ANDRÉ GOMES COELHO DE SOUZA

INFECÇÃO DE FOLHAS DE CAFEEIRO POR C*ercospora coffeicola*, IDENTIFICAÇÃO DE GENES PARA PRODUÇÃO E AUTO-RESISTÊNCIA À CERCOSPORINA E PROGRESSO DA CERCOSPORIOSE EM SISTEMAS DE CULTIVO ORGÂNICO E CONVENCIONAL

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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BIOGRAFIA

André Gomes Coelho de Souza, filho de José Leandro de Souza e Elenita Gomes Coelho de Souza, nasceu em 29 de maio de 1979, em Juiz de Fora, Estado de Minas Gerais.

Em 2000, ingressou no curso de Agronomia da Universidade Federal de Viçosa, situada em Viçosa-MG, onde graduou-se em janeiro de 2005.

Em março do mesmo ano, iniciou o curso de Mestrado em Fitopatologia na Universidade Federal de Viçosa (MG) e terminou-o em fevereiro de 2007.

Em março do ano de 2007, ingressou-se no curso de Doutorado em Fitopatologia na Universidade Federal de Viçosa. Foi contemplado com uma bolsa de doutorado sanduíche pelo CNPq para realizar atividades de pesquisa na North Carolina State University, Raleigh-NC, EUA, no período de setembro de 2009 a agosto de 2010.

SUMÁRIO

RESUMO

SOUZA, André Gomes Coelho de, D. Sc., Universidade Federal de Viçosa, Fevereiro de 2011. **Infecção de folhas de cafeeiro por *Cercospora coffeicola*, identificação de genes para produção e auto-resistência à cercosporina e progresso da cercosporiose em sistemas de cultivo orgânico e convencional.** Orientador: Luiz Antonio Maffia. Co-Orientadores: Eduardo Seiti Gomide Mizubuti e Fabyano Fonseca e Silva.

Dentre as doenças do cafeeiro, a cercosporiose, causada por *Cercospora coffeicola*, vem assumindo importância crescente ao longo dos anos. Em vista da importância e da escassez de conhecimento sobre a doença, objetivou-se: i- estudar o processo de infecção de *C. coffeicola* em folhas ; ii- identificar se os genes associados à produção da cercosporina e auto-resistência em outras espécies de *Cercospora* ocorrem em *C. coffeicola*; iii- o progresso da cercosporiose em lavouras orgânica sombreada (LOS), orgânica (LO) e convencional (LC). Para estudar o processo de infecção, inoculou-se suspensão de conídios de *C. coffeicola* nas faces adaxial e abaxial de folhas de cafeeiros, e se coletaram amostras após 4 a 168 h e ao 35º dia da inoculação. Observou-se tropismo dos tubos germinativos em direção aos estômatos, onde ocorreram tentativas de penetração. Não se observou a formação de apressórios. Após a penetração, *C. coffeicola* colonizou o parênquima lacunoso inter e intracelularmente. A esporulação ocorreu através dos estômatos e ao redor deles. Para identificar os genes associados à produção de cercosporina, avaliaram-se seis isolados provenientes de lavouras orgânicas e convencionais de cafeeiros do Estado de Minas Gerais, quanto à produção da toxina *in vitro*. Selecionou-se o isolado de maior produção para identificar genes homólogos a CTB1 e a ATR1, descritos em *C. nicotianae* como associados à produção e sensibilidade a cercosporina, respectivamente. Os genes ATR1 e CTB1 de *C. coffeicola* foram amplificados com primers específicos e não específicos, sequenciados e comparados às sequências de *C. nicotianae* disponíveis no GenBank. Adicionalmente, obteve-se a construção de GFP e a disrupção de CTB1 em *C. coffeicola*. Avaliou-se a esporulação e o crescimento micelial dos isolados mutantes, não produtores de cercosporina, em meio BDA com e sem higromicina. A esporulação e o crescimento micelial variaram entre os isolados, mas os mutantes CTB1 não diferiram significativamente do tipo selvagem. No terceiro objetivo, estudou-se o progresso da cercosporiose em três lavouras comerciais de café, orgânica sombreada (LOS), orgânica (LO) e convencional (LC), situadas em Ervália-MG, de novembro/2004 a outubro/2008.

Mensalmente, avaliou-se a incidência, severidade, desfolha e enfolhamento das folhas nos ramos das plantas selecionadas. Em geral, os maiores valores de incidência, severidade, área abaixo da curva de progresso de doença, doença máxima, desfolha e enfolhamento ocorreram nos ramos situados no terço superior e das plantas localizadas na LC. Maiores intensidades da doença, desfolha e enfolhamento ocorreram nos meses compreendidos de maio a julho, julho a setembro e outubro a janeiro, respectivamente. Por meio de análises de séries temporais, com o uso dos modelos de regressão não linear ARMA, foi possível representar a dinâmica da cercosporiose, em todos os anos e lavouras em estudo.

ABSTRACT

SOUZA, André Gomes Coelho de, D. Sc., Universidade Federal de Viçosa, February, 2011. **Coffee leaf infection by *Cercospora coffeicola*, identification of genes required for production and auto-resistance to cercosporin and cercospora leaf spot progress on organic and conventional cropping systems.** Adviser: Luiz Antonio Maffia. Co-Advisers: Eduardo Seiti Gomide Mizubuti and Fabyano Fonseca e Silva.

Coffee is considered an important commodity worldwide. Diseases are considered a limiting factor in coffee yield. Cercospora leaf spot, caused by *Cercospora coffeicola*, is considered a relevant disease in coffee crops. This study aimed at understanding: i- the *C. coffeicola* infection process on coffee leaf by microscopic analysis; and ii-identify if genes associated with cercosporin production and auto-resistance in other *Cercospora* spp. were present and had the same functions in *C. coffeicola*, and generate mutant(s) of *C. coffeicola* with differential cercosporin production; and iii- the progress of cercospora leaf spot epidemics on three different coffee production systems (organic, organic under shadow, and conventional). To the first aim both adaxial and abaxial leaf surfaces were inoculated with a conidial suspension of *C. coffeicola*. Samples were collected from 4 to 168 h, and at 35 days after inoculation. Germinated conidia showed positive tropism to stomata where attempted penetrations occurred. Appressoria were not observed. After penetration, *C. coffeicola* colonized the lacunous parenchyma both inter and intracellularly. Sporulation occurred through or around the stomata. To achieve the second aim, six *C. coffeicola* isolates from Brazilian fields representing organic and conventional production systems in the Minas Gerais state were evaluated for their ability to produce cercosporin *in vitro*. The highest producing isolate was selected to identify homologs of a polyketide synthase (CTB1) and ABC transporter (ATR1) genes involved in production and sensitivity to cercosporin in *C. nicotianae*, respectively. The *C. coffeicola CTB1* and *ATR1* genes were amplified using degenerate and standard PCR primers. These genes were sequenced and compared to *C. nicotianae* sequences available on GenBank. In addition, *C. coffeicola* was successfully transformed with a GFP construct and with a CTB1 disruption. Growth and sporulation of these disrupted strains were determined on PDA and PDA supplemented with hygromycin. The sporulation and growth were variable among isolates, and disrupted strains unable to produce cercosporin did not differ significantly from the wild type for either growth or sporulation. To achieve the

third aim, during 4 years (November/2004 – October/2008) the severity (SEV), incidence (INC), leaf fall (LF) and leaf growth (LG), were evaluated to understanding the cercospora leaf spot epidemic progress on three different coffee production systems: organic (LO), organic under shadow (LOS) and conventional (LC), at Ervália-MG, Brazil. The higher SEV, INC, LF, GL, area under disease progress curve, and maximum disease occurred under LC and upper branches. However, the opposite results occurred under LOS and lower branches. The period of greater disease intensity, LF, and GL were the months between May-July, July-September and October-January, respectively. The ARMA model from time series analysis was able to describe the cercospora leaf spot progress during all the years studied and the production systems evaluated.

INTRODUÇÃO GERAL

Entre os fatores que decrescem o potencial produtivo do cafeeiro encontram-se as doenças, como a cercosporiose, causada por *Cercospora coffeicola* Berk & Cook, que pode causar perdas de até 30% da produção (Zambolim *et al*., 1997). Nos últimos anos, em vista da expansão das lavouras cafeeiras das áreas tradicionais para as do cerrado, cujos solos geralmente têm baixa fertilidade natural e deficiência hídrica pronunciada de março a setembro, ocorreu aumento da intensidade da doença (Juliatti *et al*., 2000).

Para o gênero *Cercospora*, têm-se relatos sobre o processo de infecção em diferentes espécies de plantas, mas há uma grande variação entre as espécies. Para *C. moricola* e *C. henningsii* em amora e mandioca, respectivamente, vários tubos germinativos são formados, os qausi podem ou não formar apressório (Gupta *et al*. 1995; Babu *et al*. 2007). Em folhas de mandioca, *C. henningsii* forma tubos germinativos que ramificam para diferentes direções e ocorrem múltiplas penetrações (Babu *et al*. 2009). A penetração pode ocorrer através da epiderme, como exemplo, para *C. henningsii* em folhas de mandioca (Babu *et al*. 2009), pela epiderme e estômatos como em *C. arachidicola* em amendoim (Smith *et al*. 1992) ou somente pelos estômatos como em *C. moricola* em amora (Gupta *et al*. 1995), *C. beticola* em beterraba (Rathaiah 1976, 1977) e *C. caricis* em tiririca (Borges Neto *et al*. 1998). Apesar da importância atual da cercosporiose do cafeeiro, há carência de conhecimentos básicos sobre a doença. Pouco se sabe das etapas da patogênese de *C. coffeicola*, e as informações sobre a forma de penetração são contraditórias. Há relatos de que a penetração ocorre via estômato (Echandi, 1959; Siddiqi, 1979) e diretamente no limbo foliar (Castaño, 1956).

Fungos fitopatogênicos usam estratégias para superar as defesas impostas por seus hospedeiros. A produção de toxinas desempenha papel importante na patogenicidade desses fungos (Scheffer, 1991). Muitas espécies de *Cercospora* produzem a cercosporina, uma toxina fotoativa, que é relacionada ao parasitismo desses fungos (Berestetskiy, 2008). Na presença de luz, a cercosporina induz a produção de espécies reativas de oxigênio que danificam células da planta (Daub, 1982; Daud & Hangarter, 1983). Nos últimos anos, tem-se buscado descobrir os genes requeridos para acúmulo e ação da cercosporina. Para algumas espécies, obtiveram-se mutantes deficientes quanto à produção e à resistência de cercosporina por meio de disrupção dos genes CFP (“cercosporin facilitator protein”) em *C. kikuchii* (Callahan *et al*., 1999) e *C.*

nicotianae (Upchurch *et al*., 2005), CRG1 ("cercosporin resistance gene") e CTB1 ("cercosporin toxin biosynthesis") em *C. nicotianae* (Chen *et al*., 2007b; Choquer *et al*., 2005; Chung *et al*., 1999; Chung *et al*., 2003; Herrero *et al*., 2007), e CZK3 ("MAP kinase") em *C. zeae-maydis* (Shim & Dunkle, 2003). A disrupção dos genes CTB3, CTB5, CTB6 e CTB7 afetou a produção de cercosporina nos mutantes, os quais induziram menos lesões em plantas de fumo que o tipo selvagem, o que reforça a hipótese de que a cercosporina seja um fator de agressividade (Chen *et al*., 2007a; Dekkers *et al*., 2007). Considerando que *C. coffeicola* produz cercosporina, pode-se aventar a hipótese de que a toxina seja essencial para patogênese deste fungo no cafeeiro (Martins, 2008; Souza, 2007).

O estresse hídrico e alta insolação favorecem o aumento da intensidade da cercosporiose (Boldini *et al*., 2000). A produtividade de cafeeiros conduzidos a pleno sol é maior que a daqueles sombreados (Miranda *et al*., 1999), mas a intensidade da cercosporiose é maior nos cafeeiros a pleno sol (López-Duque & Fernández-Borrero, 1969; Almeida, 1986), bem como em plantas com deficiência nutricional (Fernández-Borrero *et al*., 1982). Portanto, é possível que lavouras de café em plantio consorciado e que não recebam insumos químicos tenham menor incidência da doença, por causa da menor insolação e da maior população de inimigos naturais. Estudos da epidemiologia comparativa podem auxiliar a entender a dinâmica da cercosporiose em diferentes sistemas de cultivo do cafeeiro. Para esses estudos, é fundamental o ajuste de modelos de progresso. Dentre esses modelos, os de séries temporais podem ser útil para a descrição de processos estocásticos (Box *et al*., 1994). Considerando o aspecto estocástico do progresso da doença, alguns autores usaram os modelos de séries temporais para descrever eventos epidemiológicos (Yang & Zeng, 1992; Hudelson *et al*., 1993; Xu *et al*., 1995; Guerin *et al*., 2001; Zauza, 2007; Holb, 2008).

Em vista da importância da cercosporiose e carência de informações sobre a doença, delineou-se este trabalho. Especificamente, objetivou-se: i- estudar os processos de germinação, penetração, colonização e a formação de fascículos de conidióforos de *C. coffeicola* em folhas de cafeeiros, utilizando-se a microscopia de luz e a microscopia eletrônica de varredura; ii- identificar se os genes já relatados para outras espécies de *Cercospora*, como requeridos para a produção e resistência a cercosporina ocorram em *C. coffeicola* e gerar mutante desse fungo deficientes na produção da toxina; iii- estudar o progresso da doença em cafeeiros conduzidos nos sistemas de cultivo convencional e orgânico (sombreado e a pleno sol), por meio de modelos de série temporal.

REFERÊNCIAS BIBLIOGRÁFICAS

Almeida, S. R. Doenças do cafeeiro. 1986. In: Rena, A. B., Malavolta, E., Rocha, M.; Yamada, T. Cultura do cafeeiro: Fatores que afetam a produtividade. Piracicaba: Instituto da Potassa e Fosfato, p.391-399.

Babu, A. M., Philip, T., Kumar, V. 2007. Development of the leaf spot fungus, *Cercospora ricinella*, on castor leaf – an SEM account. Journal of Phytopathology, 155:426-430.

Babu, A. M., Philip, T., Kariappa, B. K., Kamble, C. K. 2009. Scanning electron microscopy of the infection process of *Cercospora henningsii* on cassava leaves. Journal of Phytopathology, 157:57-62.

Berestetskiy, A. O. 2008. A review of fungal phytotoxins: from basic studies to practical use. Applied Biochemistry and Microbiology, 44:453-465.

Boldini, J. M., Souza, P. E., Pozza, E. A., Chalfoun, S. M., Faria, M. A. 2000. Incidência e severidade da cercosporiose em frutos de cafeeiro fertirrigado na região de Lavras-MG. In: 1º Simpósio de Pesquisa dos Cafés do Brasil, Poços de Caldas, MG. Resumos expandidos. Brasília, D.F. Embrapa Café, p.273-275.

Borges Neto, C. R., Silveira, E. B., Mello, S. C. M., Fontes, E. M. G. 1998. Scanning electron microscopy of the infection process of *Cercospora caricis* on purple nutsedge. Fitopatologia Brasileira, 23:169-172.

Box, G. E. P., Jenkins, G. M., Reinsel, G. C. 1994. Time Series Analysis: Forecasting and Control. San Francisco, CA, USA: Holden-Day.

Callahan, T., Rose, M., Meade, M., Ehrenshaft, M., Upchurch, R. 1999. CFP, the putative cercosporin transporter of *Cercospora kikuchii*, is required for wild type cercosporin production, resistance, and virulence on soybean. Molecular Plant-Microbe Interactions, 12:901-910.

Castaño, A. J. J. 1956. Mancha de hierro del cafeto. Boletim Informativo. Cenicafé, 82: 313-327.

Chen, H. Q., Lee, M. H., Chung, K. R. 2007a. Functional characterization of three genes encoding putative oxidoreductases required for cercosporin toxin biosynthesis in the fungus *Cercospora nicotianae*. Microbiology, 153:2781-2790.

Chen, H., Lee, M. H., Daub, M. E., Chung, K. R. 2007b. Molecular analysis of the cercosporin biosynthetic gene cluster in *Cercospora nicotianae*. Molecular Microbiology, 64:755-770.

Choquer, M., Lahey, K. A., Chen, H. L., Cao, L., Ueng, P. P., Daub, M. E., Chung, K. R. 2005. The CTB1 gene encoding a fungal polyketide synthase is required for cercosporin toxin biosynthesis and fungal virulence in *Cercospora nicotianae*. Molecular Plant-Microbe Interaction, 18:468-476.

Chung, K. R., Jenns, A. E., Ehrenshaft, M., Daub, M. E. 1999. A novel gene required for cercosporin toxin resistance in the fungus *Cercospora nicotianae*. Molecular Genetics and Genomics, 262:382-389.

Chung, K. R., Daub, M. E., Kuchler, K., Schüller, C. 2003. The CRG1 gene required for resistance to the singlet oxygen-generating cercosporin toxin in *Cercospora nicotianae* encodes a putative fungal transcription factor. Biochemical and Biophysical Research Communications, 302:302-310.

Daub, M. E. 1982. Peroxidation of tobacco membrane lipids by the photosensitizing toxin, cercosporin. Plant Physiology, 69:1361-1364.

Daub, M. E., Hangarter, R. P. 1983. Light-induced production of singlet oxygen and superoxide by the fungal toxin, cercosporin. Plant Physiology, 73:855-857.

Dekkers, K. L., You, B. J., Gowda, V. S.; Liao, H. L., Lee, M. H., Bau, H. J., Ueng, P. P., Chung, K. R. 2007. The *Cercospora nicotianae* gene encoding dual O-methyltransferase and FAD-dependent monooxygenase domains mediates cercosporin toxin biosynthesis. Fungal Genetics and Biology, 44:444-454.

Echandi, E. 1959. La chasparria de los cafetos causada por el hongo *Cercospora coffeicola* Berk. & Cooke. Turrialba, 9:54–67.

Fernández-Borrero, O., Cadena, G. G., Lopez, D. S., Buitrago, S., Arango, B. L. G. 1982. La mancha de hierro del cafeto (*Cercospora coffeicola* Berk e Cooke), biologia, epidemiologia y control. In: 10º Colloque Scientifique International Sur le Café, Salvador (Brasil), p.11-14.

Guerin, L., Froidefond, G., Xu, X.-M. 2001. Seasonal patterns of dispersal of ascospores of *Cryphonectria parasitica* (chestnut blight). Plant Pathology, 50:717-724.

Gupta, V. P., Tewari, S. K., Govindaiah, Bajpai, A. K., Datta, R. K. 1995. Observations on the surface ultrastructure of conidial stage of *Cercospora moricola* and its infection process in mulberry. Sericologia, 35:123–128.

Herrero, S., Amnuaykanjanasin, A., Daub, M. E. 2007. Identification of genes differentially expressed in the phytopathogenic fungus *Cercospora nicotianae*

between cercosporin toxin-resistant and -susceptible strains. Federation of European Microbiological Societies Microbiology Letters, 275:326-337.

Holb, I. J. 2008. Monitoring conidial density of *Monilinia fructigena* in the air in relation to brown rot development in integrated and organic apple orchards. European Journal of Plant Pathology, 120:397-408.

Hudelson, B. D., Clayton, M. K., Smith, K. P., Upper, C. D. 1993. Modeling of superimposed spatial patterns of bacterial brown spot of snap beans. Phytopathology, 83:430-438.

Juliatti, F. C., Ramos, A. S., Mendonça, F. C., Santos, C. M. 2000. Incidência e severidade da cercosporiose em lavoura cafeeira conduzida sob diferentes sistemas de irrigação e lâminas d'água. In: 1º Simpósio de Pesquisa dos Cafés do Brasil, Poços de Caldas, MG. Resumos expandidos: Embrapa Café, p.219-222.

López-Duque, S.; Fernández-Borrero, O. 1969. Epidemiologia de la mancha de hierro del cafeto (*Cercospora coffeicola* Berk. & Cooke). Cenicafé, 20:3-19.

Martins, R. B. 2008. Variabilidade de *Cercospora coffeicola* em Minas Gerais com base em compatibilidade vegetativa e produção de cercosporina. Tese de Doutorado. Viçosa-MG. Universidade Federal de Viçosa.

Miranda, E. M., Pereira, R. C. A., Bergo, C. L. 1999. Comportamento de seis linhagens de café (*Coffea arabica* L.) em condições de sombreamento e a pleno sol no estado do Acre, Brasil. Ciência e Agrotecnologia, 23:62-69.

Rathaiah, Y. 1976. Infection of sugarbeet by *Cercospora beticola* in relation to stomatal condition. Phytopathology, 66:737-740.

Rathaiah, Y. 1977. Stomatal tropism of *Cercospora beticola* in sugarbeet. Phytopathology 67:358-362.

Scheffer, R. P. 1991. Role of toxins in evolution and ecology of plant pathogenic fungi. Experientia, 47:804-811.

Shim, W. B., Dunkle, L. D. 2003. CZK3, a MAP kinase kinase kinase homolog in *Cercospora zeae-maydis*, regulates cercosporin biosynthesis, fungal development, and pathogenesis. Molecular Plant-Microbe Interaction, 16:760-768.

Siddiqi, M. A. 1979. Incidence, development and symptoms of Cercospora disease of coffee in Malawi. Transactions of the British Mycological Society, 54:415-421.

Smith, D. H., Pauer, G. D. C., Shokes, F. M. 1992. *Cercosporidium* and *Cercospora* leaf spots of peanut. In: Chaube HS, Singh US, Mukhopadhyay AN, Kumar J. Plant

Diseases of International Importance. vol. II, New Jersen, USA, Prentica Hall Inc., p. 285-304.

Souza, A. G. C. 2007. Variabilidade fisiológica de isolados de *Cercospora coffeicola*. Dissertação de Mestrado. Viçosa-MG. Universidade Federal de Viçosa.

Upchurch, R. G., Rose, M. S., Eweida, M., Zuo, W. 2005. Expression of the cercosporin transporter, CFP, in tobacco reduces frog-eye lesion size. Biotechnology Letters, 27:1543-1550.

Xu, X.-M., Butt, D. J., Ridout, U. S. 1995. Temporal patterns of airborne conidia of *Podosphaera leucotricha*, causal agent of apple powdery mildew. Plant Pathology, 44:944-955.

Yang, X. B., Zeng, S. M. 1992. Detecting patterns of wheat stripe rust pandemics in time space. Phytopathology, 82: 571-576.

Zambolim, L.; Vale, F. X. R.; Pereira, A.A.; Chaves, G. M. Café (*Coffea arábica*). In: Vale, F. X. R.; Zambolim, L. Controle de doenças de plantas: grandes culturas. Viçosa: UFV, p.83-180, 1997.

Zauza, E. A. V. 2007. Epidemiologia da ferrugem do eucalipto e resistência genética. Tese de Doutorado. Viçosa-MG. Universidade Federal de Viçosa.

Infection process of *Cercospora coffeicola* on coffee leaf

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Infection process of *Cercospora coffeicola* on coffee leaf

Abstract

Brown eye spot, caused by *Cercospora coffeicola*, is an important disease of coffee. Both adaxial and abaxial leaf surfaces were inoculated with a conidial suspension of *C. coffeicola.* Samples were collected from 4 to 168 h after inoculation and then again at 35 days. Germinated conidia showed positive tropism to stomata where attempted penetrations occurred. Appressoria were not observed. After penetration, *C. coffeicola* colonized the lacunous parenchyma both inter and intracellularly. Sporulation occurred through or around the stomata. Results from this study provide new insights into the infection process of *C. coffeicola* on coffee leaf.

Keywords: *Cercospora coffeicola*, *Coffea arabica* L., leaf spot, pathogenesis, scanning electron microscope

1 – Introduction

Coffee (*Coffea arabica* L.) is largely cultivated in Brazil, and it represents a strategic commodity to the Brazilian economy. Diseases are considered to play a significant role in reducing coffee production in Brazil. Epidemics of brown eye spot caused by *Cercospora coffeicola* Berk. & Cooke, are often reported in many coffee growing areas in Brazil. Brown eye spot is one of the oldest coffee diseases reported and was first discovered in 1881 in Jamaica (Cooke 1881). In Brazil, this disease was first reported in 1901 on coffee trees in the cities of Campinas and Araraquara in the state of São Paulo (Noack 1901). However, the first record of severe epidemics in Brazil was in 1971 in the state of Espírito Santo (Carvalho and Chalfoun 1998).

Brown eye spot may occur under both nursery and field conditions and is often considered to be more severe on plants suffering stress caused by water and/or nutrient deficiency (Fernández-Borrero et al. 1966; López-Duque and Fernández-Borrero 1969). Yield losses may reach up to 50% due to leaf drop and berry damage (Fernández-Borrero et al. 1982). In the past few years, concerns were raised due to an increase in brown eye spot severity in Brazil (Martins et al. 2008). The increase in brown eye spot intensity was mainly attributed to several factors such as the new coffee growing areas in the Cerrados region, the release of new coffee cultivars, the changes in cultural practices and alteration of the climate conditions (Juliatti et al. 2000).

The fungus infects leaves and berries. Numerous lesions appear on leaves that coalesce producing large necrotic areas. Diseased leaves fall early in the season. Round brown-purple spots are formed on berries. The lesions coalesce and become darkened, giving the berries a dried appearance. Furthermore, maturation is accelerated leading to premature berry drop and reduction in their quality (Castaño 1956). As the fungus can affect both coffee leaves and berries and that there have been several recent outbreaks of brown eye spot, the Brazilian coffee industry warrants more studies to derive effective control methods. So far, disease management is heavily based on the use of fungicides sprays, and there are no current resistant cultivars to brown eye spot. Studies on host resistance and pathogen variability do require a basic knowledge of the pathogen's life cycle, especially of its mode of infection.

