JAIME HONORATO JÚNIOR

ASPECTOS FISIOLÓGICOS E BIOQUÍMICOS DA RESISTÊNCIA DO CAFEEIRO À FERRUGEM POTENCIALIZADOS POR TRIAZOL E ESTROBILURINA

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*.

VIÇOSA
MINAS GERAIS - BRASIL
2014

Ficha catalográfica preparada pela Seção de Catalogação e Classificação da Biblioteca Central da UFV

T
H774a
2014

Honorato Júnior, Jaime, 1977-
Aspectos fisiológicos e bioquímicos da resistência do cafeeiro à ferrugem potencializados por triazol e estrobilurina / Jaime Honorato Júnior. – Viçosa, MG, 2014.
x, 75f. : il. (algumas color.) ; 29 cm.

Orientador: Laércio Zambolim.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Café - Doenças e pragas - Controle. 2. *Hemileia Vastatrix* - Controle. 3. Fungicidas sistêmicos. I. Universidade Federal de Viçosa. Departamento de Fitopatologia. Programa de Pós-graduação em Fitopatologia. II. Título.

CDD 22. ed. 633.73

JAIME HONORATO JÚNIOR

ASPECTOS FISIOLÓGICOS E BIOQUÍMICOS DA RESISTÊNCIA DO CAFEEIRO À FERRUGEM POTENCIALIZADOS POR TRIAZOL E ESTROBILURINA

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*.

APROVADA: 28 de fevereiro de 2014.

Fabrício de Ávila Rodrigues
(Coorientador)

Gleiber Quintão Furtado

Paulo Cezar Cavatte

Renata Sousa Resende

Laércio Zambolim
(Orientador)

JAIME HONORATO JÚNIOR

Eu não vejo a razão, porque Deus me ama assim.
O meu coração é duro, sou escravo, não sou ninguém!
Não consigo entender, o porquê do Seu amor,
mesmo sendo quem eu sou Ele me ama!
Estende as Suas mãos, dá proteção.
Ele me abraça... Não há razão!
(Ricardo Martins**)**

À Isabel Maria Carvalhedo Honorato,

muito amada e acalentada filha,

dedico.

AGRADECIMENTOS

Ao Deus Pai, Deus Filho e Deus Espírito Santo pelas brilhantes jóias humanas postas à disposição em meu viver: Terezinha e Ciro (*in memoriam*), avós maternos excepcionais; Arminda e João (*in memoriam*), avós paternos justos; minha animadora mãe, Cleide e meu encorajador pai, Jaime. Como aprendi e aprendo com vocês!

À jóia das jóias, estrela da manhã sempre irradiante em minha vida. Soraya, maravilhosa esposa e imprescindível amiga! Obrigado por ampliar nossa família. Boas-vindas à nossa pequena Isabel Maria!

À Universidade Federal de Viçosa, pela oportunidade em realizar este curso.

À Capes e ao CNPq, pela concessão da bolsa de estudos.

Ao Prof. Laércio Zambolim, pela orientação e confiança.

Ao Prof. Fabrício de Ávila Rodrigues por acreditar e estimular minha capacidade.

Aos pesquisadores Renata Sousa Resende e Carlos Eduardo Aucique Pérez por revisar os manuscritos.

Ao Alessandro Antonio Fortunato pelas contribuições na edição das imagens.

Aos professores do Departamento de Fitopatologia da UFV por compartilhar valiosos conhecimentos.

Aos amigos conquistados durante a caminhada acadêmica, pelos bons momentos vividos em Viçosa. Muito especialmente ao Dinho, Danilão, Little Robert, Rafa, Bira, Besouro, Cabelo, todos de fato, notáveis.

Aos colegas de laboratório: Rodrigo, Franklin, Aline Vieira, Aline Oliveira, Ueder, Henrique, Lucas, Ubiraci, Sérgio Milagres, José Cláudio, pelo convívio agradabilíssimo.

Muito Obrigado!

BIOGRAFIA

JAIME HONORATO JÚNIOR, filho de Cleide Silva Pereira Honorato e Jaime Honorato, nasceu em São Pedro do Ivaí, Paraná, em 06 de maio de 1977.

Em fevereiro de 1992, ingressou no curso Técnico em Agropecuária da Escola Média de Agropecuária Regional da Ceplac (EMARC) em Ariquemes, Rondônia, formando-se em dezembro de 1994.

Em janeiro de 1996, ingressou no curso de Agronomia da Escuela de Agricultura de la Región Tropical Húmeda (EARTH) na Costa Rica, América Central, graduando-se em 10 de dezembro de 1999.

Entre 2000 e 2007, desenvolveu atividade de pesquisa agrícola em algumas multinacionais, em especial com seringueiras, na Michelin.

Em 03 de março de 2008, iniciou o Programa de Pós-graduação em Fitopatologia, em nível de Mestrado, na Universidade Federal de Viçosa, submetendo-se à defesa de dissertação em 22 de fevereiro de 2010.

Em 01 de março de 2010, iniciou Doutorado no mesmo Programa de Pós-graduação em Fitopatologia, na Universidade Federal de Viçosa, submetendo-se à defesa de dissertação em 28 de fevereiro de 2014.

SUMÁRIO

RESUMO

HONORATO JUNIOR, Jaime, D.Sc., Universidade Federal de Viçosa, fevereiro de 2014. **Aspectos fisiológicos e bioquímicos da resistência do cafeeiro à ferrugem potencializados por triazol e estrobilurina.** Orientador: Laércio Zambolim. Coorientador: Fabrício de Ávila Rodrigues.

