	0	45*	0	Agregado
BA9F	10	0.100	55	0.000	2**	1**	0	45*	0	Agregado
BA9F	10	0.200	56	0.000	2**	1**	0	29**	0	Agregado
BA9F	10	0.100	58	0.000	3*	1**	1**	52*	0	Agregado
BA10aF	36	0.000	44	0.023	0	0	0	45**	1**	Aleatório
BA10bF	26	0.115	42	0.048	2**	0	0	6**	4**	Agregado
BA11F	35	0.028	50	0.112	8**	2**	2**	45**	34**	Agregado
BA12G	9	0.111	10	0.000	0	2**	0	0	18*	Agregado
BA12H	35	0.086	33	0.091	2**	4**	1**	19*	13*	Agregado

[a] Valores mostrados para cada parcela em cada avaliação são a proporção dos números de linhas testadas com agregação significativa (P=0,05) dividida pelo número total de linhas testadas; [b] *P=0,05; [c] **P=0,01. * parcela avaliada em dois tempos e ** parcela avaliada em cinco tempos

Tabela 3 – Estatísticas de ajuste de distribuição betabinomial e binomial para determinar o tipo de padrão espacial da murcha-de-ceratocystis em quatro áreas de coleta de brotações e 31 áreas de plantios convencionais de eucalipto sem intervenção nos estados de Minas Gerais e Bahia, Brasil

Parcelas	Tamanho da amostra	Incidência (%)	Tamanho dos '*Quadrats*'	Parâmetros estimados[a]		Ajuste da distribuição betabinomial		Ajuste da distribuição binomial		Padrão espacial
				$\hat{p}$	$\hat{\theta}$	χ^{2b}	Pr>F	χ^{2}	Pr>F	
MG1aA	1600	2,70	16	0,03	0,01	1,51	0,00	0,53	0,47	Aleatório
MG1sA	1600	50,50	16	0,50	0,07	5,04	0,75	15,80	0,01	Agregado
MG2aA	1600	12,80	16	0,13	0,02	6,68	0,08	13,20	0,00	Agregado
MG2sA	1600	39,70	16	0,40	0,32	8,44	0,65	99,80	0,00	Agregado
MG3aA	1600	15,60	16	0,16	0,08	1,18	0,88	14,50	0,00	Agregado
MG3sA	1600	22,60	16	0,23	0,05	3,83	0,57	14,70	0,01	Agregado
MG4B	1600	5,70	16	0,06	0,00	-	-	0,39	0,83	Aleatório
MG5B	1600	12,30	16	0,12	0,01	5,85	0,12	9,18	0,03	Agregado
MG6aC	480	45,80	4	0,46	0,77	14,90	0,00	173,19	0,00	Agregado
MG6bC	480	51,70	4	0,52	0,22	0,47	0,79	25,81	0,00	Agregado
MG6cC	480	62,92	4	0,51	0,83	2,10	0,35	164,72	0,00	Agregado
MG6dC	480	46,87	4	0,47	0,50	3,72	0,15	82,60	0,00	Agregado
BA1sD	360	22,22	8	0,22	0,04	1,67	0,20	1,34	0,51	Aleatório
BA2D	2912	1,16	16	0,01	0,08	0,41	0,00	5,53	0,00	Agregado
BA3D	1152	1,39	16	0,01	0,13	0,00	0,00	3,47	0,00	Agregado
BA4D	960	1,50	4	0,01	2,45	0,00	0,00	5,68	0,00	Agregado
BA5D	960	2,91	4	0,03	0,32	1,67	0,00	2,83	0,00	Agregado
BA6E*	832	40,63	4	0,41	0,30	0,74	0,69	66,85	0,00	Agregado
BA6E	832	52,28	4	0,52	0,33	1,77	0,41	83,50	0,00	Agregado