The genus *Cercospora* shows wide variation in the infection process, and even the same species shows different pattern on different hosts. *Cercospora moricola* on mulberry and *Cercospora henningsii* on cassava form several germ tubes with or

without appressoria formation (Gupta et al. 1995; Babu et al. 2007). On cassava leaves, the germ tubes of *C. henningsii* got branched and made multiple penetrations (Babu et al. 2009). Penetration may be only through epidermis as in *C. henningsii* on cassava leaf (Babu et al. 2009), through epidermis and stomata as in *Cercospora arachidicola* on peanuts (Smith et al. 1992) or only through stomata as in *C. moricola* on mulberry (Gupta et al. 1995), *Cercospora beticola* on sugarbeet (Rathaiah 1976, 1977) and *C. caricis* on purple nutsedge (Borges Neto et al. 1998).

The available information about the penetration and infection stages of *C. coffeicola* on coffee leaves is scant and somewhat contradictory. Penetration of the leaf tissue by *C. coffeicola* was reported to occur through the stomata (Echandi 1959; Siddiqi 1979) or directly (Castaño 1956). It has been reported that fungal colonization in leaves can be inter and intracellularly or strictly intracellularly (Castaño 1956; Siddiqi 1979). The present study aimed to determine the events of *C. coffeicola* penetration, colonization and sporulation on coffee leaves by light and scanning electron microscopy.

2 – Materials and Methods

2.1 – Fungus growth and conidia production

Cercospora coffeicola was directly isolated from diseased leaves and grown on Petri dishes containing potato-dextrose-agar (PDA) medium. The technique of drying the mycelial mass was used to induce conidial production (Souza et al. 2005). Three mycelial disks (0.5 mm in diameter) taken from the border of a fungus colony were transferred to 10 ml of V8 medium (200 ml V8® plus 800 ml of distilled water) in 25-ml Erlenmeyers that were kept continuously agitated (120 rpm) at 25ºC. After 4 days, the content of each Erlenmeyer was poured into Petri dishes containing water–agar at 1.5%. The dishes were kept open at 40 cm distance from white fluorescent and 40 W grow lux lamps, distributed alternately to provide light intensity of 165.3 µmol/s/m^2. Incubation conditions were 12-h photoperiod at 25ºC. After dehydration of the culture medium (approximately 4 days of incubation), 10 ml of distilled water were added to each Petri dish, the fungal colony was scratched with a glass rod, and the suspension was filtered through one layer of cheesecloth. Conidial concentration was adjusted to 2 x 10^4 conidia/ml with a haemocytometer.

2.2 – Plant inoculation with *C. coffeicola*

Conidial suspension was sprayed on two leaves of each of 32 coffee plants (cv. 'Catuaí Vermelho', 6 months-old) with a DeVilbiss sprayer and grown under greenhouse conditions. Thirty-two leaves were inoculated on the upper surface and the other 32 on the lower surface. Out of the 64 leaves, 44 were carefully detached from the plants and kept with the inoculated surface facing up inside plastic boxes (11 cm length x 11 cm width x 3 cm height) with wet sponges. Two leaves, one inoculated on the upper surface and one inoculated on the lower surface, were set in a plastic box, which was closed and kept at 25ºC with 90 ± 5% relative humidity (RH), and continuous light (40 W grow lux lamps distributed alternately to provide light intensity of 165.3 µmol/s/m^2). The remaining inoculated leaves were left attached to the plants that were kept in a dew chamber under the same conditions described earlier. After 12-h incubation, the boxes and the plants were transferred to greenhouse at 25 ± 3ºC at 70 ± 5% RH and natural light (≈385.09 µmol/s/m^2).

2.3 – Light microscopy

Thirty-five days after inoculation, four leaf samples (≈25 mm^2) were carefully collected from the lesions border with abundant fungal sporulation and transferred to glass vials containing 15 ml of a 50% (v ⁄ v) active chlorine 2.5% solution. After 24 h, leaf samples were transferred to glass slides containing a drop of lactophenol cotton blue stain. The slides were observed under a light microscope (Carl Zeiss Axio Imager A1, Göttingen, Germany) using the differential interference contrast technique.

2.4 – Scanning electron microscopy

Four leaves from the two boxes were collected at 4, 6, 8, 12, 24, 36, 48, 72, 96 and 168 h after inoculation (hai). Leaf samples kept on the plants were collected only at 35 days after inoculation (dai) when symptoms and fungal sporulation on lesions became evident. A total of 20 leaf pieces (2.5 cm^2 in size) per each sampling time were randomly taken from the leaf blade and carefully transferred to glass vials containing 15 ml of fixative (2.5% v ⁄ v glutaraldehyde in 0.1 m sodium cacodylate buffer, pH 7.2). Samples were stored at 4ºC for 10 days and then carefully washed with sodium cacodylate buffer (0.1 m), dehydrated in a graded ethanol series and critical point dried in CO_2 (Bal-tec, model CPD 030; Electron Mycroscopy Sciences, Hatfield, PA, USA). Four specimens from each sample were mounted on aluminium stubs, sputter coated with gold (Balzers Union, model FDU 010; Electron Mycroscopy Sciences, Hatfield, PA, USA), examined and photographed with a LEO scanning electron microscope (SEM) (model 1430 VP) operating at 10 kV and with working distance ranging from 10 to 30 mm. For each treatment, one stub with four specimens was examined by SEM. Leaf samples collected at 35 dai were carefully fractured with a scalpel and examined under the SEM. The identity of the pathogen on the lesions was confirmed by removing conidia and transferring them to Petri dishes containing PDA. After 7 days, the colonies growing on the media were confirmed to be *C. coffeicola* based on the morphological characteristics reported by Echandi (1959).

3 – Results

3.1 – Conidial germination and fungus penetration

A total of 20 conidia from each sampling time were observed. Conidial germination did not follow a pattern. After 4 hai, each conidium formed, on average, three germ tubes on the adaxial and abaxial leaf surfaces (Fig. 1a,b). Germ tubes were formed by different conidial cells and, occasionally, the germination tended to be bipolar (Fig. 1a,b). Conidial germination on the adaxial leaf surface started 4 h later than on abaxial surface. On leaf surfaces examined at 6, 8, 12 and 24 hai, conidia formed germ tubes, and fungal growth occurred predominantly towards the stomatal opening (Fig. 1c,d). Some germ tubes grew towards the stomata, but did not penetrate them. On some occasions, germ tubes passed over the guard cells or surrounded them (Fig. 1b–d). Penetration was observed 36 hai. The germ tubes penetrated mostly through the stomata (Fig. 1e,f) or sometimes through cracks found on the leaf epidermis (Fig. 1f). Neither appressorium formation nor direct penetration was observed for all infection sites examined.

3.2 – Fungus colonization and sporulation

At 35 dai, profuse hyphal growth of *C. coffeicola* was observed underneath the substomatic chamber from where conidiophores were formed (Fig. 2a). Intracellular and intercellular hyphae were also present under the epidermis at the lacunous parenchyma (Fig. 2b). Conidiophores emerged singly or in groups (fascicles) through or around the stomata (Fig. 3a–c). Conidiogenic cells were formed on the tips of the conidiophores (Fig. 3a). Conidiophores and conidia were more dense on the abaxial leaf surface (Fig. 3a).

3.3 – Brown eye spot symptoms on coffee leaf blades

Disease symptoms started to develop on the adaxial leaf surface by the appearance of several round brown spots surrounded by yellow halos. The brown spots enlarged and became necrotic. A mature lesion typically had a white centre with a middle brown ring and an outer yellow ring (Fig. 4a,b). Conidiophores bearing conidia were observed on the lesions (Fig. 4c). Conidia of *C. coffecicola* were hyaline, acicular to obclavate, nearly straight, truncate to subtruncate on their base, with acute tip and multiseptate.

4 – Discussion

The germination process of *C. coffeicola* has not been fully described in the literature. The present study showed that the conidia germinated mostly from the tip and ⁄ or basal cells and less frequently from the middle cells. Each conidium produced one to several germ tubes, and appressoria were not developed on the leaf surface. The formation of several germ tubes has been observed in *C. moricola* on mulberry (Gupta et al. 1995) and in *C. henningsii* on cassava (Babu et al. 2007) with or without apprressorium formation. Germ tubes of *C. coffeicola* branched in many different directions. On cassava leaves, the germ tubes of *C. henningsii* got branched and made multiple penetrations contributing to the higher aggressiveness of the pathogen (Babu et al. 2009).

The proximity of conidia of *Cercospora* spp. to somata may or may not affect their germination (Rathaiah 1977; Gupta et al.1995; Babu et al. 2007). In the present study, majority of the germ tubes present on the abaxial leaf surface tended to be directed towards the stomata while on the adaxial leaf surfaces their growth occurred randomly. The germ tubes tend to enter the nearest stoma, but sometimes the penetration occurs through a stoma situated farther. A similar pattern is reported in many other species of *Cercospora* (Dedecca 1957; Gupta et al. 1995; Rathaiah 1976). For other species of *Cercospora*, germ tube growth was extensive and random, and stomata were not necessarily penetrated by the hyphae that passed immediately beside or across from them (Echandi 1959; Rathaiah 1977; Siddiqi 1979; Babu et al. 2007). The microenvironment around the conidia and their growing germ tubes may be crucial to guarantee further growth on the leaf surface. Chemical signals such as sugars, phenolic compounds, volatile metabolites, and physical signals such as stomata and cuticle topography, may affect fungal growth on the host leaf surfaces (Dean 1997).

The penetration of *C. coffeicola* took place through the open stomata or cracks found in the epicuticular wax layer. No evidence of direct penetration was found in the present study which is in conformity with the reports of Echandi (1959) and Siddiqi (1979). However, Castaño (1956) found that penetration can occur both directly or through the stomata. As reported by Echandi (1959), no attempts of *C. coffeicola* penetration occurred on the adaxial coffee leaf surface where stomata are not present (Dedecca 1957). Penetration may be only through epidermis as in *C. henningsii* on cassava leaf (Babu et al. 2009), through epidermis and stomata as in *C. arachidicola* on

peanuts (Smith et al. 1992) or only through stomata as in *C. moricola* on mulberry (Gupta et al. 1995) and *C. beticola* on sugarbeet (Rathaiah 1976).

After penetration, *C. coffeicola* colonized the substomatal chambers and invaded the adjoining tissues. Two patterns of colonization are reported in *C. coffeicola*: strictly intracellular (Echandi 1959) and both inter and intracellular (Castaño 1956). In the present study, *C. coffeicola* colonized the leaf tissue both inter and intracellularly.

Considering the importance of brown eye spot to coffee production in Brazil and the lack of information in the literature regarding the infection process of *C. coffeicola*, the results from the present study offer novel information for a better understanding of the fungal life cycle that may help for evolving more effective disease control strategies. The study revealed that only the conidia deposited on the abaxial leaf surface were capable of penetration leading to disease development. It gives insight into the need of efficient fungicide application methods and agents of biological control targeting the lower surface of coffee leaves for an effective brown eye spot disease control.

5 – References

Babu AM, Philip T, Kumar V. (2007). Development of the leaf spot fungus, *Cercospora ricinella*, on castor leaf – an SEM account. *Journal of Phytopathology* **155**:426–430.

Babu AM, Philip T, Kariappa BK, Kamble CK. (2009). Scanning electron microscopy of the infection process of *Cercospora henningsii* on cassava leaves. *Journal of Phytopathology* **157**:57–62.

Borges Neto CR, Silveira EB, Mello SCM, Fontes EMG. (1998). Scanning electron microscopy of the infection process of *Cercospora caricis* on purple nutsedge. *Fitopatologia Brasileira* **23**:169–172.

Carvalho VL, Chalfoun SM. (1998). Manejo integrado das principais doenças do cafeeiro. *Informe Agropecuário* **19**:27–35.

Castaño AJJ. (1956). Mancha de hierro del cafeto. Boletim Informativo. *Cenicafé* **82**:313–327.

Cooke MC. (1881). *Cercospora coffeicola*. *Grevillea* **9**:99.

Dean RA. (1997). Signal pathways and appressorium morphogenesis. *Annual Review of Phytopathology* **35**:211–234.

Dedecca DM. (1957). Anatomia e desenvolvimento ontogenético de *Coffea arabica* L. var. Cramer. ***Bragantia*** **16:**315–366.

Echandi E. (1959). La chasparria de los cafetos causada por el hongo *Cercospora coffeicola* Berk. & Cooke. *Turrialba* **9**:54–67.

Fernández-Borrero O, Mestre AM, Duque SIL. (1966). Efecto de la fertilización en la incidencia de la mancha de hierro (*Cercospora coffeicola*) en frutos de café. *Cenicafé* **17**:5–16.

Fernández-Borrero O, Cadena GG, Lopez DS, Buitrago S, Arango BLG. (1982). La mancha de hierro del cafeto (*Cercospora coffeicola* Berk & Cooke), biologia, epidemiologia y control. In: 10º Colloque Scientifique International Sur le Café, Salvador (Brasil), p.11–14.

Gupta VP, Tewari SK, Govindaiah, Bajpai AK, Datta RK. (1995). Observations on the surface ultrastructure of conidial stage of *Cercospora moricola* and its infection process in mulberry. *Sericologia* **35**:123–128.

Juliatti FC, Ramos AS, Mendonça FC, Santos CM. (2000). Incidência e severidade da cercosporiose em lavoura cafeeira conduzida sob diferentes sistemas de irrigação e

lâminas d'água. In: 1º Simpósio de Pesquisa dos Cafés do Brasil, Poços de Caldas, MG. Resumos expandidos: Embrapa Café, p.219–222.

López-Duque S, Fernández-Borrero O. (1969). Epidemiología de la mancha de hierro del cafeto (*Cercospora coffeicola* Berk. & Cooke). *Cenicafé* **20**:3–19.

Martins RB, Maffia LA, Mizubuti ESG. (2008). Genetic variability of *Cercospora coffeicola* from organic and convencional coffee plantings, characterized by vegetative compatibility. *Phytopathology* 98:1205–1211.

Noack F. (1901). Die krankheiten des kaffeebaumes in Brasilien. *Zeitschrift Fur Pflanzenkrankheiten* **11**:196–203.

Rathaiah Y. (1976). Infection of sugarbeet by *Cercospora beticola* in relation to stomatal condition. *Phytopathology* **66**:737–740.

Rathaiah Y. (1977). Stomatal tropism of *Cercospora beticola* in sugarbeet. *Phytopathology* **67**:358–362.

Siddiqi MA. (1979). Incidence, development and symptoms of *Cercospora* disease of coffee in Malawi. *Transactions of the British Mycological Society* **54**:415–421.

Smith DH, Pauer GDC, Shokes FM. (1992) *Cercosporidium* and *Cercospora* leaf spots of peanut. In: Chaube HS, Singh US, Mukhopadhyay AN, Kumar J. (eds). Plant Diseases of International Importance. vol. II, New Jersen, USA, Prentica Hall Inc., p. 285–304.

Souza AGC, Martins RB, Mizubuti ESG, Maffia LA. (2005). Esporulação *in vitro* de *Cercospora coffeicola*. *Fitopatologia Brasileira* **30**:116.

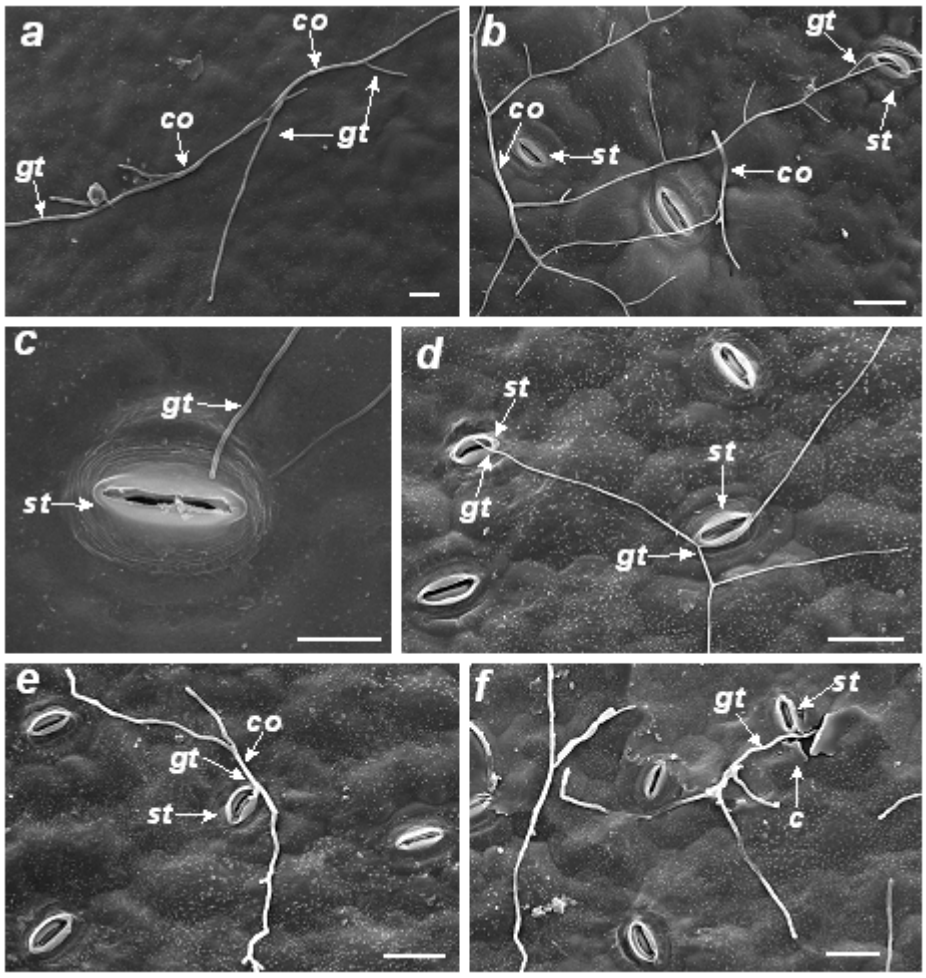

Fig. 1: Scanning electron micrographs of coffee leaves inoculated with *Cercospora coffeicola*. **a** - growth of two germ tubes from a bipolar-germinated conidium on the adaxial leaf surface 4 hours after inoculation (hai) (Bar = 10 µm); **b** - conidia producing germ tubes that crossed stomata without penetration 4 hai (Bar = 20 µm); **c** - the tip of a germ tube grows in the direction of the stomatal opening. (Bar = 10 µm); **d** - a germ tube passed over a stomatal opening and another one surrounded the ridge of a stoma without penetration (Bar = 20 µm); **e** - penetration through a stoma (Bar = 20 µm); **f** - *C. coffeicola* penetrating through cracks on the leaf epidermis (Bar = 20 µm). c = cracks; co = conidium; gt = germ tube; st = stomata.

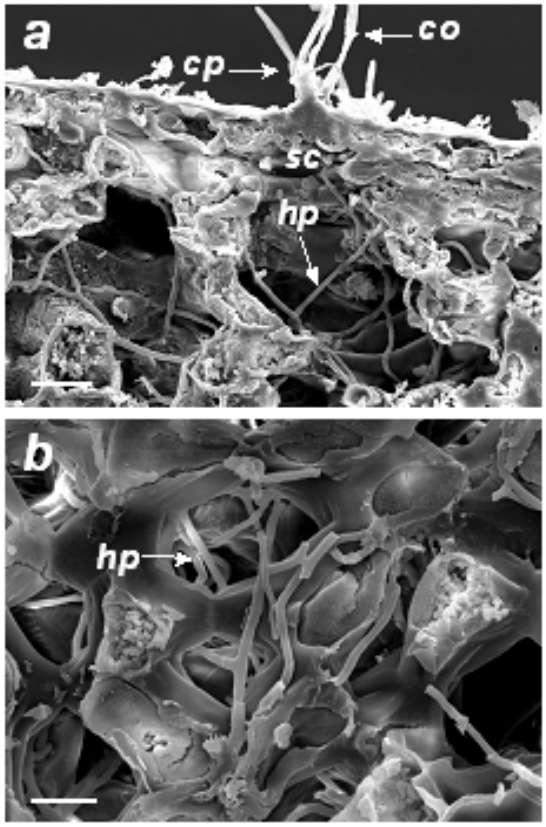

Fig. 2: Scanning electron micrographs of fractured coffee leaf samples at 35 days after inoculation with *Cercospora coffeicola*. **a** - profuse hyphal growth underneath the substomatic chamber from where conidiophores emerged (Bar = 20 µm); **b** - intracellular and intercellular hyphae in the parenchyma (Bar = 10 µm). co = conidium; sc = substomatic chamber, cp = conidiophore; hp = hyphae.

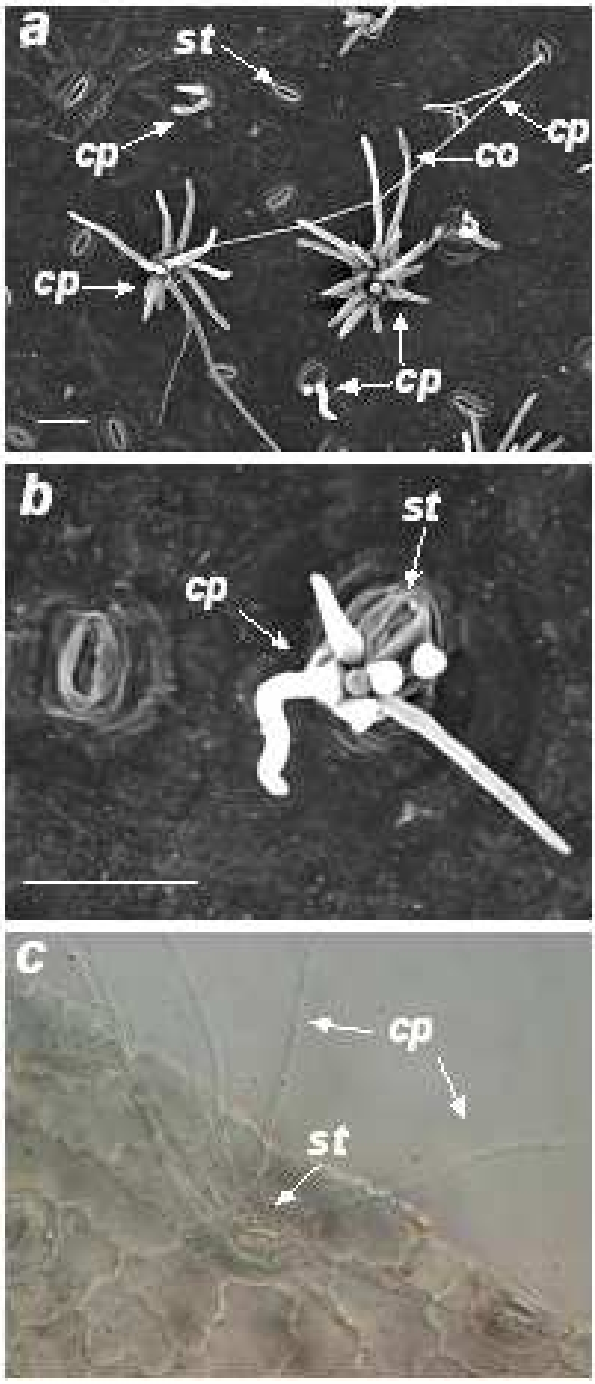

Fig. 3: Scanning electron micrographs (**a** and **b**) and differential interference contrast microscopy (**c**) of conidiophores emerging singly or in fascicles through or around stomata on the abaxial surface of coffee leaves at 35 days after inoculation with *Cercospora coffeicola* (**a** and **b** =bars are 20 µm; **c** = 400 x). co = conidium; cp = conidiophore; st = stomata.

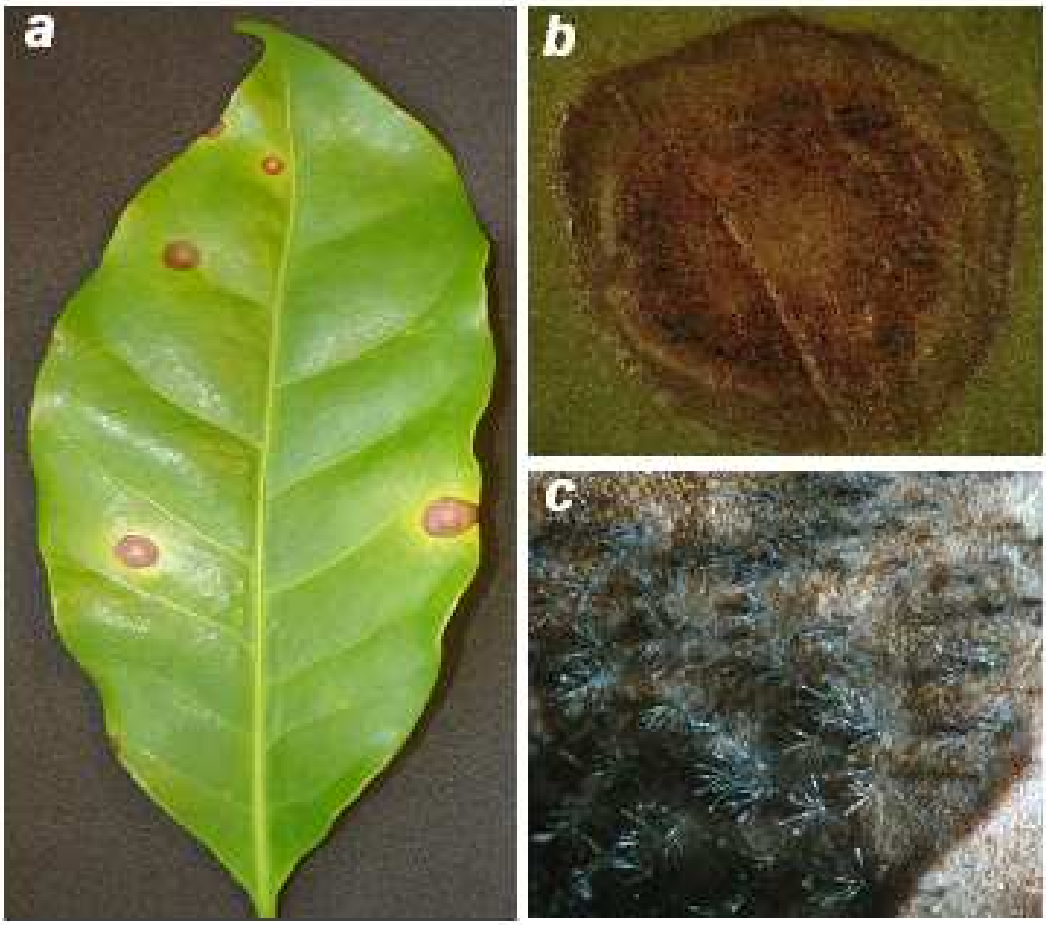

Fig. 4: Brown eye spot on coffee leaf caused by *Cercospora coffeicola*. **a** - brown round spots surrounded by yellow halos, **b** - close-up of a lesion showing intense necrotic tissue at 35 days after inoculation, **c** - fungal sporulation on the necrotic leaf tissue.