O café é a commodity mais negociada no mundo sendo o Brasil seu maior produtor. A ferrugem do cafeeiro, causada pelo fungo biotrófico *Hemileia vastatrix* é a doença mais importante do cafeeiro e pode causar redução em sua produtividade entre 35 a 50%. Muito pouco se sabe sobre este patossistema, bem como os impactos do patógeno sobre a fotossíntese da planta, a atividade de enzimas de defesa, a concentração de pigmentos vegetais e os parâmetros de fluorescência clorofila *a* sob diferentes tratamentos com fungicidas. Para contribuir com a geração de novos conhecimentos, este estudo teve como objetivos: 1) avaliar a variável F_v/F_m (Rendimento quântico máximo do fotossistema II) obtida através da imagem da fluorescência da clorofila *a* como ferramenta para diferenciar infecções pré-sintomáticas em plantas pulverizadas com epoxiconazol e piraclostrobina e 2) investigar as relações entre pigmentos fotossintéticos, atividade de enzimas de defesa e alguns parâmetros de fluorescência da clorofila *a* em folhas de café pulverizadas com epoxiconazol e piraclostrobina e inoculadas ou não-inoculadas com *Hemileia vastatrix*. Os experimentos seguiram delineamento inteiramente casualizado e foram conduzidos em casa de vegetação. As plantas de café foram cultivadas em estufa e inoculadas com urediniósporos. Epoxiconazol e piraclostrobina foram pulverizadas sobre as plantas, a fim de estudar o parâmetro F_v/F_m de fluorescência da clorofila *a*, a severidade da ferrugem do cafeeiro, a atividade de enzimas de defesa, a concentração de pigmentos fotossintéticos e outros parâmetros de fluorescência da clorofila *a*. Houve uma relação linear significativa entre severidade real e visual das áreas foliares estimadas pelo parâmetro F_v/F_m e para cada unidade adicional na severidade visual, houve um aumento de 53% na severidade real. Para os tratamentos com epoxiconazol e piraclostrobina, os sintomas da ferrugem do cafeeiro diminuíram tomando como base as imagens visual e as geradas a partir do parâmetro F_v/F_m da fluorescência da clorofila *a*. Pústulas nas folhas pulverizadas com epoxiconazol foram menores nas folhas do tratamento controle mas, maiores do que piraclostrobina. A redução nos valores do parâmetro F_v/F_m no epicentro das pústulas das folhas pulverizadas com epoxiconazol e piraclostrobina foram menores do que os do tratamento controle. A severidade da ferrugem do cafeeiro diminuiu até 40 dias após a

inoculação nas plantas pulverizadas com epoxiconazol e piraclostrobina. As atividades de catalase, peroxidases, β-1,3-glucanases e quitinases foram diferentes entre os tratamentos com plantas inoculadas e não-inoculadas. Houve redução significativa na concentração de pigmentos entre as plantas não-inoculadas e inoculadas mas, não entre as plantas pulverizadas com epoxiconazol e piraclostrobina. Os parâmetros de fluorescência da clorofila *a* foram diferentes entre plantas não-inoculadas e inoculadas e não-pulverizadas e pulverizadas com fungicidas. *Hemileia vastatrix* afetou a capacidade fotossintética de *Coffea arabica* e o parâmetro F_v/F_m de fluorescência da clorofila *a* foi capaz de mostrar esse efeito antes do aparecimento dos sintomas visuais. Alterações na fotossíntese foram detectadas em plantas não-inoculadas e inoculadas pulverizadas com epoxiconazol e piraclostrobina. Além disso, o efeito dos fungicidas sobre a fotossíntese deve ser estudado, para proporcionar mais oportunidades para a compreensão dos impactos sobre os mecanismos de defesa da planta, que levam à redução de sintomas da ferrugem do cafeeiro.

ABSTRACT

HONORATO JUNIOR, Jaime, D.Sc., Universidade Federal de Viçosa, February, 2014. **Physiological and biochemical aspects of resistance in coffee to rust potentiated by triazole and strobilurin.** Adviser: Laércio Zambolim. Co-adviser: Fabrício de Ávila Rodrigues.

Coffee is the most traded commodity in the world and Brazil is the largest producer. Coffee leaf rust, caused by the biotrophic fungus *Hemileia vastatrix* is the most important coffee disease reducing coffee yield by 35 to 50%. Too little is known about this pathosystem as well as the pathogen impacts on plant photosynthesis, defense enzymes activities, concentration of plant pigments and chlorophyll *a* fluorescence parameters under different fungicides sprayed treatments. To contribute with new knowledge generation, this study aimed: 1) to evaluate the F_v/F_m (Maximal photosystem II quantum yield) chlorophyll *a* fluorescence imaging parameter as tool to differentiate pre-symptomatic coffee leaf rust infections in plants sprayed with pyraclostrobin and epoxiconazole and 2) to investigate the relationships between photosynthetic pigments, defense enzymes activities and some chlorophyll *a* fluorescence parameters on leaves of coffee plants sprayed with epoxiconazole and pyraclostrobin and inoculated or non-inoculated with *Hemileia vastatrix*. Experiments in a completely randomized design were performed in greenhouse and coffee plants were inoculated with urediniospores. Epoxiconazole and pyraclostrobin were sprayed in coffee plants in order to study the F_v/F_m chlorophyll *a* fluorescence parameter, coffee leaf rust severity, defense enzymes activities, photosynthetic pigments concentration and other chlorophyll *a* fluorescence parameters. Results from the present study showed that the different fungicides application on soil and/or leaves of triazoles and strobilurins were efficient to control coffee leaf rust. There was a significant linear relationship between the areas of real severity and visual severity and for each additional unit in the visual severity there was an increase of 53% on the real severity. For the epoxiconazole and pyraclostrobin treatments, the coffee leaf rust symptoms decreased based on both visual and F_v/F_m images. Pustules on leaves sprayed with epoxiconazole were smaller in size than the ones on leaves of plants from the control treatment, but bigger than pyraclostrobin. Reduction on F_v/F_m values at the pustules epicenter on leaves of plants sprayed with epoxiconazole and pyraclostrobin were lower than those of the control treatment. Coffee leaf rust severity decreased until 40 days