BA7E	624	48,55	8	0,49	0,08	3,86	0,43	6,45	0,09	Agregado
BA8aF	480	24,17	16	0,24	0,01	2,87	0,03	0,53	0,53	Aleatório
BA8bF	480	18,75	16	0,19	0,09	0,38	0,54	7,63	0,10	Agregado
BA8cF	480	12,50	16	0,13	0,01	0,74	0,00	1,31	0,26	Aleatório
BA8dF	480	15,42	16	0,15	0,04	4,67	0,03	7,77	0,02	Agregado
BA8eF	480	17,08	16	0,17	0,00	-	-	2,38	0,30	Aleatório
BA8fF	480	27,50	16	0,26	0,00	-	-	3,63	0,16	Aleatório
BA8gF	480	19,79	16	0,20	0,00	-	-	0,67	0,71	Aleatório
BA8hF	480	22,29	16	0,22	0,00	-	-	1,79	0,41	Aleatório
BA8iF	480	27,17	16	0,29	0,00	-	-	1,15	0,56	Aleatório
BA8jF	480	36,46	16	0,36	0,00	-	-	0,51	0,77	Aleatório
BA9F**	480	26,70	16	0,27	0,08	0,26	0,61	2,37	0,30	Agregado
BA9F	480	26,70	16	0,27	0,08	0,26	0,61	2,37	0,30	Agregado
BA9F	480	26,70	16	0,27	0,08	0,26	0,61	2,37	0,30	Agregado
BA9F	480	29,20	16	0,29	0,07	2,53	0,11	1,64	0,44	Agregado
BA9F	480	39,60	16	0,40	0,08	1,94	0,16	1,64	0,13	Agregado
BA10aF	1920	4,17	4	0,04	0,01	0,42	0,00	0,00	0,00	Aleatório
BA10bF	1920	3,85	4	0,04	0,03	0,07	0,00	0,13	0,00	Aleatório
BA11F	1920	12,80	4	0,13	0,07	0,71	0,40	12,10	0,00	Agregado
BA12G	960	1,40	4	0,01	0,04	0,00	0,00	0,05	0,00	Aleatório
BA12H	1152	3,11	16	0,13	0,05	1,46	0,69	11,91	0,01	Agregado

[a]^p e ^θ são estimativas dos dados, [b]$\chi 2$ Teste qui-quadrado, sendo que o nível de significância é dado por Pr (convencionalmente, P>0,05 indica um ajuste aceitável). * parcela avaliada em dois tempos e ** parcela avaliada em cinco tempos

Tabela 4 – Comparação de modelos linearizados para descrever as taxas estimadas de progresso (k) da murcha-de-ceratocystis em Bocaiúva,. MG.

Modelos[a]	QMR[b]	R^{2c}	Variáveis			
			y_0		k (taxa)	
			Interseção	Erro padrão	Variável (y_1)	Erro padrão
Logaritmo	0,0773	0,6154	-1,7166	0,0445	0,0011	0,00009
Monomolecular	0,0074	0,7153	0,2032	0,0138	0,0004	0,00003
Logístico	0,1269	0,6527	-1,5134	0,0570	0,0015	0,00012
Gompertz	0,0341	0,6867	-0,5321	0,0296	0,0008	0,00006

[a] Logaritmo [y = ln (y)], Monomolecular [y = ln[1/(1-y)]], Logístico [y = ln[y/(1-y)]] e de Gompertz [y = -ln[-ln(y)]] (Campbell & Madden, 1990);

[b] Quadrado Médio do Resíduo;

[c] Coeficiente de determinação.

CONCLUSÕES GERAIS

- Apesar das introduções humanas, os estudos de "mating type"e análises genéticas sugeriram que populações locais de *C. fimbriata* formam uma única espécie biológica.
- Houve disseminação da doença por mudas contaminadas, visto que muitos do genótipos obtidos foram encontrados em diferentes regiões brasileiras.
- Não houve estruturação das populações de *C. fimbriata* em eucalipto, possilvemente devido ao trânsito de material contaminado entre as áreas.
- As áreas com vegetação do tipo Cerrado anteriormente ao plantio de eucalipto apresentaram maior variabilidade que as demais áreas analisadas.
- A maior parte das áreas apresenta padrão agregado de distribuição da doença no campo, e a baixa taxa de progresso da doença (comum para doenças poliéticas) sugere que o patógeno está presente no solo em algumas áreas.
- O padrão espacial pode ter sido influenciado pela utilização de mudas contaminadas das áreas de coleta de brotos para as áreas de plantio convencional.
- As áreas de coleta de brotos apresentaram maior incidência da doença, provavelmente em decorrência da transmissão pelas ferramentas utilizadas durante a coleta de brotos.