Genes required for production and auto-resistance to cercosporin and confirmation that CTB1 gene is required for wild type cercosporin biosynthesis in *Cercospora coffeicola*

Abstract

Brown eye spot, caused by *Cercospora coffeicola*, causes significant losses in both quality and quantity of coffee production. As many *Cercospora* spp. produce cercosporin, a photoactivated toxin thought to be involved in pathogenesis, this study aimed to determine the role of cercosporin in *C. coffeicola* pathogenesis by creating disrupted strains unable to produce the toxin. We first evaluated six *C. coffeicola* isolates from Brazilian fields representing organic and conventional production systems in the Minas Gerais state for their ability to produce cercosporin *in vitro*. Toxin production varied among isolates, ranging from 3.5 - 25.3 µM/ 5 mm plug; production was undetectable in one isolate. The highest producing isolate was selected to identify homologs of a polyketide synthase (CTB1) and ABC transporter (ATR1) genes involved in production and sensitivity to cercosporin in *C. nicotianae*, respectively. The *C. coffeicola CTB1* and *ATR1*genes were amplified using degenerate and standard PCR primers. These genes were sequenced and compared to *C. nicotianae* sequences available on GenBank. In addition, we successfully transformed *C. coffeicola* with a GFP construct and with a CTB1 disruption construct to generate six CTB1-disrupted strains using PEG-mediated protoplast transformation. Although we obtained 126 hygromycin-resistant strains, only six had a disrupted gene. Growth and sporulation of these disrupted strains were determined on PDA and PDA supplemented with hygromycin. The sporulation and growth were variable among isolates, and disrupted strains unable to produce cercosporin did not differ significantly from the wild type for either growth or sporulation.

Keywords: Coffee, leaf spot, GFP, polyketide synthase, ATR1, disruption, protoplast

1 – Introduction

Brazil is the major world producer of coffee. An important coffee disease is brown eyespot, caused by *Cercospora coffeicola*, which causes yield losses in both quality and quantity of production (40). Although the importance of the leaf spot is well known, there are no resistant cultivars to the pathogen, and disease control relies too much on fungicide sprays. Additionally, aspects related to the pathogenesis of *C. coffeicola* to coffee are not well known.

Plant pathogenic fungi use many strategies to break the defenses of their hosts. Toxins produced by plant pathogens are important to their pathogenesis (28). Many plant pathogenic *Cercospora* species produce cercosporin, a photoactivated perylenequinone toxin, which is hypothesized to be involved in the ability of these fungi to parasitize plants (4, 14). Cercosporin generates singlet oxygen and superoxide that damage plant cell membranes when irradiated by light (13, 15, 16). Cercosporin production is affected by physiological and genetic factors. Light is the most critical factor for cercosporin production (39). The ability to produce cercosporin, as well as aggressiveness and growth rate, are variable among isolates of *C. piaropi* (33). Research studies have been undertaken to discover the genes required for cercosporin accumulation and action. For some species, mutants deficient for cercosporin production and auto-resistance have been obtained by gene disruption. The genes include: *CFP* (cercosporin facilitator protein, encoding an MFS transporter) in *C. kikuchii* (5) and *C. nicotianae* (35); *CRG1* (cercosporin resistance gene, encoding a zinc cluster transcription factor), *CTB1* (cercosporin toxin biosynthesis, encoding a polyketide synthase), and *ATR1* (encoding an ABC Transporter) in *C. nicotianae* (2, 7, 8, 10, 12, 22); and *CZK3* (encoding a MAP kinase) in *C. zeae-maydis* (29). Mutants in which the genes encoding cercosporin biosynthetic enzymes were disrupted (CTB1, CTB5, CTB6, and CTB7) produced fewer lesions as compared to the wild-type when these mutants were inoculated onto tobacco leaves, demonstrating that cercosporin is an important aggressiveness factor in disease development (6, 17).

There are no reports on the genes involved in cercosporin production by *C. coffeicola*, although coffee leaf spot severity has been correlated with light. It has been shown that shading reduces fungal penetration and that fewer lesions develop on shaded leaves (18). Through studies conducted at the Universidade Federal de Viçosa (UFV) (25, 30), we found high variability in cercosporin production by isolates of *C. coffeicola*

collected in different locations of Minas Gerais state. Souza (30) also found a positive correlation between cercosporin production and aggressiveness. These studies suggest that cercosporin is an important aggressiveness factor for *C. coffeicola* infection on coffee, and that efforts to identify genes involved in cercosporin resistance may provide a novel strategy for developing coffee with resistance to the Cercospora leaf spot.

Genes that confer resistance to cercosporin have been inserted into the fungus *Cochliobolus heterostrophus* and in transgenic tobacco plants (27, 34, 35). These studies demonstrated increased cercosporin resistance in *C. heterostrophus* and also resulted in a reduction in the size of lesions following inoculation of the transgenic tobacco plants, confirming the utility of engineering with cercosporin-resistance genes.

In our research, we aimed to identify genes present in *C. coffeicola* that are involved in cercosporin production and auto-resistance to the toxin. The research was conducted in the Department of Plant Biology, North Carolina State University, from September 2009 to August 2010. Specifically, we accomplished the following:

1- Developed a genetic transformation protocol for *C. coffeicola* and confirmed transformation using green-fluorescent protein (GFP);

2- Recovered and sequenced *C. coffeicola* homologues for *CTB1* and *ATR1*, genes that are known to be associated with cercosporin production and auto-resistance in *C. nicotianae*;

3- Generated mutants of *C. coffeicola* with differential cercosporin production using *CTB1* gene disruption technology;

4- Quantified, in vitro, cercosporin production, growth rate, and sporulation of wild type and mutants isolates.

We anticipate that our results will contribute to the development of coffee plants with resistance to *C. coffeicola*, as well as to generate information that may enhance the efficient development of new fungicides.

2 – Materials and Methods

2.1 – Isolates

We used a total of six Brazilian isolates of *C. coffeicola* differing in production of cercosporin and aggressiveness. These isolates came from three Minas Gerais regions (Mata [M], Sul de Minas [S], and Triângulo [T]) and from two cropping systems (conventional [C] and organic [O]). The six isolates used were: WT-MO53, WT-SO40, WT-SC31, WT-MC56, WT-TO02, WT-TC07 (the meaning of the isolate codes are: WT = wild type; M, S, T = regions; C, O = crop systems; numbers are random) (Fig. 1). The isolates were routinely cultured on "complete medium" agar or potato dextrose agar at 25ºC in either lighted (for cercosporin production) or dark growth chambers as described (2). All analysis of variance and means comparisons were performed with SAS® v. 9.1.

2.2 – *Cercospora coffeicola* DNA isolation

The same methodology for DNA isolation was used for wild type and *CTB1* mutants. Genomic DNA was isolated from mycelia according to a protocol previously described (11). *C. coffeicola* mycelial disks (0.5 mm diameter) were placed onto the center of Petri dishes containing potato-dextrose-agar (PDA) medium, incubating at 25ºC under a 12-h photoperiod. After 12 days, nine mycelial disks (0.5 mm diameter) taken from the border of a fungus colony were transferred to 200 ml of potato-dextrose-broth (PDB) medium in 500-ml Erlenmeyer flasks that were kept under continuous agitation (150 rpm) at 25ºC in the dark. After 4 days, the mycelium was harvested by filtration, stored for 12-h at -80ºC, and lyophilized for 72h. The dry mycelium was ground in liquid nitrogen to a fine powder and placed into 1.5 ml tubes at -20ºC. To extract *C. coffeicola* DNA from the powdered mycelium we used the Fungal DNA Miniprep kit (Omega Bio-Tek).

2.3 – Amplification of an ABC Transporter (ATR1) and a polyketide synthase (CTB1) from *Cercospora coffeicola*

Standard methods for PCR amplification, cloning, and sequencing were those in current use in the Daub's laboratory as described (2, 8). PCR amplification of *C.coffeicola* homologues ATR1 and CTB1were conducted from a single isolate, WT-SO40, that had high levels of cercosporin production and aggressiveness. Since ATR1

is highly conserved, *ATR1*-specific primers derived from *C. nicotianae* sequences were used to generate multiple PCR products that were sequenced and assembled to obtain the full-length genomic copy. Amplification of ATR1PCR was performed using standard procedures in 50 µl volumes using Denville ® Taq Polymerase (Scientific, Metuchen, NJ) with the following mix: 5µl of 10x PCR buffer, 0.3 µM of dNTP, 0.3 µM of each primer, 100 ng/µl of DNA, and 2 µl Taq DNA polymerase. The standard thermal cycling program consisted of: 4 min at 94ºC; 30-40 cycles of 45 sec at 94ºC, 45 sec at 50-70ºC, and 1–5 min (varying upon the length of the expected PCR products; generally conducted as 1 min per 1.0 Kb) at 72ºC; and 10 min at 72ºC. For amplification of CTB1 from *C. coffeicola* DNA we used both CTB1-specific primers, derived from *C. nicotianae* sequences, and degenerate primers derived from highly conserved polyketide synthase sequences. The PCR was performed using standard procedures in 50 µl volumes using Apex ® Taq Polymerase (*Genesee Scientific*, San Diego, CA, USA) with the following mix: 5µl of 10x PCR buffer, 0.3 µM of dNTP, 1,5 µM of $MgCl_2$, 0.3 µM of each primer, 100ng/µl of DNA, and 1 µl Taq DNA polymerase. The standard thermal cycling program consisted of: 5 min at 94ºC; 30-40 cycles of 45 sec at 94ºC, 45 sec at 50-70ºC, and 1–7 min (varying upon length of expected PCR products; generally conducted as 1 min per 1.0 Kb) at 72ºC; and 10 min at 72ºC. Commercial kits were used according to the manufacturer's instructions unless otherwise stated. As previously mentioned, primer sequences were designed from conserved regions to the *C. nicotianae* CTB1 and ATR1 sequence available in GenBank database from the National Center for Biotechnology Information (accession numbers AAT69682.1 and ACD42872.1, respectively). PCR primers were synthesized by Integrated DNA Technologies. The PCR products were sent to Eton Bioscience for sequencing. Sequencing results and gene assembly were done using the sequence analysis software Vector NTI v.10 (Invitrogen).

2.4 –*Cercospora coffeicola* protoplast generation

For protoplast generation, we used a protocol adapted from *C. nicotianae* (20). Five plugs (0.5 mm diameter each) from the margin of a fungal culture grown on PDA were placed in a microcentrifuge tubes with 2 ml of sterile PDB with sterile glass beads and vortexed for 2 min. The ground mycelia was placed in 50ml of PDB in 125ml Erlenmeyer flasks; the flasks were covered with black plastic and kept in complete darkness at 25ºC/150 rpm. After 4 days, we ground the 50ml-culture for 10 sec in a

sterile mixer/grinder and transferred the ground culture to 200ml of PDB in 500ml flasks that were covered with black plastic. The flasks stayed in complete darkness at 25ºC/150 rpm. After 24 h, the mycelial suspension was centrifuged at 4ºC in sterile bottles for 10 min at 8,000rpm, the supernatant was discarded, and the pellet was washed by re-suspending it in 100ml of cold wash solution (1M NaCl, 10mM $CaCl_2$). The solution was centrifuged for 15 min at 8,000 rpm, the supernatant was discarded, and 25ml of enzyme mix solution (Lysing-enzyme 10 mg / ml; β-Glucuronidase 1%; Kitalase 10 mg / ml; Driselase 10mg/ ml; Vinoflow 64 mg/ml) was added to the tubes which were incubated with shaking at 30ºC/ 60 rpm during 4 h. The solutions were checked for protoplast generation every 30 min under light microscope. After the protoplasts were released, the enzymatic solution was filtered through a sterile funnel, layered with glasswool plus four layers of cheesecloth into 50 ml sterile conical tubes. The solution was centrifuged for 10 min at 5,000 rpm using a swinging rotor, and the supernatant was discarded. We re-suspended gently the protoplasts in 10-15 ml of sterile cold STC (1 M Sorbitol, 10 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$) by tapping and inverting the tubes. We centrifuged the solution at 10 min at 5,000 rpm, discarded the supernatant, and re-suspended the protoplasts gently in 1.5 ml of cold STC. The concentration of protoplasts was adjusted to 10^8 cells / ml using a haemocytometer. The protoplast solution was stored at -80ºC in a solution of four parts STC and one part of 60% PEG. The protoplasts generated were used for transformation with a GFP construct or a CTB1 disruption construct.

2.5 –*Cercospora coffeicola* transformation with Green Fluorescent Protein (GFP)

The plasmid pCT74 (24) containing the toxA constitutive promoter fused to a modified version of the green-fluorescent protein gene (sgfp) and a modified hygromycin resistance gene (HygR) was used to generate GFP transformants. *Escherichia coli* strain DH5α was transformed with pCT74 using standard transformation protocols. Briefly, tubes containing *E. coli* competent cells stored at -20ºC were thawed on ice. Approximately 200 ng/µl of the chilled pCT74 were added to a 1.5 ml microcentrifuge tube containing 50 µl of competent cells, and they were mixed gently and placed on ice for 30 min. Cells were heat-shocked by placing the tube for 1 min at 42ºC followed by chilling on ice for 5 min. After heat-shocking the cells, 950 µl of Luria broth (LB) were added, and the cells were incubated at 37º for 1 h. After the last step, we spread 100 µl of the cell suspension onto15 cm-diameter Petri dishes

containing LB solid medium with ampicillin (100 μg / ml) and incubated at 37ºC overnight in the dark. Single colonies were transferred to tubes with 5 ml of LB and 5 μl of ampicillin, incubating at 37ºC at 75 rpm overnight in the dark. For plasmid DNA extraction we used the GeneJET™ *Plasmid Miniprep Kit 0503 (Fermentas)*. The *E. coli* solution was centrifuged at 6,500 rpm/3 min/4ºC, the supernatant was discarded, and we added 250 μl RNAase and mixed. We added 250 μl of Lysis solution and incubated the mixture at 25ºC for 5 min followed by adding 350 μl of neutralization solution and centrifuging at 5 min at 13,000 x g. The supernatant was transferred to spin columns and centrifuged for 1 min at 13,000 x g followed by adding wash solution and centrifuging again twice. In the last step, we added 50 μl of deionized water at 70ºC and centrifuged 2 min at 13,000 x g, and the DNA concentration was quantified. The pCT74 vector was digested with SalI, ClaI and NcoI to verify the vector construction.

The *C. coffeicola* GFP transformation protocol was adapted from those described by Chung et al. (11) and Lorang et al. (24) and used polyethylene glycol to facilitate DNA uptake. For the transformation, were used 400 ng /μl purified plasmid to make the direct transformation into the wild-type strain protoplast. Fungal transformants were selected on regeneration minimal medium (RMM- 1 M sucrose, 0.1% Yeast Extract, 0.1% Casein) containing 125 μg/ml of hygromycin (HYG), plated in large Petri dishes (15 cm diameter) and incubated in the dark for 12 days at 25ºC. The growth of individual colonies was monitored after 3 days incubation, and the colonies were transferred to 5 cm-diameter dishes containing PDA and HYG (125 μg/ml). The green fluorescent signal was monitored in fungal colonies in culture by microscopic analyses. Microscopic analyses were performed using a Zeiss Axiophot phase-contrast microscope. Hyphae growing on media were collected from each individual colony and placed onto glass slides, covered with a cover slip, and observed using a 450–490 nm excitation filter and a 520 nm barrier filter for observation of GFP (11).

2.6 – CTB1 gene disruption

The isolates WT-SO40 and WT-SC31 (highest cercosporin production and high aggressiveness) were selected for CTB1 gene disruption experiments. A gene disruption strategy similar to the one described by Amnuaykanjanasin and Daub (2) and by Choquer et al. (8) was used to create *CTB1*-disrupted strains. A disruption vector pCTB115 containing a hygromycin-resistance gene (*HYG*) flanked by CTB1 sequences was used for *CTB1* gene replacement. The vector pCTB115 has been previously

described and used by Choquer et al. (8). To facilitate gene replacement, DNA fragments containing a split hygromycin phosphotransferase B gene (*HYG*) marker (5′ *CTB1* fused with 3′ *HYG* fragment and 5′ *HYG* fused with 3′ *CTB1* fragment) were amplified from pCTB115, and the PCR products were directly transformed into the wild-type strains according to the protocol described by Choquer et al. (8). Two different overlapping PCR fragments were amplified from pCTB115 and were used as part of the split-marker strategy to disrupt the target gene. A 2.6-kb fragment containing 5′ *CTB1* fused with 3′ *HYG* was amplified using primers 0315L (5′-ggcagtctcacagctcttgag-3′), hyg3 (5′-ggatgcctccgctcgaagta-3′); and another2.4-kb fragment containing 5′ *HYG* fused with 3′ *CTB1* was amplified with 0315L (5′-ggcagtctcacagctcttgag-3′) and hyg4 (5′-cgttgcaagaactgcctgaa-3′). The resulting PCR products were used to transform *C.coffeicola* protoplasts, and the transformed cultures were selected according to the method described above for *C. coffeicola* GFP transformation.

The transformants were screened initially for lack of red pigment production in culture according methodology described below, and *C. coffeicola* transformants that did not produce cercosporin were verified by PCR to confirm successful disruption. To PCR reaction, the primers 3R (5'-CTCCAAGAACGTTTCGCTGT-3') and OUTF (5'-CCATCTCATCTGCACT TCCGTTCTT-3') were used to amplify a fragment of CTB1 gene specific only to the intact WT sequence. The 3R primer was designed to hybridize inside of the CTB1 region (position of the 3240th nt) putatively disrupted by the disruption-cassette, whereas the OUTF primer hybridized at the 5' upstream region (-200nt). The second set of primer OUTF and HYG3 (5'GGATGCCTCCGCTCGAAGTA3') was used to amplify a region spanning the disruption construct to confirm gene disruption the CTB1 gene by the HYG gene.

2.7 – Assays to evaluate cercosporin production

To assay for cercosporin production, the transformants and the wild type were grown on PDA at 25ºC, 12 h light, conditions that induce cercosporin production by the wild type. The production was visually checked as a red pigment on the under-side of colonies 4-5 days after plating. Cercosporin production was also quantified by extracting mycelial plugs in 5N KOH. The amount of cercosporin in the extract was quantified spectrophotometrically by measuring absorbance at 480, 590 and 640 nm (39). The wild type strain used to generate the mutants as well as the other *C. coffeicola*

strains were used as controls. The experiment was in randomized complete block design with three replicates (one tube = one experimental unit).

2.8 – Assays of growth and conidia production

C. coffeicola wild type and CTB1 mutant strains were grown in Petri dishes containing PDA medium with and without HYG (125μg/ml). Each plate was inoculated with a mycelial disk (0.5 mm diameter) taken from the border of a fungal colony. Grown was assessed by measuring the colony diameter of each isolate over a 12 day period in intervals of 3 days. The technique of drying the mycelial mass was used to induce conidial production (31, 32). Three mycelial disks (0.5 mm diameter) taken from the border of a fungal colony were transferred to 10 ml of V8 medium (200 ml V8 plus 800 ml of distilled water) in 25-ml Erlenmeyer flasks that were kept continuously agitated (120 rpm) at 25ºC, 12 h light. After 4 days, the content of each flask was poured into 10 cm-diameter Petri dishes that were kept open under white fluorescent lamps, at 25ºC, 12-h photoperiod. After dehydration of the culture medium (approximately 4 days of incubation), 10 ml of distilled water were added to each Petri dish, the fungal colony was scratched with a glass rod, and the suspension was filtered through one layer of cheesecloth. The conidial concentration was evaluated with a haemocytometer. Each experiment was conducted twice, each time in a randomized complete design with three replicates (one plate= one experimental unit).

3 – Results

3.1 – Cercosporin production

Cercosporin production of wild type cultures was quantified from mycelial plugs grown on PDA as described. The isolate WT-SO40 had the highest cercosporin production, 25.17 μM/mycelial plug of 0.5 cm diameter, compared to WTZC56 where the presence of the toxin was not detectable (Fig. 2). The wild type isolate with highest cercosporin production (WT-SO40) was selected for disruption of the CTB1 gene.

3.2 – Amplification of an ABC Transporter (ATR1) and a polyketide synthase (CTB1) from *C. coffeicola*

To sequence the full-length of ATR1 from *C. coffeicola*, ATR1-specific primers were used to amplify multiple PCR products that were subsequently sequenced and assembled to obtain the full-length genomic copy. ATR1-specific primer design was facilitated by the high degree of nucleic acid and protein homology observed for ATR1 homologs of various fungal species sequences available in the GenBank database.

The sequence of ATR1 from *C. nicotianae* (accession number EU530631) was compared to publicly available homologs by using the Blastn or Blastp algorithms. Alignments resulting from these analyses were used to design ATR1-specific primers. A total of 25 primers were used to amplify multiple ATR1 fragments from *C. coffeicola* that were subsequently assembled using the software Vector NTI (data not showed). To determine the presence of the ATR1 homolog in *C. coffeicola*, the full-length (4369nt) was also amplified by PCR using ATR1 specific primers (Fig. 3). The genes from *C. coffeicola* and *C. nicotianae* were found to be 93.3 % identical, considering the sequence given by Amnuaykanjanasin and Daub (2).

We identified a 72 bp intron located between the 3778^{th} nt and the 3849^{th} nt. This intron is also found in ATR1 from *C. nicotianae* (2). The ATR1 gene from *C. coffeicola* was translated into a 1456 amino acid (aa) protein showing 96.8% identity and 97.4% similarity to the ATR1 from C. *nicotianae.* Thus, as already observed for ATR1 homologs from different fungi, ATR1 from *C. coffeicola* is also highly conserved. A premature stop codon (TGA) was found at position 313^{th} nt (105^{th} aa). We are further investigating this region of the gene to confirm the presence of the stop codon. However, this premature stop codon in *C. coffeicola* will result in a truncated and inactivated ATR1 protein (Fig. 4 A and B).

The CTB1 full-length gene was amplified by polymerase chain reaction (PCR) using a total of 37 primers (data not showed). Initially, four degenerate primers were designed from two CTB1 conserved regions [Ketosyntase (KS) and Acyl Transferase (AT)] from *C. nicotianae* and were used to amplify the CTB1 homolog from *C. coffeicola* gDNA. The products of the reactions were sequenced and used to generate new primers. Four primers outside the CTB1 gene from *C. nicotianae* were designed to obtain information on non-coding 5'- and 3'-end regions of the gene in *C. coffeicola*. The size of the CTB1 gene from *C. coffeicola* is 7044 nt, and it is 90.3% identical to CTB1 from *C. nicotianae*. The size of the CTB1 protein in *C. coffeicola* is 2196 aa and it is 98.2% similar and 97.5% identical to its counterpart in *C. nicotianae*. Similar to *C. nicotianae,* CTB1 in *C. coffeicola* showed a total of eight small introns. The CTB1 introns in *C. coffeicola* ranged from 47 to 80 bp in size and they are distributed throughout the gene, as follows: Intron 1: 294-344; Intron 2: 649-706; Intron 3:1488-1534; Intron 4: 1687-1743; Intron 5: 1793-1839; Intron 6: 4504-4583; Intron 7: 4940-5000; and Intron 8: 6314-6368 (Fig. 5 A and B). The *C. coffeicola CTB1* sequence was deposited in the GenBank database under accession number HQ173811. Alignment analysis of five conserved protein domains (keto synthase, an acyl transferase, two acyl carrier protein a thioesterase/claisen cyclase) of *C. nicotianae* (GenBank accession number AY649543) with *C. coffeicola* showed high level of conserved amino acids between these proteins (Fig. 5C).

3.3 – *Cercospora coffeicola* protoplast generation and transformation with Green Fluorescent Protein (GFP)

Due to the lack of a previously described protocol for protoplast production from *C. coffeicola*, we had to determine the optimal conditions for protoplast isolation from this fungus. We adapted the protoplast production protocol described previously for *C. nicotianae*. Briefly, the lysing step was carried in the presence of complex enzyme mix that included *β*-glucuronidase, lysing enzyme mix, kitalase, driselase, and vinoflow. High yield of protoplasts ($\approx 10^8$ protoplast/ml) were obtained from *C. coffeicola* (Fig. 6B). To first test the transformability of *C. coffeicola* protoplasts, we transformed the fungus with a GFP construct. *C. coffeicola* strains transformed with GFP (pCT74 plasmid) were analyzed by fluorescence microscopy. Out of 43 hygromycin-resistant colonies obtained, one highly expressed GFP (Fig. 6 A and C).

3.4 – CTB1 gene disruption and evaluation of cercosporin production

Previous work has described procedures to generate CTB1-disrupted strains of C. *nicotianae*. The generated mutant strains did not produce cercosporin and had lost aggressiveness. However, similar studies are lacking for *C. coffeicola*. Based on the high similarity found between the CTB1 proteins of *C. nicotianae* and *C. coffeicola*, we hypothesized that disruption constructs used to disrupt CTB1 from *C. nicotianae* would be effective in disrupting the CTB1 homolog from *C. coffeicola*. The vector pCTB115 containing a hygromycin-resistance gene (*HYG*) cassette used to disrupt CTB1 from *C. nicotianae* was also used for CTB1 gene replacement of *C. coffeicola*. We first propagated the vector pCTB115 in *E. coli*. As described in Materials and Methods, a split-marker approach was used to generate two different PCR products with overlapping hygromycin sequences. These PCR products were use directly to transform the isolates (Fig. 7).