after inoculation on plants sprayed with epoxiconazole and pyraclostrobin. Catalase, peroxidases, β-1,3-glucanases and chitinases activities were different among treatments with inoculated and non-inoculated plants. There was a significant reduction in pigments concentration between non-inoculated and inoculated treatments but not, in plants sprayed with epoxiconazole and pyraclostrobin. Chlorophyll *a* fluorescence parameters were different between non-inoculated and inoculated plants and non-sprayed and fungicides sprayed plants. *Hemileia vastatrix* affected the photosynthetic capacity of *Coffea arabica* and the F_v/F_m parameter was able to show this effect before the appearance of visual symptoms. Changes on photosynthesis were detected in non-inoculated and inoculated plants sprayed with epoxiconazole and pyraclostrobin. Also, the effect of fungicides on photosynthesis should be studied to provide more opportunities to understanding the impacts on plant defense mechanisms that lead to reduced coffee leaf rust symptoms.

INTRODUÇÃO GERAL

A ferrugem do cafeeiro (*Hemileia vastatrix* Berkeley & Broome) é a principal doença que afeta os cafezais brasileiros e pode causar perdas entre 35 e 50% (Zambolim et al., 1997; 2002). Estratégias de manejo da ferrugem são baseadas, majoritariamente, na obtenção de cultivares resistentes e no controle químico (Zambolim et al., 1997; 2002). O controle da ferrugem utilizando variedades resistentes é promissor (Zambolim et al., 1997), porém, a variabilidade fisiológica do patógeno compromete a durabilidade da resistência (van der Vossen, 2005). Em vista da dificuldade de obter resistência durável, aliado ao fato de que a maioria das áreas são plantadas com cultivares suscetíveis, além do aumento nas restrições aos produtos à base de cobre (Carvalho et al., 2002), há necessidade de alternativas para manejo da doença (Zambolim et al., 2002). Atualmente há opções de controle químico dessa doença baseadas em fungicidas de contato e sistêmicos (Mapa, 2014). Entre os sistêmicos, estrobilurinas e triazóis possuem diversas moléculas disponíveis no mercado (Zambolim et al., 2002). Também, há formulações que possuem ambas as moléculas, o que dá maiores benefícios às plantas pulverizadas (Souza et al., 2011).

Efeitos fisiológicos positivos são aqueles que promovem, em plantas pulverizadas, o aumento da biomassa, da fotossíntese e/ou da síntese de carboidratos (Venâncio et al., 1999). Os benefícios apregoados pelas empresas fabricantes de fungicidas estão relacionados ao efeito verdejante nas culturas anuais, tais como soja e trigo (Venâncio et al., 1999; 2003; Bartlett et al., 2002). Efeitos fisiológicos de diferentes fungicidas também foram verificados em videira (Saladin et al., 2003). Separar e mensurar os benefícios da aplicação destas moléculas sobre as plantas, na ocasião do controle da doença, daqueles meramente fisiológicos provocados pelo

produto tem sido objeto de estudo de muitos pesquisadores (Fagan, 2007; Rodrigues, 2009; Pinto, 2010). A ideia de que um produto por si só cause benefícios às plantas, ainda na ausência do patógeno, é muito atrativa e faz com que as empresas do setor invistam somas consideráveis para testar hipóteses relacionadas ao tema (Venâncio et al., 1999; 2003; Bartlett et al., 2002).

O estudo dos efeitos fisiológicos causados por fungicidas que afetam ferrugens não é frequente. Existem estudos desses efeitos principalmente para cereais como o trigo (Jorgensen et al., 1999; Bertelsen et al., 2001; Harvey, 2002). No entanto, a maior parte destes estudos envolve patógenos necrotróficos tais como *Septoria tritici* (Clark & Leandro, 1998), *Gaeumannomyces graminis* var. *tritici* (Jenkyn et al., 2000) e *Mycosphaerella graminicola* e *Alternaria alternata* (Bertelsen et al., 2001). Estudos relacionados com o efeito fisiológico de fungicidas na interação *Hemileia vastratix*– cafeeiro são inexistentes.

O efeito da aplicação de fungicidas na fotossíntese de algumas culturas tem sido relatado na literatura. Um fungicida de contato, por exemplo, como o cobre, inibe a fotossíntese ao destruir cloroplastos afetando, assim, a atividade do fotossistema II e a biossíntese de clorofilas (Krofta et al., 2012; Petit et al., 2012). Por sua vez, fungicidas sistêmicos como os benzimidazóis, anilidas e pirimidinas são fitotóxicos, ao passo que os azóis estimulam a fotossíntese (Petit et al., 2012). Com o aumento do estresse, vários processos fotossintéticos podem ser comprometidos na planta, levando à diminuição da fotossíntese líquida (Pn) equivalente à assimilação (A) de CO_2 (Krause & Weis, 1991). O fechamento dos estômatos ocasionado por uma redução na condutância estomática (g_s) é, com frequência, considerada com uma resposta fisiológicas precoce ao estresse, resultando na diminuição da fotossíntese líquida por meio de uma limitada disponibilidade de CO_2 no mesófilo (Krause & Weis, 1991).