Since cercosporin is red, toxin-deficient mutants are easily screened for lack of red pigmentation on PDA medium. To verify the cercosporin production from wild type and CTB1 disrupted strains, cercosporin was extracted from cultures grown in solid medium supplemented with 125 μM hygromycin (HYG). In addition to chemical analysis, a molecular analysis by PCR reaction to verify CTB1 gene disruption was undertaken. As expected, on PDA medium the wild-type had red color around of the mycelium due to cercosporin production whereas the red pigment was not observed for many transformed strains. To confirm that the strains were stable transformed strains to lack to cercosporin production, they were transferred six times to PDA dishes supplemented with HYG before we quantify the cercosporin production. Cercosporin production is readily detectable by a green-color when in the presence of 5N KOH, that is not observed in strains that fail to produce cercosporin (Fig. 8). Most regenerated cultures were hygromycin-resistant and cercosporin producer, therefore, we included two these strains in our analyses as control (MUT5 from WT-SO40 and MUT13 from WT-SC31). Out of 126 Hyg-resistant cultures obtained for both isolates, nine did not have detectable (ND) levels of cercosporin by KOH extraction method (Fig. 9). Out of 126 Hyg-resistant cultures a total of 111 strains were obtained from the highest cercosporin production isolate (WT-SO40) and 15 Hyg-resistant strains were obtained from WT-SC31. Out of 111 strains obtained from WT-SO40 and 15 from WT-SC31 wild type, only four and five strains did not produce cercosporin, respectively (Fig. 8 and 9). Cercosporin absorbance values differed between wild type and CTB1-disrupted

strains unable to produce cercosporin. The typical spectrum of cercosporin (with peaks at 480, 590 and 640 λ) extracted from wild type and strains that produced cercosporin (Fig 10A) was not observed when the toxin was extracted from the non-producing strain (Fig.10B).

Strains in which cercosporin was not detected by KOH extraction method were analyzed by PCR to confirm targeted disruption of CBT1 gene of *C. coffeicola*. The primers 3R and OUTF were used to amplify a fragment of CTB1 gene specific only to the intact WT sequence (Fig. 11A). The second set of primer OUTF and HYG3 was used to amplify a region spanning the disruption construct to confirm gene disruption the CTB1 gene by the HYG gene (Fig. 11B). Nine strains lacking cercosporin production were analyzed molecularly, and six of these strains (MUT4, MUT7, MUT12, MUT88, MUT95 and MUT98) were confirmed to be disrupted in the CTB1 gene. To the others three strains (MUT6, MUT11 e MUT27) we were not able to detect the CTB1 disruption by PCR reaction (Fig. 12).

3.5 – Growth and conidia production for wild type and CTB1 disrupted strains

Wild type and the disrupted transformants were analyzed for growth and conidial production. For the growth studies, according to the Levene's test, there was no homogeneity of variances between the two experiments to both wild type strains ($P < 0.0001$), consequently each experiment was analyzed independently. The growth for both wild type strains was not statistically different of the CTB1-disrupted strains on PDA medium for the first ($P = 0.9434$ and $P = 0.8993$ to WT-SO40 and WT-SC31, respectively) and second ($P = 0.9753$ and $P = 0.8354$ to WT-SO40 and WT-SC31, respectively) experiments. The growth at both strains on PDA supplemented with HYG was statistically different in both experiments ($P < 0.0001$). The average radial growth on PDA medium to the strains from WT-SO40 ranged from 1.64 cm (MUT27) to 1.11 cm (WT-SO40), at the first experiment, and from 2.35 cm (MUT98) to 1.80 cm (WT-SO40), at the second experiment. The average radial growth on PDA medium to the strains from WT-SC31 ranged from 1.55 cm (MUT11 and MUT12) to 1.28 cm (WT-SC31), at the first experiment, and from 2.28 cm (MUT11) to 1.79 cm (WT-SC31), at the second experiment. Radial growth on PDA supplemented with HYG was zero to both wild type strains (WTSO40 and WTSC31), at the first and second experiment. The largest radial growths on PDA supplemented with HYG to strains from WT-SO40 were 1.43 cm (MUT98) and 2.11 cm (MUT27) on the first and second experiments,

respectively. The largest radial growth on PDA supplemented with HYG to strains from WT-SC31 was 1.54 cm (MUT11) and 2.23 cm (MUT11) on the first and second experiments, respectively. Disregarding the medium and experiment, growth of disrupted strains was not statistically different independent of the wild type (Tukey's test, $\alpha = 0.05$). In both experiments, the growth of disrupted strains was not significantly affected by presence of the antibiotic HYG in the medium, whereas the wild type isolates were sensitive to HYG (Table 1).

For the sporulation experiments for both wild type strains and respective mutants, according to the Levene's test, the variances of both experiments were homogeneous ($P = 0.5243$ and $P = 0.6354$ to WT-SO40 and WT-SC31, respectively). Therefore, to compare the isolates we analyzed each experiment separately. At each experiment, the sporulation differed between strains from both wild type ($P < 0.0001$). Sporulation of strains from WT-SO40 ranged from 262,500 conidia/ml (WT-SO40) to 13,000 conidia/ml (MUT98) and 215,000 conidia/ml (MUT27) to 125,000 conidia/ml (MUT88) on the first and second experiments. Therefore, the sporulation of strains from WT-SC31 ranged from 292,500 conidia/ml (MUT12) to 14,250 conidia/ml (MUT4) and 485,000 conidia/ml (MUT11) to 170,000 conidia/ml (MUT27) on the first and second experiments. No correlation was detected between cercosporin production and conidia production to all strains from both wild type. The ability of a given isolate to sporulate varied between experiments. For example, the most- and less-sporulating isolates were MUT12 and MUT98 in the first experiment and MUT11 and MUT88 in the second, respectively (Table 2).

4 – Discussion

Many species of plant pathogenic fungi produce toxins as a trait to virulence and to establish the parasitism with their hosts (28). Specifically *Cercospora spp.* commonly produce cercosporin that is a light-activated photosensitizing toxin as a pathogenicity factor (15). Mutants of *C. nicotianae*, *C. kikuchii*, and *C. zeae-maydis* have been generated to confirm the role of cercosporin as a virulence factor (5, 8, 29). We have found evidence of the involvement of cercosporin on the virulence of *C. coffeicola*, as there was a positive correlation between cercosporin production and aggressiveness (30). To conclusively show the relatedness between cercosporin production and virulence to *C. coffeicola*, it was necessary to find the gene(s) involved in cercosporin biosynthesis and generating mutants lacking these gene(s). Therefore, we isolated a *C. coffeicola* PKS gene (*CTB1*), and an ABC transporter gene (*ATR1*). We also determined CTB1's role in fungal cercosporin biosynthesis by generating strains lacking this gene related to cercosporin production.

We found no reports about *C. coffeicola* protoplast production methodology, generation of mutants lacking cercosporin production, and on the mechanisms that coordinate fungal pathogenicity and virulence. The methods used to make transformation of some fungal species require the use of asexual conidia. However, this may pose a problem with *C. coffeicola* because of the asexual multicelled conidia the fungus produces. These multicelled conidia may become a threat in fungal transformation because it may be effective in one cell but can be masked by the presence of wild type nuclei in other cells (21). Therefore, in our study we used the protoplasts to get the transformants. We were successful in isolating protoplasts by the enzyme digestion method that digests the cell wall, using a methodology adapted from research with *C. nicotianae* (20). Considering the lack of information regarding molecular biology of *C. coffeicola*, we undertook transformation with the GFP marker to check whether this fungus is transformable, given that GFP transformation has been used with many filamentous fungi (24) to detect gene expression, protein localization, trafficking, and dynamic subcellular processes over time, as well as infection processes in the plant (37, 11, 3). We got satisfactory results with GFP transformation, and the GFP transformant was easily visualized on phase-contrast microscope due the bright green fluorescence in the mycelium. We were also able to achieve stable constitutive

expression of SGFP from *C. coffeicola* cells. Therefore we assumed that *C. coffeicola* is a transformable fungus, and GFP transformation can be used in future studies with this fungus as with gene expression.

The hypothesis about the pathway of cercosporin biosynthesis came from an earlier study that tested whether the toxin is synthesized via a polyketide pathway, although no experimental evidence was provided to support the hypothesis (26). To evaluate this hypothesis, an earlier study isolated and determined the role of a fungal polyketide gene, CTB1, in cercosporin biosynthesis by *C. nicotianae* (8). The authors found that the CTB1 is essential to cercosporin biosynthesis, and confirmed that cercosporin is biosynthesized via a polyketide pathway (8). In our study, the nucleotides and amino acid sequence of the CTB1 gene of *C. coffeicola* had strong similarity to other polyketide synthase (PKS) sequences of many fungi. The *C. coffeicola* CTB1 sequence also had strong similarities to some PKS sequences available in the GenBank database. In addition to similarity to the CTB1 protein involved in cercosporin production by *C. nicotianae* (accession number AY649543), it was also similar to the ALB1 protein involved in the conidial pigment biosynthesis in *Penicillium marneffei* and *Aspergillus fumigatus* (accession numbers XM002147681 and XM751002, respectively). The *C. nicotianae CTB1* gene consists of five catalytic domains: a keto synthase (KS), an acyltransferase (AT), a thioesterase (TE), and two acyl carrier protein (ACP) domains that are distributed along the gene, similar to other other fungal PKS genes (8, 38). These domains were also found for *C. coffeicola* and are hypothesized to be required for elongation and condensation of cyclic cercosporin. Other genes, as CTB3, CTB4, CZK3, and CFP, are also involved with cercosporin production, confirming that cercosporin biosynthesis is probably a process that involves multiple genes and signals (5, 8, 9, 17, 29).

The ATR1 gene was reported to be involved in the resistance to cercosporin (2). We were successful in isolating and sequencing the ATR1 gene from *C. coffeicola*. This gene was found to be a conserved one, as it is very similar to other ABC transporters in different fungal species. Understanding the role of genes, especially the conserved ones such as ATR1, can be very helpful in studies related to evolutionary and taxonomic characteristics of fungi. It has been shown that the ATR1 gene of *C. nicotianae* is involved in both production and resistance to cercosporin (2). The fungal resistance transporters may be divided in three groups depending on the process they are involved in: 1- those that regulate self-resistance and efflux of compounds, as Atrd of *Aspergillus*

nidulans and ATR1 of *C. nicotianae* (1, 2); 2- those involved only in protection, as SirA of *Leptosphaeria maculans* (19); and 3- those related to the secretion of endogenously produced compounds, as CTB4 of *C. nicotianae* (9). The understanding of which mechanism a fungus uses and which genes are involved in the resistance is important in the development of new fungicides and resistant cultivars. For instance Kuykendall and Upchurch (23) generated sugar beet plants resistant to cercosporin that expressed the fungal CFP. Therefore, we may foresee a development of a coffee cultivar that expresses a fungal gene conferring resistance to cercosporin. Although we are able to generate the ATR1 sequence, we believe that this copy may be inactive in *C. coffeicola* because of the presence of the premature stop codon. Anyway, our gene was similar of ATR1 gene from *C. nicotianae* that has been shown to be active (2). If our result is correct, in future studies with *C. coffeicola*, different isolates and techniques should be used to better understanding whether ATR1 gene is really inactive, a modified copy, has multiple copies on the DNA, or it is variable among isolates. We hypothesize that as *C. coffeicola* have evolved, the gene might be inactivated, either because it is not essential to the pathogen or other genes may take the same function. Given that ATR1 may be not essential to *C. coffeicola*, we believe that other genes already described as CRG1 of *C. nicotianae*(12) or CFP of *C. kikuchii*(5), may occur in *C. coffeicola* DNA to assure cercosporin resistance.

As an outcome of the gene disruption experiment, from two *C. coffeicola* isolates we were able to generate strains that do not produce cercosporin by knocking-out the CTB1 gene. Therefore, the CTB1 gene is functionally required for cercosporin biosynthesis in *C. coffeicola*. This information is the most significant finding of our study, because the results provide the first report on the cercosporin biosynthesis pathway in *C. coffeicola*. Moreover, our results confirm the essentiality of this gene to cercosporin production, as found previously with *C. nicotianae* (8). Those authors were able to generate ctb1-disrupted strains that did not produce cercosporin and with significantly reduced virulence when inoculated onto tobacco plants. The hypothesis of involvement of cercosporin production in pathogen virulence comes from an earlier study in which mutants of *C. kikuchii* were generated through UV mutagenesis, although the gene involved in the process was unknown (36). Recently, cercosporin biosynthesis has been studied through the generation of disrupted strains from different species and genes. For example, CFP and CZK3 genes were studied to generate disrupted strains with less cercosporin production and less virulence than the wild type

isolates of *C. kikuchii* and *C. zeae-maydis*, respectively (5, 29). Subsequently, considering that the CTB gene cluster is comprised of eight encoding genes, CTB1-8, studies have been undertaken to understand the function of all genes in *C. nicotianae*. By generating the disrupted CTB3, CTB4, CTB5, CTB6, and CTB7, it was shown that these genes are involved in the cercosporin biosynthesis and, moreover, that CTB4 is also involved in the secretion of the toxin outside the mycelium (6, 9, 17). However, in some cases when the gene is disrupted, the strains can behave differently depending of the gene. For example, with CTB3 disruption, a yellow/brown pigment from the mutants accumulated in PDA medium, and was suggested to be an intermediate product of cercosporin biosynthesis (17). Our results provide an additional information confirming the importance of the CTB1 gene in cercosporin production in *C. coffeicola* as has been shown in *C. nicotianae*. Given the importance of *Cercospora spp.* in agriculture, it is worth understanding cercosporin biosynthesis, mostly because it has been consistently found that there is a correlation between cercosporin production ability and virulence of these pathogens (5, 8, 29, 30). Regarding cercosporin biosynthesis, we believe that our results are conclusive to support the hypothesis that cercosporin is biosynthesized via a polyketide pathway.

The relationship between growth and cercosporin production has been studied for different pathogenic *Cercospora spp.* (9, 36). In our study, growth in vitro was not different between strains that either produce cercosporin or not. A similar result was reported when CTB4- and CTB3-disrupted strains of *C. nicotianae* were compared, and the disrupted strains produced less cercosporin although they had a similar growth as the wild type (9, 17). Similarly, disruption of the CFP gene of *C. kikuchii* led a loss of 95% of the cercosporin production as compared as the wild type isolate with no loss of growth. In contrast, *C. kikuchii* UV-mutants unable to produce cercosporin grew more than the wild type on solid medium, although the growth on liquid medium did not differ (36). The authors considered that growth may be not a pathogenicity factor, because no cercosporin producer and nonpathogenic isolates had similar growth on liquid medium than cercosporin producers and pathogenic isolates (36). Similarly, in *C. zeae-maydis,* disruption strains for the CZK3 gene that are defective in cercosporin production also had higher vegetative growth than the wild type (29). Therefore, we can conclude that the effect of loss cercosporin production on the growth is variable among *Cercospora spp*. and this is dependently of the disrupted gene.

Cercospora spp. sporulate profusely on the lesions to generate secondary inoculum that accounts for biological fitness and survival. There are reports of an isolate being a non-cercosporin producer and having reduced sporulation (5, 29, 36), although in other studies *C. nicotianae* CTB1 and CTB3 mutants lacking in cercosporin biosynthesis sporulated similarly to the wild type strain (8, 17). We did not find a significant difference in sporulation between producing and non-cercosporin producing isolates of *C. coffeicola*. Considering the relevance of sporulation on disease progress on the field, it would be interesting to quantify both cercosporin production and sporulation on the host tissue to verify if there is any relation between them.

The data we presented demonstrate that *C. coffeicola* wild type isolates differ in the production of cercosporin from 25.17μM/plug to undetectable levels. A protoplast preparation and transformation protocol for *C. coffeicola* was developed by modifying existing protocols for *C. nicotianae*. We found that wild type isolates of *C. coffeicola* were hygromycin-sensitive when using a concentration of 125 μg/ml. As confirmed by fluorescent microscopy, we successfully transformed *C. coffeicola* protoplasts with the green fluorescent protein (GFP). Two *C. nicotianae* homologs, *CTB1* and *ATR1*, were identified and sequenced in *C. coffeicola*. Sequence analysis indicated a high degree of homology for both genes to *C. nicotianae* genes. A split marker strategy was used to generate *ctb1*-disrupted strains in *C. coffeicola*. Out of nine lacking detectable levels of cercosporin, six were confirmed to be disrupted by molecular analysis. When compared to the wild type, both growth and sporulation *in vitro* of *C. coffeicola* CTB1-disrupted strains were not significantly influenced by the lack of cercosporin production. This is the first report on the cercosporin biosynthesis pathway in *C. coffeicola*. Following previous reports that hypothesize the cercosporin toxin is a factor of pathogenesis, we will further test the aggressiveness of ctb1-disrupted strains to coffee plants and try to confirm the role of cercosporin in *C. coffeicola* aggressiveness. We expect that the molecular genetics results gained in this work will be used to advance studies on *C. coffeicola* and its interactions with coffee plants, and may provide practical uses for our knowledge for better control of Cercospora leaf spot.

5 – References

1- Andrade, A. C., Van Nistelrooy, J. G., Peery, R. B., Skatrud, P. L., and De Waard, M. A. 2000. The role of ABC transporters from *Aspergillus nidulans* in protection against cytotoxic agents and in antibiotic production. Molecular Genetics and Genomics, 263:966–977.

2- Amnuaykanjanasin, A., and Daub, M. E. 2009. The ABC transporter ATR1 is necessary for efflux of the toxin cercosporin in the fungus *Cercospora nicotianae*. Fungal Genetics and Biology, 46:146-158.

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

4- Berestetskiy, A. O. 2008. A review of fungal phytotoxins: from basic studies to practical use. Applied Biochemistry and Microbiology, 44:453-465.

5- Callahan, T., Rose, M., Meade, M., Ehrenshaft, M., and Upchurch, R. 1999. *CFP*, the putative cercosporin transporter of *Cercospora kikuchii*, is required for wild type cercosporin production, resistance, and virulence on soybean. Molecular Plant-Microbe Interactions, 12:901-910.

6- Chen, H. Q., Lee, M. H., and Chung, K. R. 2007. Functional characterization of three genes encoding putative oxidoreductases required for cercosporin toxin biosynthesis in the fungus *Cercospora nicotianae*. Microbiology, 153:2781-2790.

7- Chen, H., Lee, M. H., Daub, M. E., and Chung, K. R. 2007. Molecular analysis of the cercosporin biosynthetic gene cluster in *Cercospora nicotianae.* Molecular Microbiology, 64:755-770.

8- Choquer, M., Dekkers, K. L., Chen, H.-Q., Cao, L., Ueng, P. P., Daub, M. E., and Chung, K. R. 2005. The *CTB1* gene encoding a fungal polyketide synthase is required for cercosporin toxin biosynthesis and fungal virulence in *Cercospora nicotianae*. Molecular Plant-Microbe Interaction, 18:468-476.

9- Choquer, M., Lee, M. H., Bau, H. J., and Chung, K. R. 2007. Deletion of a MFS transporterlike gene in *Cercospora nicotianae* reduces cercosporin toxin accumulation and fungal virulence. Federation of European Biochemical Societies Letters, 581:489-494.

10- Chung, K. R., Jenns, A. E., Ehrenshaft, M., and Daub, M. E. 1999. A novel gene required for cercosporin toxin resistance in the fungus *Cercospora nicotianae*. Molecular Genetics and Genomics, 262:382-389.

11- Chung, K. R., Ehrenshaft, M., and Daub, M. E. 2002. Functional expression and cellular localization of cercosporin-resistance proteins fused with the GFP in *Cercospora nicotianae*. Current Genetics, 41:159–167.

12- Chung, K. R., Daub, M. E., Kuchler, K., and Schüller, C. 2003. The CRG1 gene required for resistance to the singlet oxygen-generating cercosporin toxin in *Cercospora nicotianae* encodes a putative fungal transcription factor. Biochemical and Biophysical Research Communications, 302:302-310.

13- Daub, M. E. 1982. Peroxidation of tobacco membrane lipids by the photosensitizing toxin, cercosporin. Plant Physiology, 69:1361-1364.

14- Daub, M. E. 1987. Resistance of fungi to the photosensitizing toxin, cercosporin. Phytopathology, 77:1515-1520.

15- Daub, M. E., and Hangarter, R. P. 1983. Light-induced production of singlet oxygen and superoxide by the fungal toxin, cercosporin. Plant Physiology, 73:855-857.

16- Daub, M. E., Herrero, S. and Chung, K. R. 2005. Photoactivated perylenequinone toxins in fungal pathogenesis of plants. Federation of European Microbiological Societies *Microbiology* Letters, 252:197-206.

17- Dekkers, K. L., You, B. J., Gowda, V. S.; Liao, H. L., Lee, M. H., Bau, H. J., Ueng, P. P., and Chung, K. R. 2007. The *Cercospora nicotianae* gene encoding dual O-methyltransferase and FAD-dependent monooxygenase domains mediates cercosporin toxin biosynthesis. Fungal Genetics and Biology, 44:444-454.

18- Echandi, E. 1959. La chasparria de los cafetos causada por el hongo *Cercospora coffeicola* Berk and Cooke. Turrialba, 9:54-67.

19- Gardiner, D. M., Cozijnsen, A. J., Wilson, L. M., Pedras, M. S., and Howlett, B. J. 2004. The sirodesmin biosynthetic gene cluster of the plant pathogenic fungus *Leptosphaeria maculans*. Molecular Microbiology, 53:1307-1318.

20- Gwinn, K. D., and Daub, M. E. 1988. Regenerating protoplasts from *Cercospora* and *Neurospora* differ in their response to cercosporin. Phytopathology, 78:414-418.

21- Gwinn, K. D., and Daub, M. E. 1989. Techniques for isolation of mutants using protoplasts from *Cercospora nicotianae*. Mycologia, 81:322-324.

22- Herrero, S., Amnuaykanjanasin, A., and Daub, M. E. 2007. Identification of genes differentially expressed in the phytopathogenic fungus *Cercospora nicotianae* between

cercosporin toxin-resistant and -susceptible strains. Federation of European Microbiological Societies *Microbiology* Letters, 275:326-337.

23- Kuykendall, L. D., and Upchurch, R. G. 2004. Expression in sugar beet of the introduced cercosporin toxin export (CFP) gene from *Cercospora kikuchii*, the causative organism of purple seed stain in soybean. Biotechnology Letters, 26:723-727.

24- Lorang, J. M., Tuori, R. P., Martinez, J. P., Sawyer, T. L., Redman, R. S., Rollins, J. A., Wolpert,T. J., Johnson, K. B., Rodriguez, R. J., Dickman, M. B., and Ciuffetti, L. M. 2001. Green fluorescent protein is lighting up fungal biology. Applied and Environmental Microbiology, 67:1987-1994.

25- Martins, R. B. 2008. Variabilidade de *Cercospora coffeicola* em Minas Gerais com base em compatibilidade vegetativa e produção de cercosporina. Tese de Doutorado. Viçosa-MG. Universidade Federal de Viçosa.

26- Okubo, A., Yamazak, S., and Fuwa, K. 1975. Biosynthesis of cercosporin. Agricultural Biological Chemistry, 39:1173-1175.

27- Panagiotis, M., Kritonas, K., Irini, N. O., Kiriaki, C., Nicolaos, P., and Athanasios, T. 2007. Expression of the yeast cpd1 gene in tobacco confers resistance to the fungal toxin cercosporin. Biomolecular Engineering, 24:245-251.

28- Scheffer, R. P. 1991. Role of toxins in evolution and ecology of plant pathogenic fungi. Experientia, 47:804-811.

29- Shim, W. B., and Dunkle, L. D. 2003. *CZK3*, a MAP kinase kinase kinase homolog in *Cercospora zeae-maydis*, regulates cercosporin biosynthesis, fungal development, and pathogenesis. Molecular Plant-Microbe Interaction, 16:760-768.

30- Souza, A. G. C. 2007. Variabilidade fisiológica de isolados de *Cercospora coffeicola*. Dissertação de Mestrado. Viçosa-MG. Universidade Federal de Viçosa.

31- Souza, A. G. C., Martins, R.B., Mizubuti, E.S.G., and Maffia, L.A. 2005. Esporulação in vitro de *Cercospora coffeicola*. In: XXXVIII Congresso Brasileiro de Fitopatologia. Fitopatologia Brasileira, 30:116.

32- Souza, A. G. C., Rodrigues, F. A., Maffia, L. A., and Mizubuti, E. S. G. 2011. Infection process of *Cercospora coffeicola* on coffee leaf. Journal of Phytopathology, 159:6-11.

33- Tessmann, D. J., Charudattan, R., and Preston, J. F. 2008. Variability in aggressiveness, cultural characteristics, cercosporin production and fatty acid profile of *Cercospora piaropi*, a biocontrol agent of water hyacinth. Plant Pathology, 57:957-966.

34- Upchurch, R. G., Rose, M. S., Eweida, M., and Callahan, T. M. 2002. Transgenic assessment of *CFP*-mediated cercosporin export and resistance in a cercosporin-sensitive fungus. Current Genetics, 41:25-30.

35- Upchurch, R. G., Rose, M. S., Eweida, M., and Zuo, W. 2005. Expression of the cercosporin transporter, *CFP*, in tobacco reduces frog-eye lesion size. Biotechnology Letters, 27:1543-1550.

36- Upchurch, R. G., Walker, D. C., Rollins, J. A., Ehrenshaft, M., and Daub, M. E. 1991. Mutants of *Cercospora kikuchii* altered in cercosporin synthesis and pathogenicity. Applied and Environmental Microbiology, 57:2940-2945.

37- Walker, D., Hunt, H., and Hager, G. L. 1999. Using inducible vectors to study intracellular trafficking of GFP-tagged steroid/nuclear receptorsin living cells. Methods, 19:386-393.

38- Yang, G., Rose, M., Turgeon, B. G., and Yoder, O. C. 1996. A polyketide synthase is required for fungal virulence and production of the polyketide T-toxin. Plant Cell 8:2139-2150.

39- You, B. J., Lee, M. H., and Chung, K. R. 2008. Production of cercosporin toxin by the phytopathogenic *Cercospora* fungi is affected by diverse environmental signals. *Canadian Journal of Microbiology, 54:259-269.*

40- Zambolim, L., Vale, F. X. R., Pereira, A. A., and Chaves, G. M. 1997. Café (*Coffea arabica*). In: Vale, F. X. R., and Zambolim, L., eds. Controle de doenças de plantas: grandes culturas. Viçosa: UFV, p.83-180.

Table 1. Average radial growth (diameter in cm) of wild type and respective *ctb1* disrupted transformant cultures grown on potato dextrose agar (PDA) and PDA supplemented with hygromycin (PDA+HYG) medium, in two experiments.

Wild Type	Strain	First experiment		Second experiment	
		PDA*	PDA+HYG*	PDA*	PDA+HYG*
WT-SO40	WT-SO40	1.11 A	0.00 B	1.80 A	0.00 B
	MUT95**	1.26 A	0.95 A	1.92 A	1.30 A
	MUT88**	1.47 A	1.18 A	2.00 A	1.80 A
	MUT98**	1.59 A	1.43 A	2.35 A	2.03 A
	MUT27	1.64 A	1.31 A	2.22 A	2.11 A
WT-SC31	WT-SC31	1.28 A	0.00 B	1.79 A	0.00 B
	MUT6	1.44 A	1.36 A	2.19 A	2.13 A
	MUT7**	1.52 A	1.40 A	2.09 A	2.09 A
	MUT4**	1.53 A	1.44 A	2.10 A	2.22 A
	MUT11	1.55 A	1.54 A	2.28 A	2.23 A
	MUT12**	1.55 A	1.51 A	2.20 A	2.22 A

*Values with different letters in the same column of a wild type strain indicate significantly different growth (Tukey's test, $\alpha = 0.05$).

** Strains that showed lack of cercosporin production and CTB1 gene disrupted by PCR reaction.

Table 2. Average sporulation (conidia/ml) of wild type and respective CTB1-disrupted-strains, in two experiments.

Wild Type	Strain	First experiment*	Second experiment*
WT-SO40	WT-SO40	262500 AB	145000 A
	MUT27	215000 B	215000 A
	MUT95**	27500 C	145000 A
	MUT88**	18000 C	125000 A
	MUT98**	13000 C	175000 A
WT-SC31	MUT12**	292500 A	170000 C
	MUT7**	282500 AB	217500 C
	MUT6	225000 ABC	342500 B
	MUT11	165000 C	485000 A
	WT-SC31	16750 D	362500 B
	MUT4**	14250 D	402500 AB

*Values with different letters in the same column of a wild type strain indicate significantly different sporulation (Tukey's test, $\alpha = 0.05$). For the statistical analysis, the sporulation values were transformed to log (sporulation).
**Strains that showed lack of cercosporin production and CTB1 gene disrupted by PCR reaction.

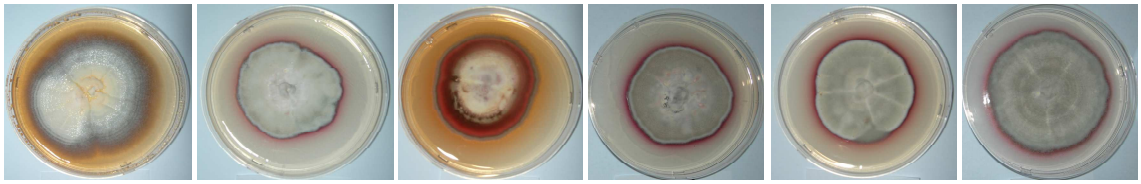

Fig. 1. Isolates of *C. coffeicola* collected from three Minas Gerais regions in Brazil (Mata, Sul de Minas, and Triângulo) from two cropping systems (conventional and organic). The isolates differ in cercosporin production and aggressiveness. The *C. coffeicola* isolates were grown in PDA dishes for 12 days at 25ºC and 12 h of light.

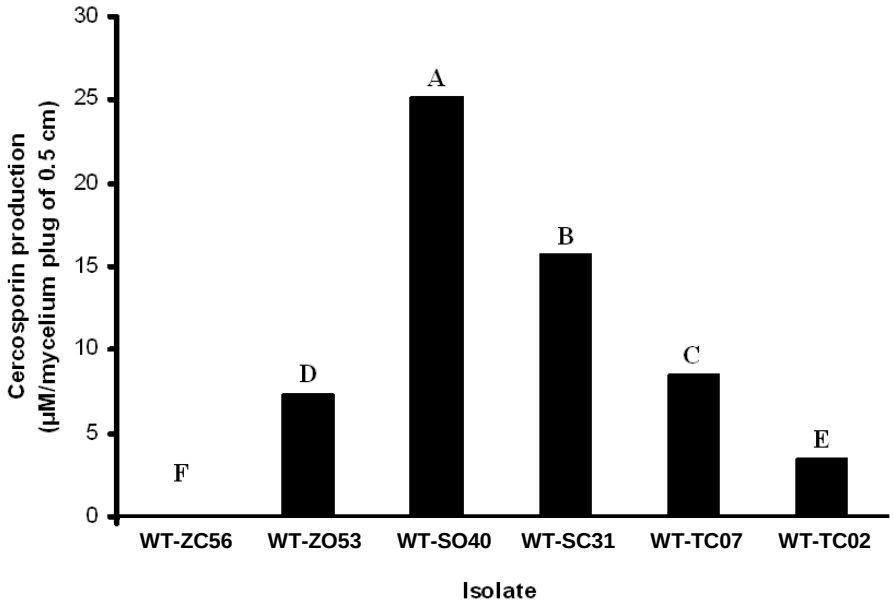

Fig. 2. Average cercosporin production of the *C. coffeicola* isolates grown on PDA (12 days, 25ºC, 12h light). Bars with different letters indicate significantly different toxin production (Tukey test, α=0.05).

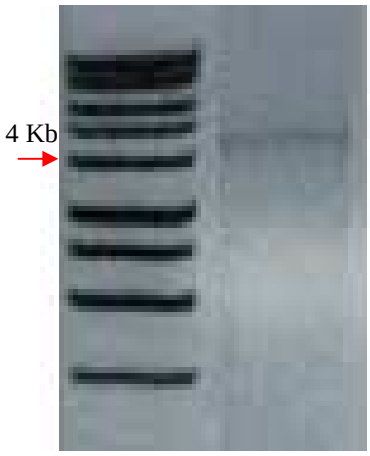

Fig. 3. PCR amplification of ATR1 transporter from *C. coffeicola* strain WT-SO40, the highest cercosporin producer. *ATR1*-specific primers were used to generate the PCR product.

(A)

atgccttccctccaacggtcagggtcgtgggtcgcacctgggcctggcagcggatcaggagaagaggtctaccgcacgctcagcaatgctatctcgagaacattcagcg
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(B)

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Fig. 4. *Cercospora coffeicola* ATR1 nucleotide and protein sequence. A- Complete ATR1 nucleotide sequence from *C. coffeicola*. The premature stop codon and an intron are highlighted in gray and black, respectively; B- ATR1 amino acid sequence. Stop codon is highlighted in gray.

(A)

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(B)

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(C)

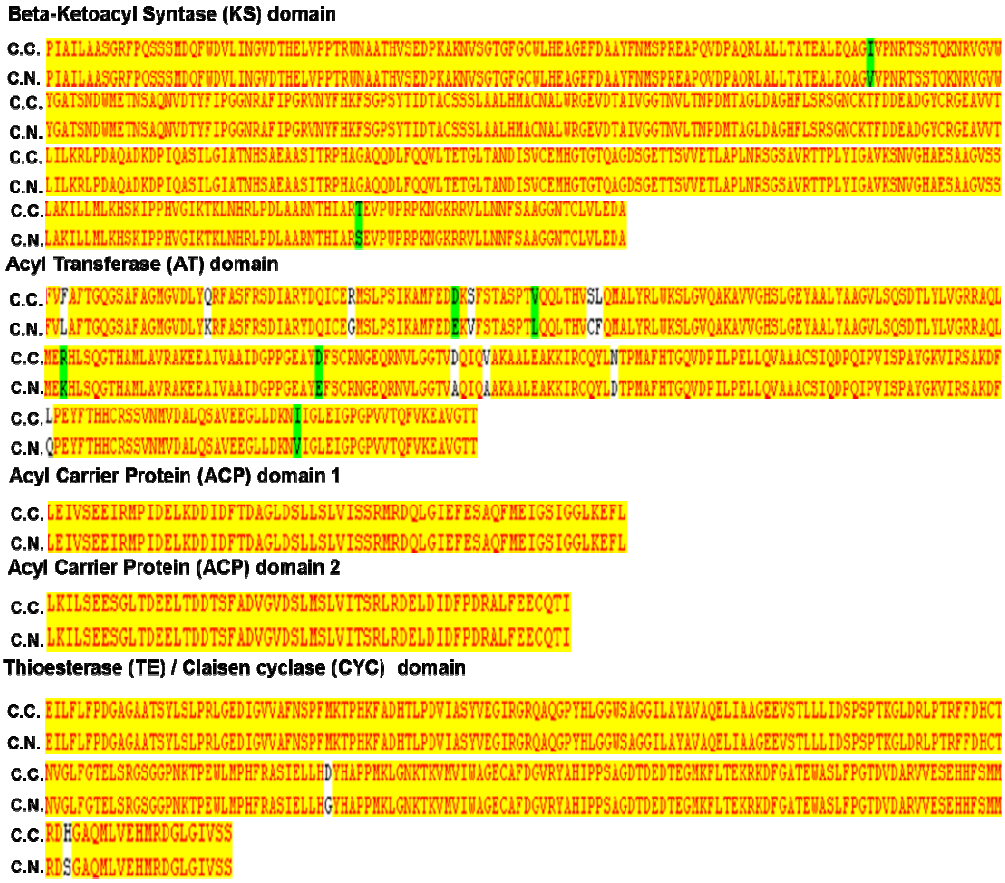

Fig. 5. A- Sequence of cercosporin polyketide synthase gene (CTB1) from *C. coffeicola* with introns highlighted in black; B- CTB1 amino acids sequence from *C. coffeicola*. C- Alignments of *Cercospora coffeicola* (C.C.) CTB1 with conserved protein domains (keto synthase, an acyl transferase, two acyl carrier protein a thioesterase/claisen cyclase) of *C. nicotianae* (C.N.) (GenBank accession number AY649543). Conserved, similar and distinct amino acids for all peptides are highlighted in yellow, green and white color, respectively.

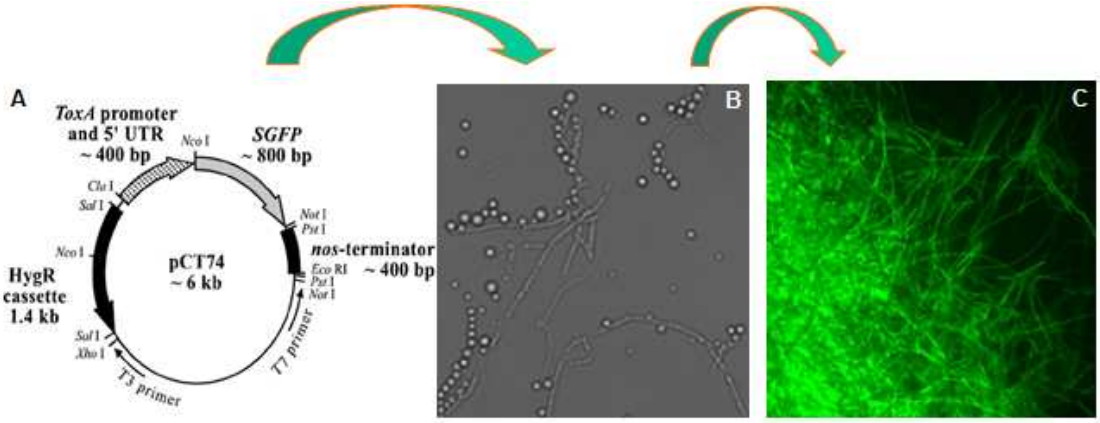

Fig. 6. (A) Fungal transformation plasmid containing the GFP gene and a hygromycin (HYG) resistance selectable marker (HygR) from Lorang et al. (24). (B) Light microscopy of *C. coffeicola p*rotoplasts obtained by enzymatic removal of cell walls of a mycelial culture (C) Mycelium of *C. coffeicola* transformed with GFP emitting green fluorescence under fluorescence microscopy.

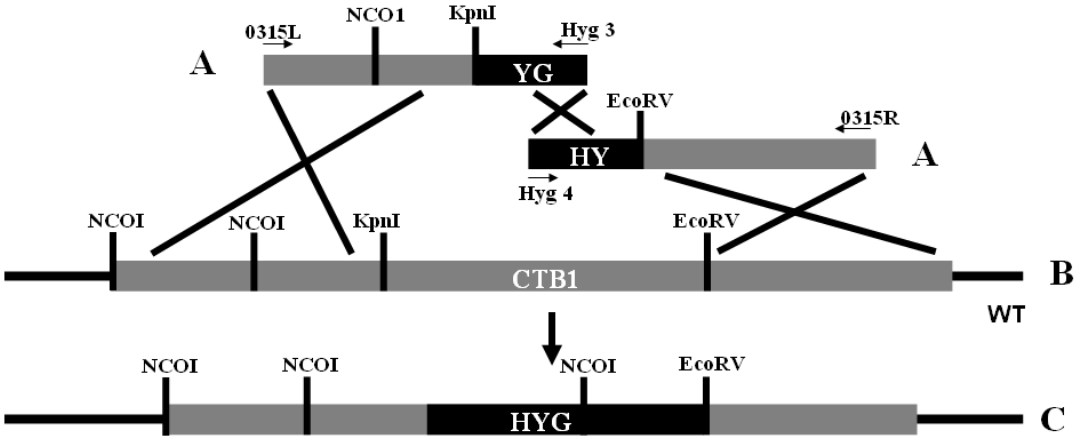

Fig. 7. Split marker strategy to disrupt *CTB1* in *C. coffeicola.* (A) Two truncated *CTB1* fragments fused with overlapping HYG sequences (HY/YG) were obtained by PCR amplification from the pCTB115 vector (Choquer et al., 2005) harboring the disruption construct. Primers 0315L/Hyg3 and 0315R/Hyg4 were used for PCR amplification. (B) Partial restriction map of the *CTB1* locus (WT= Wild Type). (C) Map of a *ctb1*-disrupted strain. Diagrams adapted from *C. nicotianae* (8).

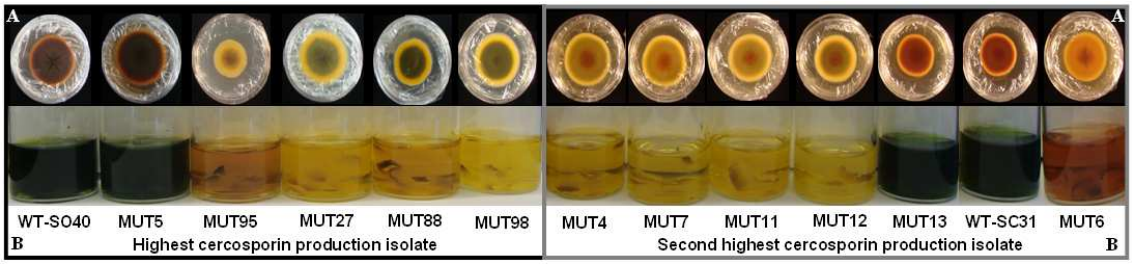

Fig. 8. Cercosporin production of wild type (WT) and 11 Hyg-resistant transformants of two isolates of *C. coffeicola*. (A) Colonies grown in PDA with HYG (12 days, 25ºC, 12h light); (B) For each culture, cercosporin was extracted from mycelial plugs in 5N KOH. The presence of cercosporin is reflected by the green color in KOH. The MUT5, MUT27, MUT88, MUT95 and MUT98 strains came from the highest cercosporin production isolate (WT-SO40). The MUT4, MUT6, MUT7, MUT11, MUT12 and MUT13 strains came from second highest cercosporin production isolate (WT-SC31). The MUT5 and MUT13 are cercosporin producers and Hyg-resistant.

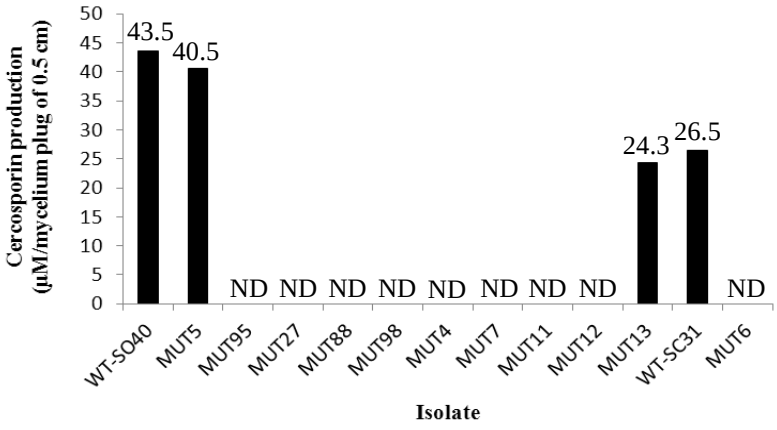

Fig. 9. Average values of cercosporin production in the wild type (WT) and transformed strains (MUT) quantified by spectrophotometry. The absorbance was measured in KOH extraction solution. Out of 126 hyg-resistant cultures obtained for both isolates, nine did not have detectable (ND) levels of cercosporin. The MUT5, MUT27, MUT88, MUT95 and MUT98 strains came from the highest cercosporin production isolate (WT-SO40). The MUT4, MUT6, MUT7, MUT11, MUT12 and MUT13 strains came from second highest cercosporin production isolate (WT-SC31).

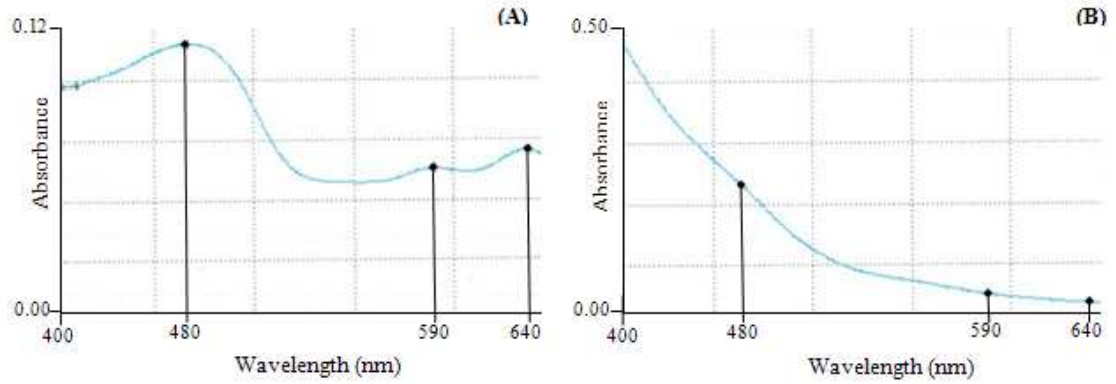

Fig. 10. Absorbance spectrum of cercosporin extracted from the wild type (A) and a transformed strains unable to produce cercosporin (B). Each colony was grown in PDA with HYG (12 days, 25ºC, 12h light), and cercosporin was extracted from mycelial plugs in 5N KOH. The presence of cercosporin is reflected by absorption peaks at 480, 590, and 640 nm. In the disrupted strains, the presence of interfering pigments prevented quantification at 480 nm.

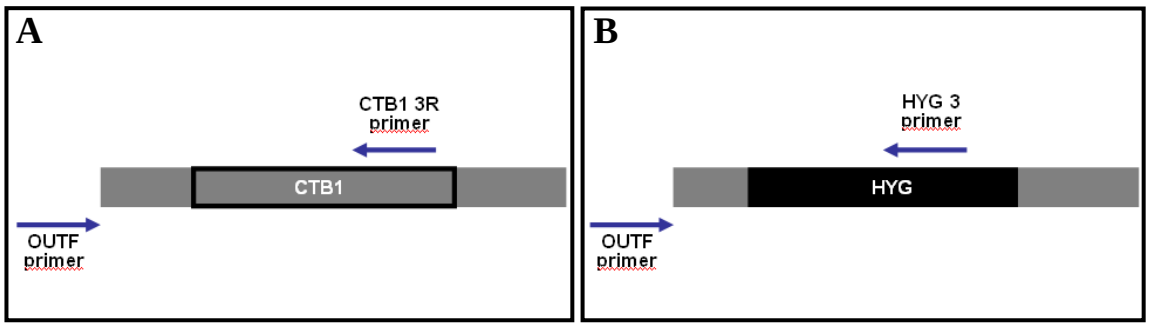

Fig. 11. Illustration of the CTB1 gene (gray bar) and HYG gene (Black bar), primers and primer annealing region on the genes used to make the PCR reaction to verify the CTB1 disrupted strains. A- Primers 3R and OUTF used to amplify a fragment of CTB1 gene specific. B- Primers OUTF and HYG3 used to amplify a region spanning the disruption construct to confirm gene disruption the CTB1 gene by the HYG gene.

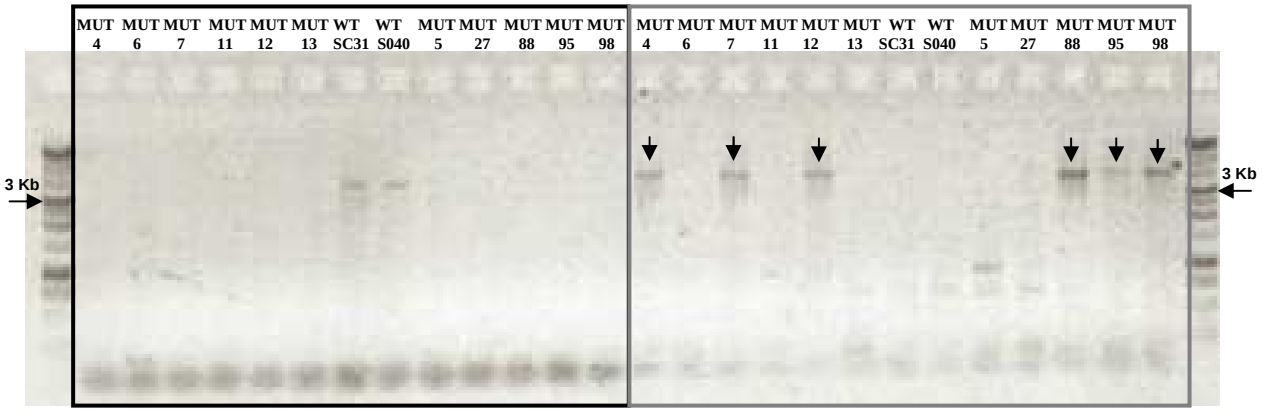

Fig. 12. Molecular analysis of *C. coffeicola* wild type (WT) and transformed cultures (MUT). Protoplasts were transformed with the two PCR fragments. Regenerated colonies were selected for both HYG resistance and lack of cercosporin production. DNA was extracted and strains were analyzed by PCR disruption of the *CTB1* locus. Two sets of primers (OUTF and 3R= 3.5 Kb; OUTF and HYG3= 4.0 Kb, showed in black and gray color box, respectively) were used to identify disrupted strains. The MUT4, MUT6, MUT7, MUT11, MUT12 and MUT13 strains came from second highest cercosporin production isolate (WT-SC31). The MUT5, MUT27, MUT88, MUT95 and MUT98 strains came from the highest cercosporin production isolate (WT-SO40). Amplified bands in the gray panel indicate presence of the disrupted gene. Arrows indicate disrupted mutants.

Progresso da cercosporiose em cafeeiros sob diferentes sistemas de produção em Ervália-MG

RESUMO

O cultivo orgânico vem crescendo nos últimos anos em muitas culturas, como na do café. Neste sistema de cultivo, um fator limitante à produção é a ocorrência de doenças, como a cercosporiose, causada por *Cercospora coffeicola*. Apesar da importância crescente da doença, os estudos são escassos e muitas vezes não conclusivos, principalmente, no que se refere à epidemiologia da doença em diferentes sistemas de cultivo. Assim, objetivou-se avaliar o progresso da cercosporiose em três lavouras comerciais de café: orgânica sombreada (LOS), orgânica (LO) e convencional (LC), situadas em Ervália-MG, de novembro/2004 a outubro/2008. Mensalmente, avaliaram-se a incidência e severidade da doença, desfolha e enfolhamento em ramos de plantas marcadas em cada lavoura. Em geral, maiores valores de incidência, severidade, área abaixo da curva de progresso de doença, doença máxima, desfolha e enfolhamento ocorreram nos ramos situados no terço superior e nas plantas localizadas na LC. Maiores valores de intensidade da doença, desfolha e enfolhamento ocorreram nos período de maio-julho, julho-setembro e outubro-janeiro, respectivamente. Por meio de análises de séries temporais, com o uso dos modelos de regressão não linear ARMA, representou-se a dinâmica da cercosporiose, em todos os anos e lavouras em estudo. Conclui-se que, na LOS e no terço inferior das plantas, ocorreram as condições menos favoráveis à ocorrência da cercosporiose, e que os modelos de séries temporais foram eficientes em descrever o progresso da doença, durante todo o período do estudo.