A fotossíntese é um dos processos fisiológicos mais estudados e melhor compreendidos pelos pesquisadores (Bernacchi et al., 2013). Modelos bioquímicos detalhados da fotossíntese incluem reações à luz, transporte de prótons e elétrons e reações enzimáticas (Bernacchi et al., 2013). Além do mais, funções regulatórias também têm sido recentemente melhor entendidas (Laisk et al., 2006; Bernacchi et al., 2013). Muitos trabalhos sobre flutuações na fotossíntese após aplicações de fungicidas em várias culturas reportam modificações tanto nas trocas gasosas como na fluorescência da clorofila *a* (Krugh & Miles, 1996; van Iersel & Bugbee, 1996; Untiedt & Blanke, 2004; Xia et al., 2006).

Desta forma, o presente estudo busca contribuir na elucidação dos possíveis efeitos de estrobilurinas e triazóis na resistência do cafeeiro à ferrugem com base na melhoria da capacidade fotossintética. Neste trabalho objetivaram-se: i. as relações entre fluorescência da clorofila *a*, pigmentos e enzimas de defesa em plantas de café infectadas por *H. vastatrix*, pulverizadas com epoxiconazole e piraclostrobina (Cap. 1); e ii. o potencial da análise de imagem da fluorescência da clorofila *a* para identificação de infecções latentes de *H. vastatrix* em folhas de café pulverizadas ou não com epoxiconazol e piraclostrobina (Cap. 2).

REFERÊNCIAS

Bartlett DW, Clough JM, Godwin JR, Hall AA, Hamer M, Parr-Dobrzanski B. (2002) Review: The strobilurins fungicides. Pest Management Science 58:649-662.

Bernacchi CJ, Bagley JE, Serbin SP, Ruiz-Vera UM, Rosenthal DM, Vanloocke A. (2013) Modelling C3 photosynthesis from the chloroplast to the ecosystem. Plant, Cell and Environment 36:1641-1657.

Bertelsen JR, Neergaard E de, Smedegaard-Petersen V. (2001) Fungicidal effects of azoxystrobin and epoxiconazole on phyllosphere fungi, senescence and yield of winter wheat. Plant Pathology 50:190-250.

Carvalho VL, Cunha RL, Chalfoun SM. (2002) Manejo ecológico das principais doenças do cafeeiro. Informe Agropecuário 23:101-114.

Clark B, Leandro L. (1998) Physiological, disease control and yield effects of kresoxim-methyl. The Agronomist 2:15-17.

Fagan EB. (2007) A cultura da soja: modelo de crescimento e aplicação de estrobilurina. 84 p. Tese (Doutorado em Agronomia) - Escola Superior de Agricultura "Luiz de Queiroz", ESALQ, Piracicaba.

Harvey IC. (2002) Epidemiology and control of leaf and awn spot of barley caused by *Ramularia collo-cygni*. New Zealand Plant Protection 55:331-335.

Jenkin JF, Bateman GL, Gutteridge RJ, Edwards SG. (2000) Effects of foliar sprays of azoxistrobina on take-all in wheat. Annals of Applied Biology 137:99.

Jorgensen LN, Henriksen KE, Nielsen GC. (1999) Adjusting thresholds for Septoria control in winter wheat using strobilurins. In: GINKEL, M. V.; McNAB, A.; KRUPINSK, Y. Septoria and Stagonospora diseases of control: a compilation of global research. México: CIMMYT, p. 173-175.

Krause GH, Weis E. (1991) Chlorophyll fluorescence and photosynthesis: the basics. Annual Review of Plant Physiology and Plant Molecular Biology 42:313-349.

Krofta K, Pokorný J, Kudrna T, Ježek J, Pulkrábek J, Křivánek J, Bečka D. (2012) The effect of application of copper fungicides on photosynthesis parameters and level of elementary copper in hops. Plant, Soil and Environment 58:91-97.

Krugh BW, Miles D. (1996) Monitoring the effects of five ‘‘nonherbicidal’’ pesticide chemicals of terrestrial plants using chlorophyll fluorescence. Environmental Toxicology Chemistry 15:495-500.

Laisk A, Eichelmann H, Oja V. (2006) C3 photosynthesis in silico. Photosynthesis Research 90:45-66.

Mapa. Ministério da Agricultura, Pecuária e Abastecimento. Disponível em http://www.agricultura.gov.br. Acesso em 09 de janeiro de 2014. 2014.

Petit AN, Fontaine F, Vatsa P, Clément C, Vaillant-Gaveau N. (2012) Fungicide impacts on photosynthesis in crop plants. Photosynthesis Research 111:315-326.

Pinto TLF. (2010) Fungicida foliar à base de estrobilurina, produção e potencial fisiológico de sementes de soja. 117 p. Tese (Doutorado em Fitotecnia) - Escola Superior de Agricultura “Luiz de Queiroz”, ESALQ, Piracicaba.

Rodrigues MAT. (2009) Avaliação do efeito fisiológico do uso de fungicidas na cultura de soja. 197 p. Tese (Doutorado em Fitotecnia) - Escola Superior de Agricultura “Luiz de Queiroz”, ESALQ, Piracicaba.

Saladin G, Magné C, Clément C. (2003) Effects of fludioxonil and pyrimethanil, two fungicides used against *Botrytis cinerea*, on carbohydrate physiology in *Vitis vinifera* L. Pest Management Science 59:1083-1092.

Souza AF, Zambolim L, Jesus Júnior VC, Cecon PR. (2011) Chemical approaches to manage coffee leaf rust in drip irrigated trees. Australasian Plant Pathology 40:293-300.