Palavras chave: *Cercospora coffeicola*, séries temporais, cultivo orgânico

Progress of cercospora leaf spot at different coffee production systems at Ervália-MG

Abstract

Organic crop production systems has been increasing in the last years, specially in coffee. A limiting yield factor in coffee organic system are diseases, as the cercospora leaf spot, caused by *Cercospora coffeicola*. Although the importance, studies on the disease are scarce or not conclusive. Therefore, this study aimed to understand epidemics of cercospora leaf spot epidemic on three coffee production systems: organic (LO), organic under shadow (LOS), and conventional (LC), Ervália-MG, Brasil, from November/2004 to October/2008. Disease severity (SEV) and incidence (INC), leaf fall (LF) and leaf formation (LG), were evaluated to understand cercospora leaf spot epidemics. Higher values of SEV, INC, LF, GL, area under disease progress curve, and maximum disease occurred at both LC and upper branches, whereas lower values of all variables occurred at both LOS and lower branches. Higher values of disease intensity, LF, and GL occurred in the period between May-July, July-September and October-January, respectively. The ARMA model from time series analysis described the cercospora leaf spot progress throughout the study period.

Keywords: *Cercospora coffeicola*, time series, organic system

1 – Introdução

Entre os fatores que reduzem a produtividade do cafeeiro encontram-se as doenças, como a cercosporiose, causada por *Cercospora coffeicola* Berk & Cook, que pode causar perdas de até 30% (Zambolim *et al*., 1997). Os sintomas da cercosporiose, nas folhas, incluem manchas necróticas, com centro de coloração clara, circundadas por um anel de coloração marrom arroxeado de bordos amarelecidos, e, nos frutos, manchas escuras e com aspecto ressecado (López-Duque & Fernández-Borrero, 1969; Zambolim *et al*., 1997). Nos últimos anos, em vista da expansão das lavouras cafeeiras das áreas tradicionais para as do cerrado, cujos solos geralmente têm baixa fertilidade natural e deficiência hídrica pronunciada de março a setembro, ocorreu aumento da intensidade da cercosporiose (Juliatti *et al*., 2000).

O controle da cercosporiose baseia-se, principalmente, no uso de fungicidas cúpricos e triazol. No entanto, o uso irracional desses produtos pode levar a impactos ambientais e sociais e aumentar o custo de produção. Com isso, nos últimos anos, ocorreu aumento do número de lavouras que adotam o sistema orgânico de produção. Em algumas culturas, como na do café, sob cultivo orgânico, tem-se observado redução na intensidade de doenças (Workneh *et al*., 1993; Abbasi *et al*., 2002; Santos *et al*., 2008). Com a expansão da cafeicultura orgânica e conscientização quanto aos impactos mencionados, tem-se buscado desenvolver estratégias alternativas de controle da doença para diminuir o uso de fungicidas e métodos de manejo que possam ser usados nas lavouras orgânicas (Pereira *et al.*, 2008; Botelho *et al.*, 2009; Galdeano *et al.*, 2010).

Apesar de a cercosporiose do cafeeiro ter sido estudada há décadas e ser importante atualmente, os conhecimentos sobre o progresso da doença são escassos e não conclusivos, principalmente em se tratando do cultivo orgânico. Para se fazer o uso adequado das estratégias de manejo, é essencial o entendimento da epidemiologia da cercosporiose, inclusive dos fatores que podem contribuir para a ocorrência da doença. O estresse hídrico e a alta insolação favorecem o aumento da intensidade da doença (Boldini *et al*., 2000). Apesar de a produtividade de cafeeiros conduzidos a pleno sol ser maior que a daqueles sombreados (Miranda *et al*., 1999), a intensidade da doença é maior nos cafeeiros a pleno sol (López-Duque & Fernández-Borrero, 1969; Almeida, 1986; Salgado *et al*., 2007), bem como em plantas com deficiência nutricional (Fernández-Borrero *et al*., 1982). Espera-se, portanto, que lavouras de café em plantio consorciado e que não recebam agrotóxicos, tenham menor incidência da cercosporiose,

por causa da menor insolação e da maior população de inimigos naturais. Entretanto, muitas das afirmativas carecem de rigor científico para avaliações conclusivas. Nesse contexto, demandam-se mais estudos epidemiológicos sobre a cercosporiose.

Estudos de epidemiologia comparativa podem auxiliar a entender a dinâmica da doença em diferentes sistemas de cultivo do cafeeiro. Para esses estudos, o ajuste de modelos de progresso tem papel importante, pois são relevantes para detectar e descrever o padrão temporal de ocorrência de doenças. Dentre esses modelos, os de séries temporais podem ser úteis em descrever a dinâmica de doenças (Box et al., 1994). Os modelos tradicionais usados para descrever o progresso da doença não são eficazes para descrever doenças que tenham um comportamento atípico de ocorrência ao longo da estação de crescimento da cultura, como é o caso da cercosporiose do cafeeiro. Nesse aspecto, os modelos de séries temporais são uma alternativa para descrever o comportamento de doenças que não possuem um padrão de ocorrência ao longo da estação de crescimento da cultura e, adicionalmente, podem gerar parâmetros que auxiliem à interpretação biológica do ciclo da doença. Os modelos de séries temporais, diferentemente dos modelos tradicionais de progresso de doença, consideram que os erros não são independentes, pois se avalia o mesmo individuo (planta) é repetidamente ao longo do tempo, o que possibilita melhores ajustes e descrição da doença (Box et al., 1994). Considerando-se estes aspectos associados às vantagens dos modelos de séries temporais para descrição a dinâmica de doença e o aspectos estocástico do progresso de doenças, estes modelos ainda não são amplamente usados em fitopatologia. Entretanto, alguns autores usaram os modelos de séries temporais para descrever eventos associados a epidemias de doenças (Yang & Zeng, 1992; Hudelson *et al*., 1993; Xu *et al*., 1995; Guerin *et al*., 2001; Zauza, 2007; Holb, 2008).

Espera-se que a dinâmica da cercosporiose seja diferenciada entre os sistemas de cultivo do cafeeiro. Assim, esse estudo objetivou obter informações que possam contribuir para o manejo racional da doença, principalmente considerando as perdas crescentes causadas pela doença e o número de produtores que adotam o sistema orgânico de cultivo. Especificamente, objetivou-se estudar o progresso da cercosporiose em lavouras orgânica sombreada (LOS), orgânica (LO) e convencional (LC), situadas em Ervália-MG, no período de novembro/2004 a outubro/2008, por meio de epidemiologia comparativa, com o uso de diferentes técnicas de análise, com ênfase em modelos de séries temporais.

2 – Material e Métodos

2.1 – Caracterização das lavouras de café

Conduziu-se o estudo por quatro anos consecutivos, de novembro-2004 a outubro-2008, em lavouras comerciais de café localizadas no Sítio Boa Vista, Ervália, Minas Gerais, situado à altitude de 827 m, latitude de 20º54'52" S e longitude de 42º37'20" W. Nesta região, o período chuvoso ocorre de outubro a janeiro e o da estiagem de março a agosto (dados não mostrados). Na área experimental, de relevo acidentado e solo do tipo argissolo, cultivam-se plantas de café da variedade 'Catuaí Vermelho'. Na propriedade, há três lavouras, distintas quanto ao sistema de cultivo: i- convencional (LC), com 0,6 ha, cafeeiros de 8 anos, no espaçamento de 2,0 x 1,5 m; usam-se insumos químicos (calcário dolomítico, adubo N-P-K, 20-05-20, oxicloreto de cobre, supermagro e calda viçosa); ii- orgânica sombreada (LOS), com 0,5 ha, cafeeiros de 16 anos, no espaçamento de 2,5 x 1,5 m; 4 anos antes do início e durante o experimento, cultivavam-se os cafeeiros em consórcio com bananeiras plantadas nas entrelinhas da lavoura, com espaçamento de 2,5 x 3,5 m; e iii- orgânica (LO), com 0,8 ha, plantas de 20 anos, no espaçamento de 2,5 x 1,8 m; 4 anos antes do início e durante o experimento, cultivavam-se bananeira nas entrelinhas, espaçadas de 15,0 x 3,5 m. Para manejo de doenças e correção do solo nas LOS e LO, usaram-se supermagro, calda viçosa, incorporação de restos das bananeiras, esterco de curral e cama de galinha. A floração e granação transcorreram de setembro-outubro e janeiro-abril, respectivamente, com colheita era manual realizada de maio a junho. Para cada período de avaliação, em média, efetuaram-se cinco e duas pulverizações de calda viçosa nas lavouras convencional e orgânicas, respectivamente.

2.2 – Delineamento experimental

Para estudar o progresso da cercosporiose, considerou-se o delineamento de blocos casualizados com quatro repetições em esquema de parcela subdividida no tempo (período de 12 meses), sendo os blocos representados pelos quatro pontos cardeais. Os fatores considerados foram lavoura, terço e tempo (subparcela).

Em cada lavoura, efetuou-se caminhamento em W para a seleção das dez plantas avaliadas. Considerou-se cada planta como uma unidade experimental. Em cada planta, marcaram-se 12 ramos, igualmente distribuídos nos terços inferior (I), médio (M) e superior (S).

2.3 – Progresso da cercosporiose e enfolhamento do cafeeiro

Considerou-se como um ano de avaliação o período de novembro a outubro. Mensalmente, avaliou-se a intensidade da doença (incidência e severidade) e se quantificou o número de folhas em cada ramo selecionado. Para a incidência, considerou-se uma folha como doente quando nela havia pelo menos uma lesão típica de cercosporiose. Para a severidade, adotou-se uma escala diagramática, com cinco notas: 1- 0%; 2- >0 a 3%; 3- >3 a 6%; 4- >6 a 12%; 5- >12 a 25% de área foliar doente (Oliveira *et al*., 2001). Para as análises estatísticas, usou-se o valor intermediário compreendido entre o limite inferior e superior de cada nota. Em cada ramo, avaliaram-se, também, o enfolhamento (proporção de folhas que surgiram em relação à avaliação anterior) e a desfolha (proporção de folhas que caíram em relação à avaliação anterior).

Plotaram-se os valores médios de incidência, severidade, enfolhamento e desfolha em função do tempo, para obter curvas de progresso da doença, e de enfolhamento durante o período de avaliação. Estimaram-se os pontos de máxima incidência e de máxima severidade por ano, para cada lavoura, em cada terço. Para cada fator, integrou-se a dinâmica temporal da epidemia da cercosporiose pela área abaixo da curva de progresso de doença (AACPD) para incidência (AACPDI) e severidade (AACPDS), calculadas segundo Shaner & Finney, 1977.

2.4 – Análise dos dados

Realizou-se análise descritiva dos gráficos de incidência, severidade, enfolhamento e desfolha em função do tempo, para cada lavoura dentro de cada terço. Submeteram-se os valores de AACPD à análise de variância (ANOVA) e se adotou o teste de Tukey ($\alpha = 0,05$) para comparação de médias.

Para descrever o progresso da incidência e da severidade da cercosporiose, modelaram-se as médias obtidas para cada nível dos fatores lavoura e terço com um modelo de regressão não linear cuja variável explicativa foi o tempo. O modelo em questão, obtido de Box *et al*. (1994), é:

$$y_i = a + b\,sen\left(\frac{c\pi x_i}{12}\right) + e_i, \text{para} \begin{cases} x_i \leq 12 \\ 12 < x_i \leq 24 \\ 24 < x_i \leq 36 \\ 36 < x_i \leq 48 \end{cases},$$

em que: ″i″ é o i-ésimo mês, com ″i″ variando de 1 a 48, sendo que para o 1º período de avaliação ″i″ variou do 1º ao 12º meses; para o 2º período ″i″ variou do 13º ao 24º meses; para o 3º período ″i″ variou do 25º ao 36º meses; e para o 4º período ″i″ variou

do 37º ao 48º meses; ″y_i″ é a variável dependente (incidência ou severidade); ″a″ representa a média de y_i; ″b″ representa a distância da média até o valor máximo (pico); pode-se obter o valor máximo pela soma de ″a″ e ″b″; ″c″ representa a metade do número de ciclos da epidemia; ″x_i″ representa o tempo em meses; e ″e_i″ o erro aleatório. Para a descrição detalhada dos parâmetros do modelo, efetuou-se um esquema (Figura 1).

Como se obtiveram as medidas de intensidade subsequentemente nos mesmos ramos ao longo do tempo, os erros associados ao modelo não foram considerados independentes, e portanto, foram modelados por um processo autoregressivo (p) de médias móveis (q), ARMA (p,q). Neste processo, assume-se que o valor do erro em um tempo i é dependente dos valores nos tempos anteriores ($e_{i-1, \ldots, e_{i-p}}$), e que o novo erro (que representa efeitos de outras variáveis exceto o tempo) também apresenta a mesma dependência ($a_{i-1, \ldots, a_{i-q}}$), de forma que o termo a_i passa ser considerado o verdadeiro erro (ruído branco) associado ao modelo utilizado (Box *et al.*, 1994). Este processo é dado por:

$$e_i = \phi_1 e_{i-1} + \cdots + \phi_p e_{i-p} + \theta_1 a_{i-1} + \cdots + \theta_q a_{i-q} + a_i\,, \qquad a_i \sim N(0, \sigma^2)$$

Ajustou-se o modelo em questão por meio do procedimento MODEL do software SAS® v. 9.1, o qual utiliza o método dos quadrados mínimos generalizados para modelos de regressão não linear com o processo iterativo de Gauss-Newton. A qualidade de ajuste dos modelos foi verificada por meio do coeficiente de determinação, dado por:

$$R^2 = 1 - \frac{SQE}{SQTotal} = r(y, \hat{y})^2$$

Adicionalmente, plotaram-se os valores dos resíduos em função dos valores preditos para checar a independência dos erros.

Como já relatado, a estimativa do pico, denotado a partir de agora por $\hat{p}$, é dada por $\hat{p} = \hat{a} + \hat{b}$. Assim, comparou-se este parâmetro por meio do método da sobreposição de intervalos de confiança de 95% entre as diferentes lavouras (LO, LC e LOS) e terços (S, M e I) em cada ano. O intervalo de confiança é dado por:

$\hat{p} \pm 1{,}96\sqrt{\hat{V}(\hat{p})}$, sendo $\hat{V}(\hat{p}) = \hat{V}(\hat{a}) + \hat{V}(\hat{b}) + 2cov(\hat{a}, \hat{b})$.

As estimativas dos picos entre, por exemplo, duas lavouras serão consideradas estatisticamente diferentes se o limite inferior do intervalo para o pico de uma das lavouras for maior que o limite superior do outra, ou seja, se não houver sobreposição.

3 – Resultados

3.1 – Área abaixo da curva de progresso da doença (AACPD) para incidência e severidade

Para cada ano, por meio dos valores da AACPD para incidência (AACPDI) e severidade (AACPDS), observaram-se diferenças entre as lavouras, bem como entre os terços (Tabela 1). Detectou-se interação significativa entre os fatores lavoura e terço, no 1º (P = 0,0284) e 4º anos (P = 0,0003). No 2º e 3º anos, observou-se significância (P < 0,0001) apenas para o efeito de lavoura, com maiores valores da AACPDI e AACPDS na LC. No 3º ano, os valores de AACPDS não diferiram entre LC e LO. No 1º ano, os valores de AACPDI e AACPDS no terço inferior nas lavouras LC e LO não diferiram, enquanto nas demais combinações, os valores na LC diferiram dos da LO e LOS (Tabela 1).

Estudou-se o efeito do terço da planta, dentro de cada ano e lavoura. No terço superior e médio, ocorreram os maiores valores de AACPDI e AACPDS no 1º e 4º anos. Apenas na combinação 4º ano e LC, os valores de AACPDI e AACPDS no terço superior foram diferentes daqueles do terço inferior e médio. Nas LO e LOS, não se observou o efeito do fator terço para os 1º e 4º anos de avaliação (Tabela 1).

3.2 – Progresso da Cercosporiose e enfolhamento do cafeeiro

A doença ocorreu em todas as lavouras e anos, mas a intensidade variou ao longo dos anos. Houve tendência de dinâmica similar nos 1º e 4º anos e baixa intensidade no 2º e 3º anos (Figura 2). No 1º ano, a intensidade de doença aumentou de forma contínua, do início das avaliações (novembro) até maio, principalmente na LC. Maiores valores de intensidade da doença ocorreram no 1º ano, em todas as lavouras, sendo os valores de máxima incidência 26,43; 15,75 e 8,84% e severidade 5,78; 2,44 e 0,86% nas LC, LO e LOS, respectivamente. Entre as lavouras, os maiores valores de incidência e severidade ocorreram na LC e menores na LOS (Figura 2).

Quanto ao terço das plantas, a intensidade da cercosporiose foi maior nas folhas do terço superior e menor nas do terço inferior (Figura 2). Em geral, a intensidade foi menor no período de novembro a dezembro e maior de maio a julho. Em algumas combinações de ano, lavoura e terço, a intensidade foi maior de novembro a dezembro e tenderam a zero nos demais meses, como no 3º ano na LOS, independente do terço, e para severidade na LO nos terços médio e inferior (Figura 2). Em abril, detectaram-se

picos de severidade no 2º ano nos terços médio e superior da LO, no 3º ano no terço inferior da LC, 4º ano no terço superior da LOS e de incidência no 3º ano no terço inferior da LC (Figura 2 A, B, C e F). Entretanto, mais tarde, em agosto, é que se detectaram os picos de máxima incidência no 2º ano nos terços superior e inferior da LOS, 4º ano nos terços médio e inferior da LOS, e severidade no 2º ano no terço inferior da LOS, 4º ano no terço inferior da LO e LOS (Figura 2 C, D, E e F).

O enfolhamento dos cafeeiros foi mínimo de abril a setembro principalmente, e o máximo foi de outubro a janeiro, nos diferentes anos, lavouras e terços (Figura 3 A, B e C). Para algumas combinações de ano, lavoura e terço, detectou-se tendência diferente, com ocorrência de um segundo pico de enfolhamento fora da época descrita anteriormente, como no 3º ano no terço médio na LO e LOS, quando o máximo ocorreu em abril, e no 4º ano, quando o pico no terço inferior da LC, LO e LOS foi em março (Figura 3 B e C). O máximo de enfolhamento para a LC, LO e LOS foi no 4º ano, em todos os terços, entre todos os anos, exceto no terço inferior para a LOS, onde o máximo ocorreu no 2º ano. Maior enfolhamento ocorreu para a LC em todas as combinações de ano e terço, com exceção do 3º ano no terço médio, em que o máximo foi na LOS (Figura 3 A, B e C).

A desfolha foi maior nos meses de junho a setembro. Além de ocorrer nesses meses, observou-se um pico precoce, em abril, no 3º ano no terço médio na LO e LOS e um tardio no primeiro mês do 2º ano, novembro, no terço superior da LC referente ao final do ciclo do 1º ano (Figura 3 D, E e F). Em geral, a desfolha foi maior na LC e menor na LOS, mas foi maior no 4º ano no terço médio e inferior na LOS e no 1º ano no terço superior da LO (Figura 3 D, E e F). Em geral, na LOS observaram-se os menores índices de desfolha (Figura 3 D, E e F).

3.3 – Modelagem da doença

O modelo ARMA de regressão não linear adotado foi eficiente em descrever o progresso da cercosporiose. Adaptaram-se as funções matemáticas para cada combinação de ano, lavoura e terço, para modelar as dinâmicas da severidade e incidência da doença. Geraram-se 72 equações; na maioria delas, os valores de R^2 foram superiores a 60 %. As estimativas dos três parâmetros (a, b e c) dos modelos estão apresentadas na Tabela 2. Com o modelo, além de se descrever a dinâmica temporal da doença, obteve-se a estimativa de máxima intensidade, o que possibilitou comparar a dinâmica da cercosporiose nas diferentes condições de cultivo (Tabela 2).

Com o gráfico plotado a partir da função usada, observou-se tendência similar de progresso da doença próximo aos dados observados (Figuras 4 e 5). Em geral, com os três parâmetros estimados, foi possível modelar a dinâmica da incidência e severidade, nas combinações de ano, lavoura e terço. Em certas situações, houve variação na intensidade da doença, e a exatidão do modelo foi menor. Como exemplos, têm-se os picos de severidade e incidência nos 1º e 4º anos no terço superior e médio da LC, a dinâmica da severidade no 2º ano da LC no terço superior e a dinâmica da incidência, no 1º ano dos terços inferior e superior da LO e no 2º ano do terço médio da LC (Figuras 4 e 5). No 2º e 3º anos, as estimativas da incidência e severidade da doença tenderam a zero nas LO e LOS (Figuras 4 e 5). Em algumas situações, mesmo havendo aparente diferença, os picos de máxima incidência e severidade, não se detectou diferença significativa, em vista da variabilidade nos valores da intensidade da doença entre as folhas avaliadas, o que gerou um intervalo de confiança amplo (Figuras 6 e 7).

Em geral, os valores do pico de severidade e de incidência foram maiores para a LC e menores para a LOS. Considerando a LC, os valores do pico de severidade foram significativamente maiores que os das demais lavouras no 2º ano, no terço superior, e 4º ano no terço médio e inferior. Para incidência, os picos ocorreram nos 1º, 2º e 4º anos do terço superior, 1º ano do terço médio e 2º anos do terço inferior (Figuras 6 e 7). Os valores de pico da LOS foram significativamente menores somente no 1º ano de avaliação, independente do terço (Figuras 6 A, E e I ; e 7 A, E e I). Barras de intervalo de confiança se sobrepuseram para as LC, LO e LOS, quanto à máxima severidade em todos os terços do 3º ano e nos terço inferior e médio do 2º ano; incidência houve sobreposição e essas não se diferiram no terço médio para o 2º e 3º anos e terço inferior no 4º (Figuras 6 C, F, G, J e K ; e 7 F, G e L).

4 – Discussão

Apesar da importância da cercosporiose do cafeeiro, há carência de informações sobre a doença, principalmente quanto à sua dinâmica temporal. Apesar de o ajuste de um modelo que descreva o progresso é importante para melhor compreensão da doença, não se obtiveram relatos de modelo efetivo em descrever a dinâmica temporal da cercosporiose do cafeeiro. Este fato, provavelmente, se deve ao comportamento atípico da cercosporiose do cafeeiro ao longo dos anos, o que dificulta o ajuste de modelos tradicionais de progresso de doença, como o de Gompertz e o logístico. Para descrever a dinâmica temporal de epidemias da cercosporiose, nos sistemas convencional e orgânico a pleno sol e sombreado, usaram-se a AACPD, curva de progresso da doença e modelos não lineares. A intensidade da cercosporiose durante a estação favorável a doença aumentou gradativamente ao longo do tempo, o que leva a inferir sobre a ocorrência de ciclos secundário do patógeno, conforme esperado para epidemias de cercosporiose (Vereijssen *et al*., 2007) e já observado para a cercosporiose do cafeeiro (Santos et al., 2008).

Na LC, ocorreram condições mais favoráveis ao progresso da cercosporiose que nas lavouras que adotam o sistema orgânico de produção. Maiores valores de AACPD, intensidade e de pico da doença ocorreram na LC. Em outros estudos, obtiveram-se resultados similares: na Costa Rica, a intensidade de doença foi maior na lavoura convencional que na orgânica (Samayoa & Sanchez, 2000). Em dois anos de avaliação, maiores valores de AACPDI média e de incidência em frutos ocorreram em sistema convencional que no orgânico (Santos et al., 2008). A incidência máxima da doença foi de 15% em lavouras convencionais e 3% em orgânica (Teixeira *et al.*, 2005). Poder-se-ia esperar menor intensidade da doença em sistema convencional de produção, em vista do uso intensivo de fungicidas, o que ocorreu com a cercosporiose do sabugueiro, causada por *C. depazoides* (Holb *et al.*, 2009). Entretanto, na presente situação, o produtor pulverizou calda viçosa na lavoura orgânica, apesar de ser em menor intensidade que na convencional.

Em geral, na LO a intensidade da doença foi maior que na LOS. Provavelmente, este efeito deveu-se à radiação, pois a exposição ao sol foi menor na LOS. Há relatos de que a exposição de cafeeiros ao sol é mais favorável à ocorrência da cercosporiose (Echandi, 1959; López-Duque & Fernández-Borrero, 1969; Lamouroux *et al.*, 1995). *Cercospora* spp., como *C. coffeicola*, produzem a cercosporina, toxina fotoativa que

induz à produção de radicais livres que desestruturam tecidos do hospedeiro (Daub *et al.*, 2005; Martins, 2008). A habilidade de produzir a cercosporina é considerada como um fator de agressividade de isolados de *C. coffeicola* (Souza, 2007). Considerando o observado neste estudo e as informações disponíveis na literatura, pode-se aventar a hipótese de que a maior intensidade da doença, observada nas lavouras com maior exposição ao sol, como LC, LO e no terço superior, deveu-se, provavelmente, à maior agressividade de *C. coffeicola* em vista da maior produção de cercosporina nesta condição. Para alguns autores, a maior intensidade da cercosporiose em cafeeiros expostos ao sol deve-se ao fato de estas plantas estarem mais sujeitas ao déficit hídrico e, concomitantemente, ao estresse nutricional, condições também descritas como favoráveis à ocorrência da cercosporiose (Echandi, 1959; Talamini *et al.*, 2001; Salgado *et al.*, 2007; Santos *et al.*, 2008). Portanto, acredita-se que o fato de a intensidade da doença ter sido maior nas plantas mais expostas ao sol deveu-se à interação de variáveis ambientais como precipitação, umidade relativa, radiação solar, temperatura e molhamento foliar.

Especificamente, na LC a ferrugem do cafeeiro, causada por Hemileia *vastatrix*, e outras doenças do cafeeiro mantiveram-se em níveis baixos durante os anos de avaliação (dados não mostrados). Portanto, o fato de a intensidade da cercosporiose e desfolha terem sido maiores na LC reforça a importância da cercosporiose como fator de desfolha do cafeeiro. Esse fato é conhecido dos produtores e de outros pesquisadores (Phiri *et al*., 2001; Santos *et al*., 2008). Acredita-se que o processo que leva à desfolha prematura esteja associado ao aumento na produção de etileno nas folhas infectadas por *C. coffeicola*, como determinado para *C. arachidicola* em plantas de amendoim (Ketring & Hassan, 1982). No presente estudo, o período de maior desfolha foi de junho a setembro, que coincide com o período de maior intensidade da cercosporiose, maio a julho, o que reforça a hipótese de que a desfolha deva-se à ocorrência da doença nas folhas. A época de maior intensidade da cercosporiose coincide com o período de escassez de chuvas e após a granação, o que pode favorecer a doença, em vista de as plantas estarem mais sujeitas a estresses hídrico e nutricional (Santos *et al*., 2008). Já se relatou que a incidência máxima da cercosporiose ocorre em períodos similares aos observados neste trabalho, maio a julho e maio a setembro, que coincidem com o período de estiagem (Talamini *et al*., 2001, 2003). Em algumas combinações de ano, lavoura e terço, a intensidade máxima de doença ocorreu precocemente, similarmente ao que ocorreu em lavoura de cafeeiro consorciada com ingazeiros, em que o máximo

de incidência ocorreu em março (Salgado *et al*., 2007). Características locais de precipitação, temperatura e de nutrição podem levar a estas situações.

O enfolhamento máximo dos cafeeiros ocorreu de outubro a janeiro e, o mínimo, de abril a setembro. A época de menor intensidade de doença, novembro a dezembro, coincidiu com o período de maior enfolhamento, o que pode se dever a dois fatos: 1- a intensidade da doença foi menor em vista da presença de novos tecidos sadios demandando a ocorrência de novos ciclos de infecções; ou 2- a época das chuvas estar se iniciando e com ela, os novos ciclos de infecção, como ocorre para a ferrugem do cafeeiro (Teixeira *et al*., 2005; Santos *et al*., 2008). A dinâmica da cercosporiose variou ao longo dos quatro anos de avaliação, em todas as lavouras, apesar de ter sido similar nos dois primeiros anos. Tal fato já foi relatado e pode estar relacionado ao ciclo bienal de produção do cafeeiro (Santos *et al*., 2008).

Em vista do comportamento da dinâmica da cercosporiose nas lavouras cafeeiras deste estudo ao longo das estações de cultivo, não foi possível usar os modelos de crescimento rotineiramente usados para descrever e inferir sobre as doenças (Xu, 2006; Jagger & Richards, 2007; Contreras-Medina *et al*., 2009). Portanto, pela primeira vez, usou-se um modelo de série temporal para representar o progresso da cercosporiose do cafeeiro. Com o modelo de regressão não linear para séries temporais, efetuou-se descrição adequada do progresso da doença e usaram-se os parâmetros estimados para comparar os diferentes fatores estudados. Portanto, como em outros trabalhos (Yang & Zeng, 1992; Hudelson *et al*., 1993; Xu *et al*., 1995; Guerin *et al*., 2001; Holb, 2008), os modelos ARMA foram eficientes em modelar o progresso da epidemia.

O melhor ajuste obtido com o modelo de série temporal, provavelmente, deve-se ao fato de que os erros associados ao modelo não são independentes, isto é, a avaliação num tempo é dependente da avaliação em tempos anteriores (Box *et al*., 1994). Este aspecto é importante, pois as avaliações ocorreram sempre nos mesmos ramos da mesma planta, ou seja, a intensidade de doença de um dado momento depende da observação anterior. Além disso, o modelo de séries temporal é constituído por parâmetros que têm uma interpretação prática do evento em estudo (Box *et al*., 1994). Os resultados obtidos co a interpretação dos gráficos de incidência e severidade predita foram similares àqueles obtidos com a análise descritiva. Ademais, os meses que se observaram os picos de doença estimados pelos parâmetros do modelo de séries temporais ocorreram no mesmo período da máxima intensidade observado com a análise descritiva.

Os modelos de séries temporais, ainda não amplamente usados em fitopatologia, foram usados para descrever a dinâmica de determinados eventos: flutuação temporal de esporos de *Podosphaera leucotricha* e *Monilinia fructigena* (Xu *et al.*, 1995; Holb, 2008), dispersão temporal de ascósporos de *Cryphonectria parasitica* em plantas de castanheira (Guerin *et al*., 2001), padrão espacial de *Puccinia striiformis* em plantas de trigo (Yang & Zeng, 1992), dispersão espacial de *Pseudomonas syringae* pv. *syringae* em feijoeiro (Hudelson *et al*., 1993) e progresso da ferrugem do eucalipto (Zauza, 2007). Os autores usaram modelos de séries temporais para inferir sobre a época de maior movimentação de esporos, ocorrência da doença, efeito de estratégia de manejo e observar a ocorrência ou não de sazonalidade da doença. No entanto, adotaram diferentes modelos e estruturas de ordens dos erros,como AR (auto-regressivo), MA (médias móveis), ARMA (auto-regressivo com médias móveis), ARIMA (auto-regressivo integrado de médias móveis), SARIMA (auto-regressivo integrado de médias móveis que incorpora o comportamento de sazonalidade), segundo o objetivo e o patógeno em estudo (Yang & Zeng, 1992; Hudelson *et al*., 1993; Xu *et al*., 1995; Guerin *et al*., 2001; Zauza, 2007; Holb, 2008). Um fator determinante para que os modelos de séries temporais sejam eficientes em descrever a dinâmica de doenças é a escolha correta do modelo e da estrutura de ordem dos erros (Box *et al*., 1994). Em muitos experimentos epidemiológicos, efetuam-se avaliações repetidas em um mesmo indivíduo (planta, haste, caule, ramo, folha, flor, semente e fruto), as quais,, geralmente, transcorrem em intervalos constantes ao longo do tempo. Assim, podem-se ajustar modelos de séries temporais, para que se possa descrever o processo estocástico de interesse (Box *et al*., 1994). Portanto, acredita-se que no futuro, estes modelos tornem-se mais aplicados para descrever doenças cuja dinâmica não seja representada pelos modelos de crescimento, hoje comumente adotados em epidemiologia.

Neste estudo, efetuaram-se análises quantitativas e comparativas de epidemias da cercosporiose em lavouras de cafeeiros conduzidas nos sistemas de cultivo convencional e orgânico a pleno sol e sombreado. Com as técnicas e modelos aqui adotados, foi possível entender a dinâmica da cercosporiose e definir as condições de manejo cultural relacionadas à menor intensidade da doença. Estes resultados são importantes para ampliar o conhecimento da doença e para avaliar estratégias de manejo da mesma. Ademais, com o uso da análise de série temporal, efetuou-se uma abordagem inovadora para descrever a dinâmica da epidemia da cercosporiose do cafeeiro. Espera-se que este tipo de análise possa ajudar em trabalhos futuros que

envolvam eventos estocásticos em patossistemas. Entretanto, demandam-se mais pesquisas para se determinar como os fatores ambientais interferem na dinâmica de epidemias. De qualquer forma, neste estudo visualizou-se que a adoção de dada prática de cultivo afeta o progresso da cercosporiose do cafeeiro. Essa informação, aliada ao conhecimento do efeito do ambiente e de informações sobre medidas de controle, pode vir a ser aplicada no manejo integrado da cercosporiose do cafeeiro em lavouras comerciais dessa importante cultura.

5 – Referências Bibliográficas

Abbasi, P. A., Al-Dahmani, J., Sahin, F., Hoitink, H. A. J., Miller, S. A. 2002. Effect of compost amendments on disease severity and yield of tomato in organic and conventional production systems. Plant Disease, 86:156-161.

Almeida, S. R. Doenças do cafeeiro. 1986. In: Rena, A. B., Malavolta, E., Rocha, M.; Yamada, T. Cultura do cafeeiro: Fatores que afetam a produtividade. Piracicaba: Instituto da Potassa e Fosfato, p. 391-399.

Boldini, J. M., Souza, P. E., Pozza, E. A., Chalfoun, S. M., Faria, M. A. 2000. Incidência e severidade da cercosporiose em frutos de cafeeiro fertirrigado na região de Lavras-MG. In: 1º Simpósio de Pesquisa dos Cafés do Brasil, Poços de Caldas, MG. Resumos expandidos. Brasília, D.F. Embrapa Café, p. 273-275.

Botelho, D. M. S., Pozza, E. A., Alves, E., Neto, A. E. F., Barbosa, J. P. R. A. D., Castro, D. M. 2009. Aspectos, anatômicos e fisiológicos de mudas de cafeeiro (*Coffea arábica* L.) com cercosporiose (*Cercospora coffeicola* Berk &Cook) adubadas com ácido silíco. Coffee Science, 4:93-99.

Box, G. E. P., Jenkins, G. M., Reinsel, G. C. 1994. Time Series Analysis: Forecasting and Control. San Francisco, CA, USA: Holden-Day.

Contreras-Medina, L. M., Torres-Pacheco, I., Guevara-González, R. G., Romero-Troncoso, R. J., Terol-Villalobos, I. R., Osornio-Rios, R. A. 2009. Mathematical modeling tendencies in plant pathology. African Journal of Biotechnology, 8:7399-7408.

Daub, M. E., Herrero, S., Chung, K. R. 2005. Photoactivated perylenequinone toxins in fungal pathogenesis of plants. Federation of European Microbiological Societies Microbiology Letters, 252:197-206.

Echandi, E. 1959. La chasparria de los cafetos causada por el hongo *Cercospora coffeicola* Berk. & Cooke. Turrialba, 9:54-67.

Fernández-Borrero, O., Cadena, G. G., Lopez, D. S., Buitrago, S., Arango, B. L. G. 1982. La mancha de hierro del cafeto (*Cercospora coffeicola* Berk e Cooke), biologia, epidemiologia y control. In: 10º Colloque Scientifique International Sur le Café, Salvador (Brasil), p. 11-14.

Galdeano, D. M., Guzzo, S. D., Patrício, F. R. A., Harakava, R. 2010. Proteção do cafeeiro contra cercosporiose por acibenzolar-S-metil e proteína harpina. Pesquisa agropecuária brasileira, 45:686-692.

Guerin, L., Froidefond, G., Xu, X.-M. 2001. Seasonal patterns of dispersal of ascospores of *Cryphonectria parasitica* (chestnut blight). Plant Pathology, 50:717-724.

Holb, I. J. 2008. Monitoring conidial density of *Monilinia fructigena* in the air in relation to brown rot development in integrated and organic apple orchards. European journal of plant pathology, 120:397-408.

Holb, I. J., Gáll, J. M., Fodor. 2009. Effect of production system and pruning on temporal development of *Cercospora depazeoides* and on berry yield in black elderberry orchards. Plant Disease, 93:625-631.

Hudelson, B. D., Clayton, M. K., Smith, K. P., Upper, C. D. 1993. Modeling of superimposed spatial patterns of bacterial brown spot of snap beans. Phytopathology, 83:430-438.

Jagger, M., Richards, K. 2007. Temporal analysis I: Quantifying and comparing epidemics. In: Madden, L. V., Hughes, G., Van den Bosch, F. The study of plant disease epidemics. APS Press, St. Paul, MN, p. 63-116.

Juliatti, F. C., Ramos, A. S., Mendonça, F. C., Santos, C. M. 2000. Incidência e severidade da cercosporiose em lavoura cafeeira conduzida sob diferentes sistemas de irrigação e lâminas d'água. In: 1º Simpósio de Pesquisa dos Cafés do Brasil, Poços de Caldas, MG. Resumos expandidos: Embrapa Café, p. 219-222.

Ketring, D. L., Melouk, H. A. 1982. Ethylene production and leaflet abscission of three peanut genotypes infected with *Cercospora arachidicola* Hori. Plant Physiology, 69: 789-792.

Lamouroux, N., Pellegrin, F., Nandris, D., Kohler, F. 1995. The *Coffea arabica* fungal pathosystem in New Caledonia: Interactions at two diferente spatial scales. Journal of phytopathology, 143:403-413.

López-Duque, S., Fernández-Borrero, O. 1969. Epidemiologia de la mancha de hierro del cafeto (*Cercospora coffeicola* Berk. & Cooke). Cenicafe, 20:3-19.

Martins, R. B. 2008. Variabilidade de *Cercospora coffeicola* em Minas Gerais com base em compatibilidade vegetativa e produção de cercosporina. Tese de Doutorado. Viçosa-MG. Universidade Federal de Viçosa.

Miranda, E. M., Pereira, R. C. A., Bergo, C. L. 1999. Comportamento de seis linhagens de café (*Coffea arabica* L.) em condições de sombreamento e a pleno sol no estado do Acre, Brasil. Ciência e Agrotecnologia, 23:62-69.

Oliveira, C. A., Pozza, E. A., Oliveira, V. B., Santos, R. C., Chaves, Z. M. 2001. Escala diagramática para avaliação da severidade de cercosporiose em folhas de cafeeiro. In: 2º Simpósio Brasileiro de Pesquisa dos Cafés do Brasil, Vitória, ES. Anais: Embrapa Café, p. 1151-1157.

Pereira, R. B., Alves, E., Ribeiro Junior, P. M., Resende, M. L. V., Lucas, G. C., Ferreira, J. B. 2008. Extrato de casca de café, óleo essencial de tomilho e acibenzolar-S-metil no manejo da cercosporiose do cafeeiro. Pesquisa agropecuária brasileira, 43:1287-1296.

Phiri, N. A., Hillocks, R. J., Jeffries, P. 2001. Incidence and severity of coffee diseases in smallholder plantations in northern Malawi. Crop Protection, 20:325-332.

Salgado, B. G., Macedo, R. L. G., Carvalho, V. L., Salgado, M., Venturin, N. 2007. Progresso da ferrugem e da cercosporiose do cafeeiro consorciado com grevílea, com ingazeiro e a pleno sol em Lavras, MG. Ciência e Agrotecnologia, 31:1067-1074.

Samayoa, J. O. J., Sanchez, V. G. 2000. Enfermedades foliares en café orgânico y convencional. Turrialba, 58:9-19.

Santos, F. S., Souza, P. E., Pozza, E. A., Miranda, J. C., Barreto, S. S., Theodoro, V. C. 2008. Progresso da cercosporiose (*Cercospora coffeicola* Berkeley &Cooke) em cafeeiros sob cultivos orgânico e convencional. Suma phytopathologica, 34:48-54.

Shaner, G., Finney, R. E. 1977. The effect of nitrogen fertilization on the expression of slow-mildewing resistance in knox wheat. Phytopathology, 67:1051-1056.

Souza, A. G. C. 2007. Variabilidade fisiológica de isolados de *Cercospora coffeicola*. Dissertação de Mestrado. Viçosa-MG. Universidade Federal de Viçosa.

Talamini, V., Souza, P. E., Pozza, E. A., Silva, A. M, Bueno Filho, J. S. S. 2001. Progresso da ferrugem e da cercosporiose em cafeeiro (*Coffea arabica* L.) em diferentes lâminas de irrigação e diferentes parcelamentos de adubação. Ciência e Agrotecnologia, 25:55-62.

Talamini, V., Pozza, E. A., Souza, P. E., Silva, A. M. 2003. Progresso da ferrugem e da cercosporiose em cafeeiro (*Coffea arabica* L.) com diferentes épocas de início e parcelamentos da fertirrigação. Ciência e Agrotecnologia, 27:141-149.

Teixeira, H., Maffia, L. A., Mizubuti, E. S. G. 2005. Progresso de doenças em sistemas convencional e orgânicos de produção de café. In: XXXVIII Congresso Brasileiro de Fitopatologia, 2005, Brasília-DF. Fitopatologia Brasileira, 30S:146.

Vereijssen, J., Schneider, J. H. M., Jeger, M. J. 2007. Epidemiology of cercospora leaf spot on sugar beet: Modeling disease dynamics within and between individual plants. Phytopathology, 97:1550-1557.

Workneh, F., Van Bruggen, A. H. C., Drinkwater, L. E., Shennan, C. 1993. Variables associated with corky root and Phytophthora root rot of tomatoes in organic and conventional farms. Phytopathology, 83:581-589.

Xu, X. 2006. Modelling and interpreting disease progress in time. In: The epidemiology of plant disease. Cooke, B. M., Gareth Jones, D., Kaye, B. Springer, Dordrecht, The Netherlands, p. 215-238.

Xu, X.-M., Butt, D. J., Ridout, U. S. 1995. Temporal patterns of airborne conidia of *Podosphaera leucotricha*, causal agent of apple powdery mildew. Plant Pathology, 44, 944-955.

Yang, X. B., Zeng, S. M. 1992. Detecting patterns of wheat stripe rust pandemics in time space. Phytopathology, 82: 571-576.

Zambolim, L.; Vale, F. X. R.; Pereira, A. A.; Chaves, G. M. 1997. Café (*Coffea arábica*). In: Vale, F. X. R.; Zambolim, L. Controle de doenças de plantas: grandes culturas. Viçosa: UFV, p. 83-180.

Zauza, E. A. V. 2007. Epidemiologia da ferrugem do eucalipto e resistência genética. Tese de Doutorado. Viçosa-MG. Universidade Federal de Viçosa.

Tabela 1: Valores médios da área abaixo da curva de progresso para incidência (AACPDI) e severidade (AACPDS), referentes aos quatro anos de avaliação da cercosporiose em lavouras convencional (LC), orgânica (LO) e orgânica sombreada (LOS), em ramos dos terços superior (S), médio (M) e inferior (I) de cafeeiros.

Ano	Terço	AACPDI			AACPDS		
		Lavoura			Lavoura		
		LC	LO	LOS	LC	LO	LOS
1*	S	140,06 Aa	71,73 Ba	32,75 Ba	24,76 Aa	10,16 Ba	4,05 Ba
	M	141,46 Aa	71,40 Ba	24,39 Ca	23,49 Aa	12,98 Ba	5,83 Ba
	I	75,19 Ab	67,67 Aa	19,59 Ba	11,18 Ab	7,26 Aa	1,95 Ba
2**	ns	45,57 A	12,44 B	10,15 B	4,45 A	1,43 B	1,20 B
3**	ns	22,65 A	12,31 B	3,99 C	2,15 A	1,39 A	0, 46 B
4*	S	104,44 Aa	27,77 Ba	13,32 Ba	15,94 Aa	2,78 Ba	1,80 Ba
	M	68,83 Ab	16,36 Ba	15,88 Ba	8,44 Ab	1,50 Ba	2,14 Ba
	I	41,19 Ab	11,43 Ba	10,30 Ba	3,98 Ac	1,35 Ba	0,90 Ba

*Para os anos 1 e 4, médias seguidas da mesma letra maiúscula na linha e minúscula na coluna dentro do mesmo ano e variável (AACPDI ou AACPDS), não diferem entre si (Teste de Tukey, α=0,05).

**Para os anos 2 e 3, os efeitos de terço e da interação terço-lavoura não foram significativos (ns). Para cada um desses anos, para cada variável (AACPDI ou AACPDS), médias seguidas da mesma letra maiúscula na linha, não diferem entre si (Teste de Tukey, α=0,05).

Tabela 2: Estimativas dos parâmetros (a, b, c), ordens das estruturas de erros autoregressivos (p) e médias móveis (q), ARMA(p;q), e coeficiente de determinação ajustado (R^2) para modelos não lineares de progresso de incidência e severidade da cercosporiose nos ramos de terços superior (S), médio (M) e inferior (I) de cafeeiros em lavouras convencional (LC), orgânica (LO) e orgânica sombreada (LOS), de 2004/05 a 2007/08 (anos 1 a 4).

Terço	Lavoura	Ano	Severidade					Incidência				
			Parâmetros			ARMA	R^2	Parâmetros			ARMA	R^2
			a	b	c	(p;q)		a	b	c	(p;q)	
S	LC	1	0,51	3,85	0,72	1;1	0,97	12,09	13,33	-2,08	2;2	0,95
		2	0,29	0,80	1,60	1;1	0,55	4,15	4,37	1,59	1;1	0,82
		3	0,04	0,13	0,94	1;1	0,79	0,66	1,10	1,00	2;1	0,88
		4	1,51	1,69	-2,04	1;1	0,85	5,49	22,17	-0,98	1;1	0,75
	LO	1	0,71	2,17	0,81	1;1	0,80	3,70	13,30	0,71	1;1	0,86
		2	0,14	0,19	2,81	1;1	0,82	1,14	1,06	-2,02	2;1	0,93
		3	0,10	0,12	3,02	1;1	0,48	1,85	1,76	-0,90	1;1	0,83
		4	0,22	0,13	5,03	2;2	0,49	2,60	3,08	5,01	1;1	0,77
	LOS	1	0,09	0,62	0,66	1;1	0,86	2,80	3,19	-1,98	1;1	0,80
		2	0,10	0,13	1,55	2;2	0,89	0,08	1,49	-0,88	2;2	0,95
		3	0,05	0,02	4,74	1;1	0,59	0,73	0,74	1,30	1;1	0,52
		4	0,18	0,19	2,92	1;1	0,79	1,38	2,03	-2,65	1;1	0,95
M	LC	1	0,32	3,36	0,77	1;1	0,96	5,46	25,44	0,64	1;1	0,95
		2	0,44	0,52	1,58	3;3	0,79	2,83	1,08	1,43	1;1	0,77
		3	0,23	0,28	1,00	2;1	0,55	0,76	2,35	0,99	1;1	0,73
		4	1,44	1,48	-2,06	2;1	0,86	5,77	4,82	-2,10	1;1	0,83
	LO	1	0,75	2,53	0,78	1;1	0,85	2,73	11,90	0,72	2;1	0,97
		2	0,16	0,12	2,89	1;1	0,66	1,09	0,57	1,09	1;1	0,83
		3	0,16	0,13	1,27	1;1	0,46	2,12	-1,98	0,91	1;1	0,54
		4	0,13	0,17	2,44	2;2	0,91	0,81	0,60	5,12	2;2	0,80
	LOS	1	0,08	0,59	0,66	1;1	0,95	0,44	3,29	0,67	1;1	0,91
		2	0,07	0,09	1,38	2;2	0,66	0,37	0,37	1,34	2;2	0,83
		3	0,11	0,06	0,50	2;1	0,90	0,96	-0,40	2,24	1;1	0,49
		4	0,18	0,17	2,42	1;1	0,85	0,58	1,61	2,27	2;2	0,84
I	LC	1	0,16	1,71	0,90	1;1	0,95	0,58	10,33	0,84	1;1	0,92
		2	0,77	0,62	1,36	1;1	0,63	3,00	2,12	1,58	1;1	0,78
		3	0,36	0,46	0,45	1;1	0,59	1,87	2,73	0,79	2;2	0,92
		4	0,38	0,34	2,40	1;1	0,84	2,56	2,06	2,40	2;1	0,84
	LO	1	0,14	1,20	0,96	1;1	0,93	4,52	13,80	0,79	1;1	0,65
		2	0,15	-0,10	1,62	1;1	0,61	1,62	-0,78	1,58	1;1	0,54
		3	0,38	0,02	0,42	1;2	0,70	3,91	3,20	-0,92	1;1	0,95
		4	0,11	0,02	2,48	1;1	0,83	1,30	0,73	2,50	2;1	0,78
	LOS	1	0,07	0,35	0,84	1;1	0,84	0,46	3,15	0,83	1;1	0,81
		2	0,20	0,11	1,17	2;2	0,64	1,21	0,50	1,35	2;2	0,50
		3	0,14	-0,05	1,04	1;1	0,59	1,45	-0,58	1,04	1;1	0,63
		4	0,08	0,08	2,44	1;1	0,72	1,29	1,49	0,15	1;1	0,63

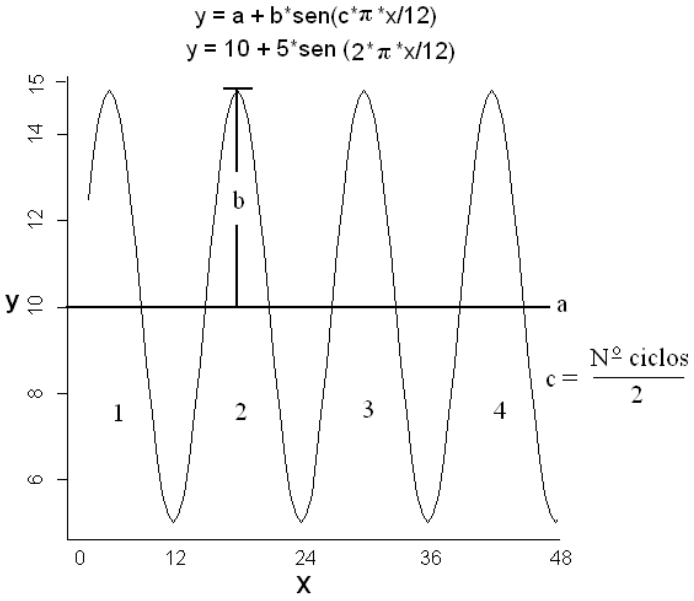

Figura 1: Ilustração do modelo não linear adotado para descrever o progresso da incidência e severidade (y) da cercosporiose do cafeeiro, ao longo de 48 meses (x).