Untiedt R, Blanke MM. (2004) Effects of fungicide and insecticide mixtures on apple tree canopy photosynthesis, dark respiration and carbon economy. Crop Protection 23:1001-1006.

van der Vossen HAM. (2005) State-of-the-art of developing durable resistance to biotrophic pathogens in crop plans, such as coffee leaf rust. In: Zambolim, L., Zambolim, E. M., Várzea, V. M. P. (Eds.). Durable resistance to coffee leaf rust. Suprema Gráfica e Editora, Visconde do Rio Branco, Brasil, pp. 1-29.

van Iersel MW, Bugbee B. (1996) Phytotoxic effects of benzimidazole fungicides on bedding plants. Journal of the American Society for Horticultural Science 121:1095-1102.

Venâncio WS, Rodrigues MAT, Begliomini E, Souza NL. (2003) Physiological effects of strobilurin fungicides on plants. Publicatio UEPG, Ciências Exatas e da Terra, Ciências Agrárias e Engenharias 9:59-68.

Venâncio WS, Zagonel J, Furtado EL, Souza NL. (1999) Novos fungicidas. I - produtos naturais e derivados sintéticos: estrobilurinas e fenilpirroles. In: Luz WC, Fernandes JM, Prestes AM, Picinini EC (Eds). Revisão Anual de Patologia de Plantas 7:103-155.

Xia XJ, Huang YY, Wang L, Huang LF, Yu YL, Zhou YH, Yu JQ. (2006) Pesticides-induced depression of photosynthesis was alleviated by 24-epibrassinolide pretreatment in *Cucumis sativus* L. Pesticide Biochemistry and Physiology 86:42-48.

Zambolim L, Vale FXR, Costa H, Pereira AA, Chaves GM. (2002) Epidemiologia e controle integrado da ferrugem-do-cafeeiro. In: Zambolim, L. (Ed.). O Estado da Arte de Tecnologias de Produção de Café. UFV - Viçosa, MG, Brasil. p.369-450.

Zambolim L, Vale FXR, Pereira AA, Chaves GM. (1997) Café (*Coffea arabica* L.), Controle de Doenças. In: Vale FXR, Zambolim L. (Eds.). Controle de Doenças de Plantas: Grandes Culturas, Vol. 1. Suprema Gráfica e Editora, Visconde do Rio Branco, Brasil, pp.83-140.

CHAPTER 1 - EFFECT OF TRIAZOLE AND STROBILURIN FUNGICIDES IN COFFEE LEAVES INFECTED BY *Hemileia vastatrix* DETERMINED BY CHLOROPHYLL *a* FLUORESCENCE IMAGING

Jaime Honorato Júnior[1], Laércio Zambolim[1], Henrique Silva Silveira Duarte[2], Carlos Eduardo Aucique-Pérez[1], Fabrício Ávila Rodrigues[1]

Author's addresses: [1]Universidade Federal de Viçosa, Departamento de Fitopatologia, Viçosa, Minas Gerais State, Zip Code 36570-000, Brazil; [2]Paraná Federal University, Plant Protection Department, SCA-DFF, Curitiba, Paraná State, Zip Code 80035-050, Brazil.

(correspondence to L. Zambolim. E-mail: zambolim@ufv.br and F. A. Rodrigues. E-mail:fabricio@ufv.br)

Abstract

Coffee is the most traded commodity in the world and Brazil is the largest producer. Coffee leaf rust, caused by the biotrophic fungus *Hemileia vastatrix* is the most important coffee disease reducing coffee yield by 35 to 50%. F_v/F_m chlorophyll *a* fluorescence imaging parameter was used to differentiate pre-symptomatic coffee leaf rust infections in plants sprayed with pyraclostrobin and epoxiconazole. Coffee plants were grown in greenhouse and urediniospores were inoculated. Fungicides were sprayed and F_v/F_m chlorophyll *a* fluorescence parameter was captured. There was a significant linear relationship between the areas of real severity and visual severity and for each additional unit in the visual severity there was an increase of 1.53 units on the real severity. For the epoxiconazole and pyraclostrobin treatments, the coffee leaf rust symptoms decreased based on both visual and F_v/F_m images. Pustules on leaves sprayed with epoxiconazole were smaller in size than the ones on leaves of plants from the control treatment, but bigger than pyraclostrobin. Reduction on F_v/F_m values at the pustules epicenter on leaves of plants sprayed with epoxiconazole and pyraclostrobin were greater than those of the control treatment. This was expected and reflects the importance of these fungicides to slow coffee leaf rust progress. *Hemileia vastatrix* affected the photosynthetic capacity of *Coffea arabica* and the F_v/F_m parameter was able to show this effect before the appearance of visual symptoms.

Keywords: *Coffea arabica* L., coffee rust, photosynthesis, sistemic fungicides.

1-Introduction

After crude oil, coffee is the most traded commodity in the world (Vega et al., 2003; Crumley, 2013). Currently, Brazil is the largest coffee producer and in 2012 contributed with one-third of the world's coffee production (3.05 million tonnes) (Conab, 2013). Approximately, 75% of this production came from *Coffea arabica* and the remainder from *C. canephora* (conilon coffee) (Conab, 2013). Although Brazil exports approximately 30% of its annual production, the mean yield is only 1.49 ton per hectare, about 40% less than the most productive regions of the country (Conab, 2013). Diseases are one of the most important factors that limit coffee yield and the Coffee Leaf Rust (CLR), caused by the biotrophic fungus *Hemileia vastatrix* Berkeley & Broome, is of greatest importance (Waller et al., 2007).

The effect of CLR in coffee trees is due to leaves premature fall, branches death and low photosynthesis rates that reduces coffee yield in the following year by 35 to 50% (Zambolim et al., 1992, 1999; Waller et al., 2007). The disease reduces significantly the ability of leaves for CO_2 fixation that is necessary for sugars formation required in the reproductive phase (de Ribou et al., 2013). CLR symptoms are manifest as pale yellow spots on the abaxial side of the leaves (Kushalappa and Eskes, 1989). The spots develop into orange-yellow pustules that often coalesce with other ones (Kushalappa and Eskes, 1989; Zambolim et al., 1999). On the adaxial side of the leaves, yellow chlorotic spots corresponding to the pustules producing urediniospores on the abaxial leaf surface are often observed (Kushalappa and Eskes, 1989). Disease development makes the spots become necrotic and causes leaves fall (Zambolim et al., 1999). The chemical control of CLR in Brazil is basically performed with triazoles and strobilurins sprayed on the leaves and/or applied into the soil at different times throughout the growing season (Zambolim et al., 1999; 2002). Also, there are

formulations with both molecules, which provide further benefits (e.g. green effect) to the sprayed plants (Bartlett et al., 2002; Venâncio et al., 2003).

The CLR severity assessment must be precise and accurate (Capucho et al., 2011). Estimates of disease severity, most often the percentage of leaf area infected, are used for many purposes such as to evaluate control measures, determine varietal resistance, assess efficiency of fungicides and develop a crop loss model with the use of methods to determine the quality of assessments and improve their precision and accuracy (Nutter et al., 1993; Vale et al., 2004; Madden et al., 2007; Bock et al., 2010).

Normally, the quantification of foliar disease severity caused by fungal infection has relied on methods such as the standard area diagram based on visible symptoms (Vale et al., 2004; Madden et al., 2007). For instance, a standard area diagram set to estimate CLR severity was devised using photographs of leaves with six diseases severities ranging from 2.5 to 80% of diseased foliar area (Capucho et al., 2011). Although easy and quick to perform, this method only assesses readily visible symptoms and can be affected by the subjectivity of the observers (Capucho et al., 2011). One way to reduce observer subjectivity is using digital image analysis to quantify disease severity based on changes in leaf colors due to disease symptoms (Vale et al., 2003).

This is a major advantage in studying localized stress responses such as those caused by plant diseases with longer incubation period, where there are irregular infected areas on a leaf while other parts of the same leaf remain apparently healthy (Baker, 2008; Rolfe and Scholes, 2010). Chlorophyll *a* fluorescence imaging may be used to quantify the effects of foliar diseases on photosynthesis as a non-invasive, nondestructive and highly sensitive probe (Schreiber et al., 1986; Baker, 2008; Rolfe and Scholes, 2010; Fiorani et al., 2012; Mahlein et al., 2012). Chlorophyll *a*

fluorescence imaging is based that light energy absorbed by chlorophyll molecules in photosystem II (PSII) can either be re-emitted as detectable fluorescence used for photosynthesis (photochemical quenching, q_p) or lost as heat (non-photochemical quenching, NPQ) (Maxwell and Johnson, 2000). Increased energy used in one of those processes leads to decreased energy used in the other two (Baker, 2008). Many chlorophyll fluorescence parameters can be measured (Baker and Oxborough, 2004; Baker, 2008). The ratio of variable and maximum fluorescence of dark-adapted tissue (F_v/F_m) is the most widely used chlorophyll *a* fluorescence parameter (Baker, 2008). In healthy leaves, F_v/F_m is close to 0.8 and lower F_v/F_m values indicate that a proportion of PSII reaction centers were damaged, which is known as photoinhibition and is often observed on stressed plants (Müller et al., 2001; Baker and Oxborough, 2004). F_v/F_m parameter is useful for assess the plant photochemical capacity because it act as a stress indicator for plants (Maxwell and Johnson, 2000; Müller et al., 2001; Baker and Oxborough, 2004; Baker, 2008).

Many necrotrophic, hemibiotrophic and biotrophic fungi (Meyer et al., 2001; Berger et al., 2004; Swarbrick et al., 2006; Chaerle et al., 2007; Behr et al., 2010), bacterias (Berger et al., 2007; Rodriguez-Moreno et al., 2008; Iqbal et al., 2012) and virus (Balachandran et al., 1994; Chaerle et al., 2004; Perez-Bueno et al., 2006) causing foliar diseases have been imaged using chlorophyll *a* fluorescence. These studies have focused on factors such as detecting the infections before the visible symptoms occur (Chou et al., 2000; Bonfig et al., 2006; Berger et al., 2007) and comparing compatible versus incompatible host-pathogen interactions (Bonfig et al., 2006; Swarbrick et al., 2006; Berger et al., 2007). However, only Swarbrick et al. (2006) and Tung et al. (2013) used chlorophyll *a* fluorescence images to quantify the amount of infected tissue.

The aim of this study was to use chlorophyll *a* fluorescence imaging based on the F_v/F_m parameter to determine areas on coffee leaves with altered chlorophyll *a* fluorescence and to differentiate pre-symptomatic tissue from healthy tissue during CLR development. The same approach was used to determine the impacts of CLR on coffee leaves plants sprayed with a strobilurin (pyraclostrobin) and a triazole (epoxiconazole), known to benefit the physiology of several plant species (Bartlett et al., 2002), in comparison to non-sprayed coffee plants.

2-Materials and Methods

2.1-Coffee plants growth

Coffee seeds (cv. "Catuaí Vermelho IAC 144") were sowed in a moist sand bed for 60 days. After this period, coffee plants were transplanted to plastic pots containing 1kg of a mix of soil, manure and sand (2.5:1:0.5 proportions). Soil pH was corrected by adding 1.5 g of dolomitic limestone to each pot 30 days before seedlings transplant. Coffee plants were fertilized at three days after transplant with 25 mL of nutritive solution (Novais et al., 1991) and then at each 7 days until the end of the experiment.