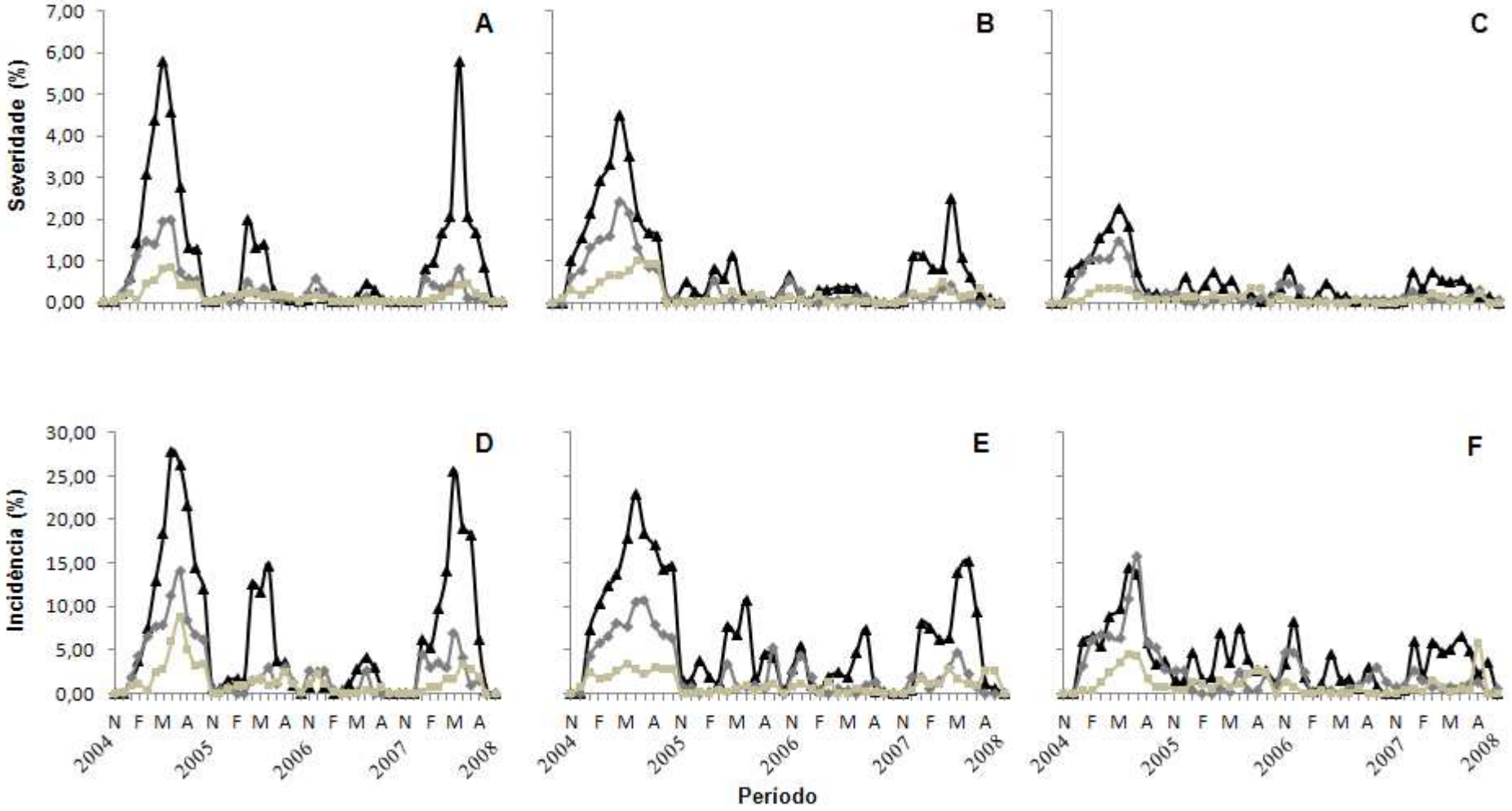

Figura 2: Médias de severidade (A, B e C) e incidência (D, E e F) da cercosporiose do cafeeiro em ramos dos terços superior (A e D), médio (B e E) e inferior (C e F), em lavouras convencional ( ), orgânica ( ) e orgânica sombreada ( ). No eixo referente a período, as letras N, F, M e A referem-se, respectivamente, a novembro, fevereiro, maio e agosto, entre 2004 e 2008.

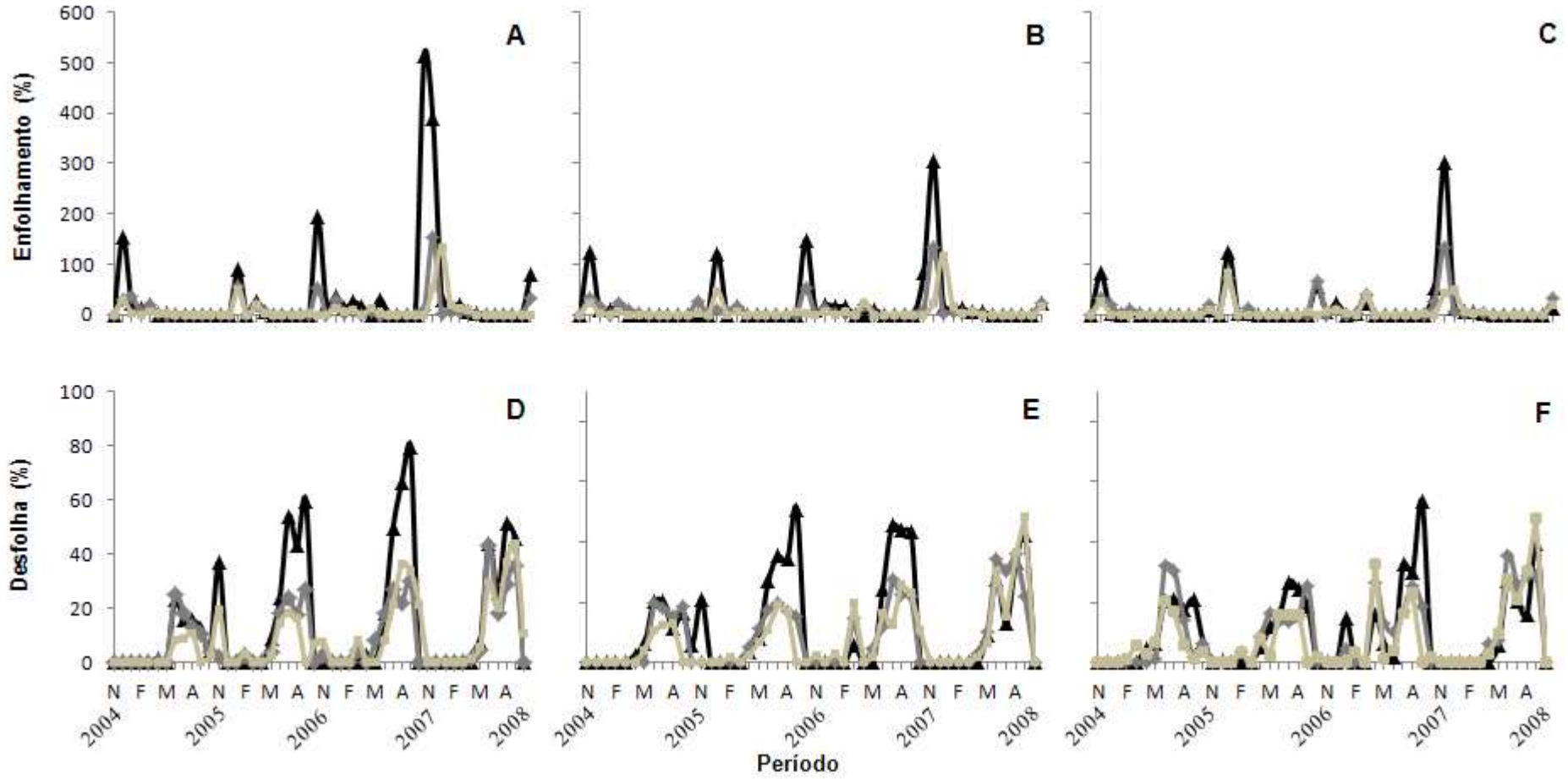

Figura 3: Percentagem de enfolhamento e desfolhadas do cafeeiro, em ramos dos terços superior (A e D), médio (B e E) e inferior (C e F), em lavouras convencional ( ), orgânica ( ) e orgânica sombreada ( ). No eixo referente a período, as letras N, F, M e A referem-se, respectivamente, a novembro, fevereiro, maio e agosto, entre 2004 e 2008.

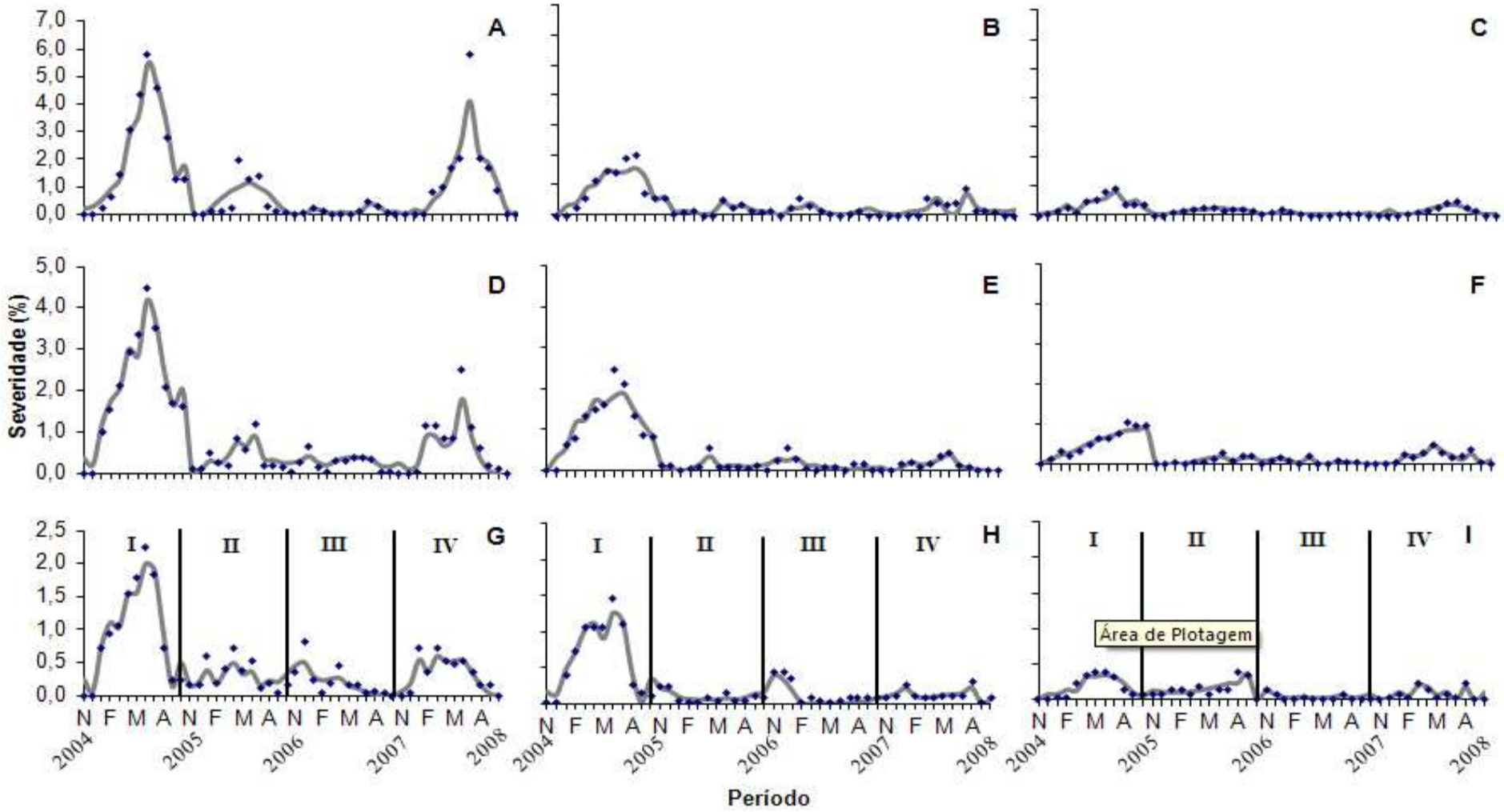

Figura 4: Severidade da cercosporiose, observada (◆) e predita (—) pelos modelos não lineares referentes aos anos I (2004/05), II (2005/06), III (2006/07) e IV (2007/08) de avaliação em ramos dos terços superior (A, B e C), médio (D, E e F) e inferior (G, H e I) de cafeeiros em lavouras convencional (A, D e G), orgânica (B, E e H) e orgânica sombreada (C, F e I). No eixo referente a período, as letras N, F, M e A referem-se, respectivamente, a novembro, fevereiro, maio e agosto. Os modelos foram ajustados para cada período de forma independente.

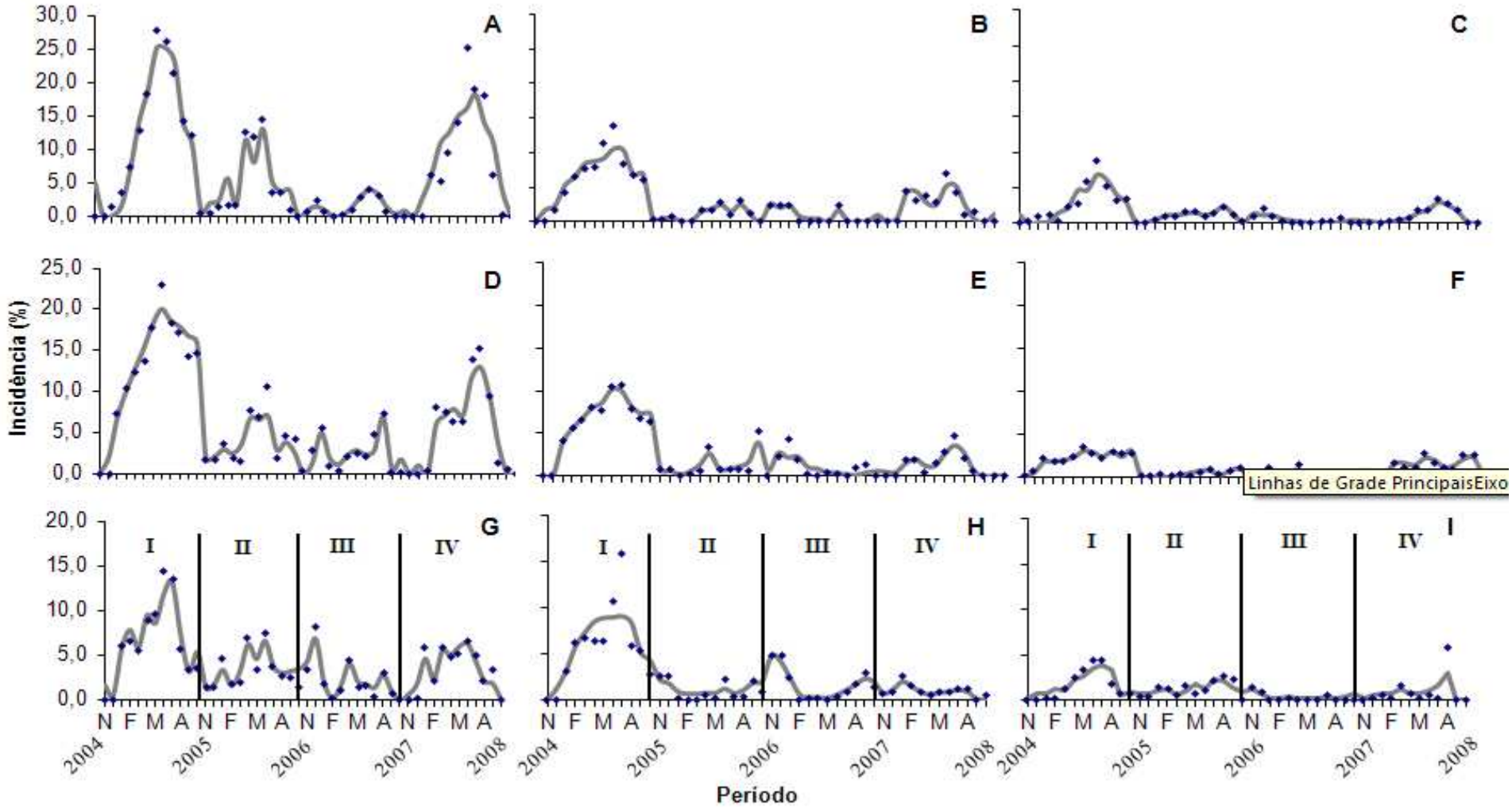

Figura 5: Incidência da cercosporiose, observada (◆) e predita (—) pelos modelos não lineares referentes aos anos I (2004/05), II (2005/06), III (2006/07) e IV (2007/08) de avaliação em ramos dos terços superior (A, B e C), médio (D, E e F) e inferior (G, H e I) de cafeeiros em lavouras convencional (A, D e G), orgânica (B, E e H) e orgânica sombreada (C, F e I). No eixo referente a período, as letras N, F, M e A referem-se, respectivamente, a novembro, fevereiro, maio e agosto. Os modelos foram ajustados para cada período de forma independente.

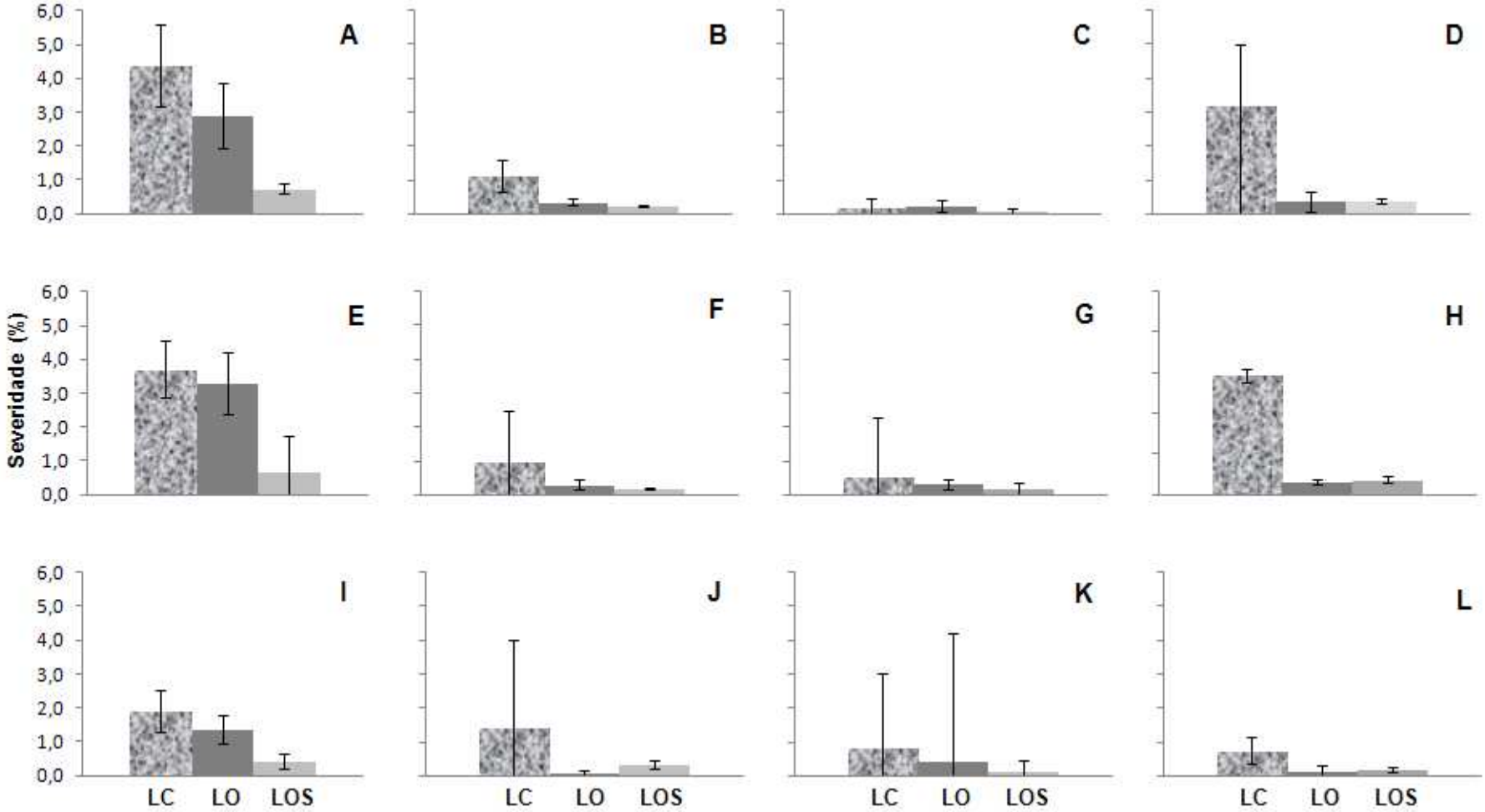

Figura 6: Estimativas da intensidade máxima (soma das estimativas dos parâmetros a e b) de severidade referentes aos anos I, 2004/05 (A, E e I), II, 2005/06 (B, F e J), III, 2006/07 (C, G e K) e IV, 2007/08 (D, H e L) de avaliação em lavouras convencional (LC), orgânica (LO) e orgânica sombreada (LOS), em ramos dos terços superior (A, B, C e D), médio (E, F, G e H) e inferior (I, J, K e L) de cafeeiros. Estimativas cujos limites dos intervalos de confiança assintóticos de 95% se sobrepõem dentro do mesmo gráfico não são estatisticamente diferentes.

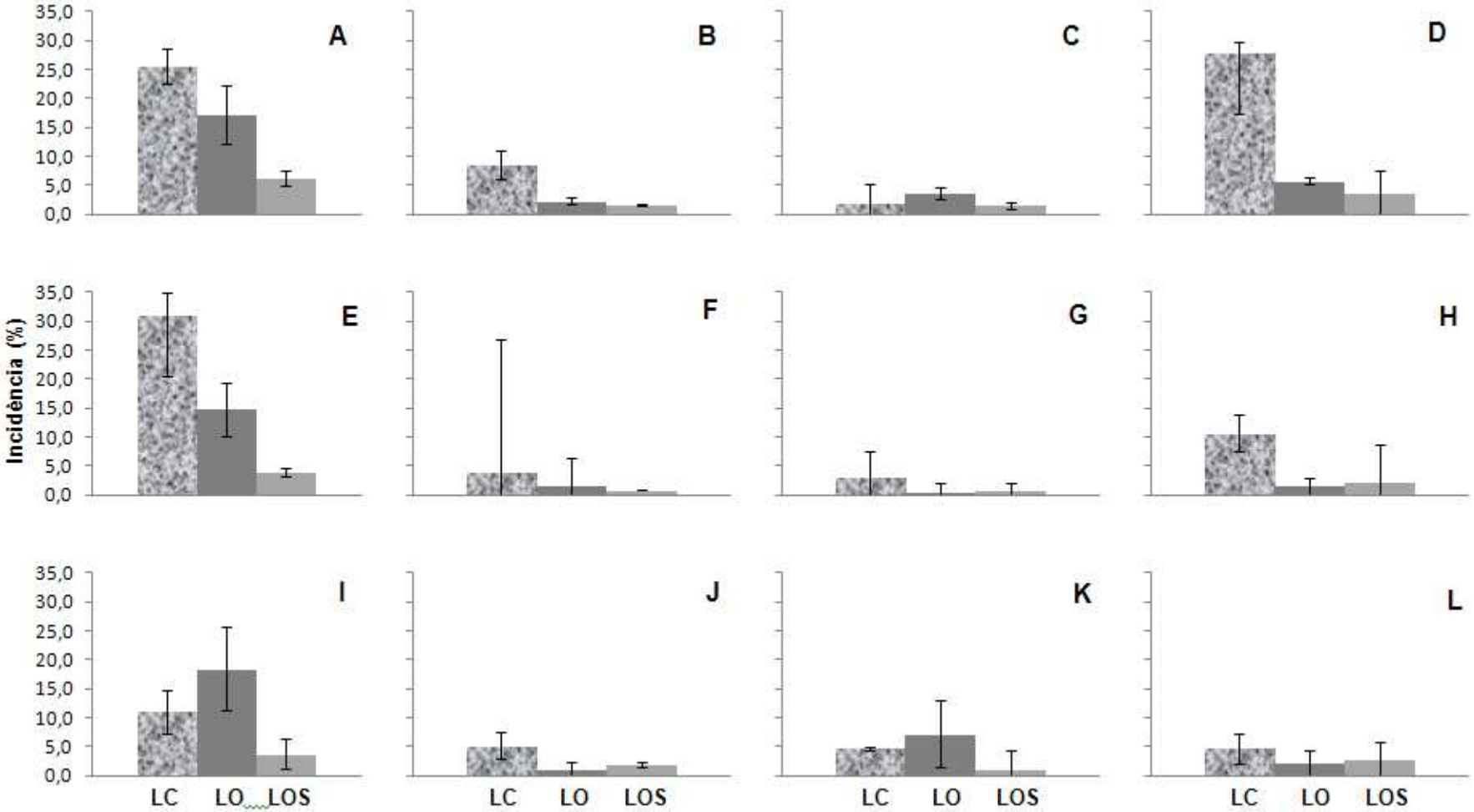

Figura 7: Estimativas da intensidade máxima (soma das estimativas dos parâmetros a e b) de incidência referentes aos anos I, 2004/05 (A, E e I), II, 2005/06 (B, F e J), III, 2006/07 (C, G e K) e IV, 2007/08 (D, H e L) de avaliação em lavouras convencional (LC), orgânica (LO) e orgânica sombreada (LOS), em ramos dos terços superior (A, B, C e D), médio (E, F, G e H) e inferior (I, J, K e L) de cafeeiros. Estimativas cujos limites dos intervalos de confiança assintóticos de 95% se sobrepõem dentro do mesmo gráfico não são estatisticamente diferentes.

Conclusões Gerais

Em vista dos resultados obtidos, concluiu-se que:

1- os tubos germinativos de *C. coffeicola* tiveram tropismo positivo para os estômatos, onde ocorreu a penetração, sem a formação de apressórios;
2- *Cercospora coffeicola* colonizou, inter e intracelularmente o parênquima lacunoso;
3- a esporulação de *C.coffeicola* ocorreu através e ao redor dos estômatos;
4- os seis isolados do tipo selvagem de *C. coffeicola* variaram quanto à produção de cercosporina; para um desses, não se detectou a produção da toxina;
5- os genes ATR1 e CTB1 de *C. coffeicola* amplificados tiveram similaridade com as sequências disponíveis no GenBank para *C. nicotianae;*
6- obteve-se sucesso na construção de GFP e na disrupção do gene CTB1 em *C. coffeicola*;
7- isolados do tipo selvagem e mutante CTB1 não diferiram significativamente quanto à esporulação e crescimento micelial;
8- a incidência e severidade da cercosporiose e desfolha e enfolhamento do cafeeiro foram maiores em ramos do terço superior e em cafeeiros da lavoura convencional;
9- maiores valores de intensidade da doença, desfolha e enfolhamento ocorreram de maio a julho, julho a setembro e de outubro a janeiro, respectivamente; e
10- a análise de séries temporais, com o uso dos modelos de regressão não linear ARMA, representaram, efetivamente, o progresso da cercosporiose do cafeeiro.