Three sets of three months-old plants (each set with three plants) were transferred to a growth chamber (relative humidity 90 ± 5% at 22 ± 2ºC and 12 h light (32.13 µmoles/m^2/s)) before being inoculated with *H. vastatrix*.

2.2-Coffee leaf rust fungus, inoculation and fungicides spraying

Urediniospores were collected from coffee leaves with pustules in the field and their viability was assessed before plants inoculation. For this purpose, a total of 125 µL of a suspension of urediniospores was transferred to five Petri dishes containing BDA (potato-dextrose-agar) media and was homogeneously distributed on each dish using a Drigalsky glass stick. Petri dishes were kept in complete darkness in a growth chamber at 25°C. After 24 h, lactophenol was added to the plates to stop urediniospore germination. Two hundred urediniospores were randomly examined from each Petri dish under a microscope (Carl Zeiss Axio Imager A1) at 400 × magnification. A urediniospore was considered germinated when the germ tube was longer than its diameter. The percentage of urediniospores germination was always bigger than 30 ± 2.5% (Capucho et al., 2009) for use in all experiments.

Inoculation was performed on the abaxial side of the first pair of expanded leaves with a suspension of urediniospores (1 mg/leaf) with a camel hair brush (Silva et

al., 2002). Distilled water was then sprayed onto the inoculated leaf surface and plants were kept for 48 h in a dark moist chamber (relative humidity of 95 ± 5%, 24 ± 1°C). Subsequently, the plants were transferred to growth chamber (relative humidity 90 ± 5% at 22 ± 2ºC and 12 h photoperiod with fluorescent light (32.13 µmoles/m^2/s)) until the end of the experiment.

Four days after inoculation (dai), plants were sprayed with the fungicides pyraclostrobin (199.5 g a.i. ha^{-1}) and epoxiconazole (75 g a.i. ha^{-1}). These fungicides were applied on plants canopies with a manual backpack sprayer (Jacto PJH, JD-12P spray nozzle) gauged for applying a spray volume of 400 L/ha. Control treatment consisted of inoculated plants sprayed with distilled water.

2.3-Chlorophyll a fluorescence (Chl*a*F) image capture

The fluorescence imaging parameters were determined by using the Imaging-PAM M-Series chlorophyll fluorometer and the software version 2.32 ImagingWIN (Heinz Walz GmbH, Effeltrich, Germany) between 2:00h and 6:00h am. The sensor system consisted of 44 LED's-lamp high power (450 nm) required to apply fluorescence excitation, actinic illumination and saturation pulses. These LEDs are arranged in pairs with each pair featuring a red (660 nm) and a near-infrared (780 nm) LED. A CCD camera with 640 × 480 resolutions pixels located above the plants canopies at a distance of 18.5 cm give an image area of 10 × 13 cm. The determination protocol began with the adaptation of plants to darkness for 45 min (Baker, 2008), then leaves were exposed to a light pulse intensity of 0.5 µmol m^{-2} s^{-1} (1 Hz), establishing the minimum fluorescence image (F_0). Next, a saturating pulse of blue light (470 nm) of 2400 µmol m^{-2} s^{-1} intensity (10 Hz) was delivered for at 0.8 s allowed obtaining the maximum fluorescence image (F_m). By calculating F_0 and F_m, the software performed the calculation and provided maps with the fluorescent quantum efficiency (F_v/F_m = (F_m-

F_0)/F_m) (Baker, 2008). Coffee plants were taken out of the incubation chamber to the laboratory for Chl*a*F imaging at each evaluation time.

2.4-Experiment 1

A total of 75 coffee leaves infected with *H. vastatrix* and presenting different visual severity levels were collected and photographed. First of all, the visual severity values were determined using discriminant functions by QUANT software (Vale et al., 2003). Then, for the same leaves, the real severity estimated by F_v/F_m parameter was quantified by QUANT software. Then, a linear regression analysis between the two measurements obtained from the same leaf area was performed to assess their relationship. The virtual severity (real severity minus visual severity) was analyzed by graphical analysis.

2.5-Experiment 2

This experiment was arranged in a completely randomized design with three replications and consisted of inoculated plants sprayed with two fungicides (pyraclostrobin and epoxiconazole) and non-sprayed plants (control). Each experimental unit corresponded to a plastic pot containing one plant from which one pair of expanded leaves was inoculated. A total of 9 plants were used in each experiment (3 plants per each treatment). Visual and real severity assessments were performed at 2, 5, 10, 26 and 40 dai. The disease progress curves were plotted for the visual, real and virtual disease severities for pyraclostrobin, epoxiconazol and control treatments. Data from CLR severity (visual, real and virtual) was used to calculate the area under rust progress curve (AURPC) according to Shaner and Finney (1977). The experiment was repeated once. The data for all of the variables were subjected to an analysis of variance (ANOVA) and the means from the treatments were compared by Tukey's test ($P \leq 0.05$) using the SAS software (SAS Institute Inc., Cary, NC).

2.6-Experiment 3

F_v/F_m values were obtained by transect function from ImagingWIN software version 2:32 (Heinz Walz GmbH, Effeltrich, Germany) of 50 pustules (10 mm each) obtained from leaves sprayed with epoxiconazole and pyraclostrobin as well as from leaves non-sprayed (control treatment) after 40 dai. The pixel values were adjusted to represent the distance of 10 mm in the real leaf image. The standard error was calculated for each point and the line graph was drawn for each treatment.

3-Results

3.1-Experiment 1

The leaf area containing necrosis and chlorosis of 75 inoculated leaves at 40 dai estimated based on the F_v/F_m images was compared with the proportion of leaf area containing pustules of the same leaf to determine the relationship between F_v/F_m and the visual severity. It was considered as real disease, the image treated with the QUANT software using the F_v/F_m parameter. This parameter indicates all areas in which the whole leaf Chl*a*F was altered and indirectly damage to the photosynthetic apparatus and may, therefore, be an indicator of the amount of non-apparent disease. There was a significant linear relationship between the areas of real severity and visual severity (R^2 = 0.89) (Fig. 1). Some of the variation was likely caused by underestimations of the areas of necrosis from the F_v/F_m images due to occasional merging of necrotic and chlorotic areas at 40 dai and also by some variation in the sizes of the visible pustules. The real severity was higher than visual severity, which can be confirmed by the positive values of the virtual lesions (Fig. 2). The equation of linear regression showed that for each additional unit in the visual severity there was an increase of 1.53 units on the real severity (Fig. 1).

3.2-Experiment 2

Images obtained from the analysis of 30 coffee leaves infected with *H. vastatrix* sprayed with epoxiconazole and pyraclostrobin and non-sprayed leaves (control) shown increase in CLR symptoms over time (Fig. 3). In the control treatment, symptoms were more evident in both visual and F_v/F_m images (Fig. 3-A). On the other hand, for the epoxiconazole and pyraclostrobin treatments, the CLR symptoms decreased based on both visual and F_v/F_m images (Fig. 3-B and C).

Epoxiconazole and pyraclostrobin were efficient to reduce the CLR progress. The CLR severity curves obtained based on the analysis of the visual and F_v/F_m images demonstrated increase in CLR at 10 dai (Fig. 4-A). The same pattern was obtained for epoxiconazole and pyraclostrobin, however, CLR severity obtained from different images did not exceed 4 and 2% for epoxiconazole and pyraclostrobin, respectively (Figs. 4-B and C). The AURPC values (real, virtual and visual) were high for the control treatment when compared to the epoxiconazole and pyraclostrobin treatments regardless of the type of severity analyzed (Fig. 5).

3.3-Experiment 3

Pustules on leaves of non-sprayed plants were larger and had lower F_v/F_m values in their epicenter (center of the pustule) (Fig. 6). Pustules on leaves sprayed with epoxiconazole were smaller in size than the ones on leaves of plants from the control treatment, but bigger than pyraclostrobin. Similarly, the reduction on F_v/F_m values at the pustules epicenter on leaves of plants sprayed with epoxiconazole (0.564 ± 0.021) and pyraclostrobin (0.639 ± 0.012) were greater than those of the control treatment (0.170 ± 0.053).

4-Discussion

Many photosynthetic parameters can deduce the amount of disease in leaves infected by pathogens. However, a more common measurement is the F_v/F_m, which was used to image the foliar symptoms caused by *Colletotrichum lindemuthianum* in *Phaseolus vulgaris* (Meyer et al., 2001), *Puccina polysora* in *Zea mays* (Duraes et al., 2001), *Botrytis cinerea* in *Solanum lycopersicum* (Berger et al., 2004) and *Colletotrichum orbiculare* in *Nicotiana benthamiana* (Tung et al., 2013). However, this study is the first one to determine some Chl*a*F parameters for coffee leaves sprayed with the fungicides pyraclostrobin and epoxiconazole and infected with *H. vastatrix*. Many studies used F_v/F_m and NPQ parameters to infer the performance of photosynthetic processes such as carbon fixation. F_v/F_m and/or NPQ parameters were selected because of their biological relevance. Therefore, parameters such as F_v/F_m and NPQ would be expected to be more appropriate for detection and quantification of foliar diseases. For this reason, this study used F_v/F_m to infer the CLR severity on leaves of coffee plants and its variations when they were sprayed with the epoxiconazole and pyraclostrobin.

Epoxiconazole and pyraclostrobin are molecules widely used to control CLR (Zambolim et al., 1999, 2002). Epoxiconazole is a triazole fungicide that inhibits one specific enzyme, C14-demethylase, which plays a role in sterol production (Akers et al., 1990). Sterols, such as ergosterol, are needed for membrane structure and function, making them essential for the development of functional cell walls (Akers et al., 1990). Therefore, these fungicides result in abnormal fungal growth and eventually death (Akers et al., 1990). Pyraclostrobin is a strobilurin fungicide which has *β*-methoxyacrylate compounds that inhibit fungi respiration by binding to the Qo site of the cytochrome bc1 complex located in the inner mitochondrial membrane (Wiggins and Jager, 1993; Bartlett et al., 2002). There are many reports highlighting the

physiological benefits of spraying epoxiconazole and pyraclostrobin on plants grown under field conditions. Among these benefits are those related to increase in photosynthesis (Hong et al., 1995; Kasele et al., 1995; Panneerselvam et al., 1997; Gopi et al., 2005) and leaf chlorophyll concentration (Grossmann et al., 1999; Buchenauer and Rohner, 1981; Gao et al., 1988; Muthukumarasamy and Panneerselvam, 1997; Gopi et al., 1999; Kishorekumar et al., 2006). Probably, reduction on CLR severity by epoxiconazole and pyraclostrobin was due to the direct effect of these fungicides on fungal cell wall.

The CLR severity on leaves of plants based on visual symptoms was lower in contrast with the values used to F_v/F_m estimate the virtual severity. This information is very relevant to the field given that the level of disease detected visually may represent a higher level of severity because the virtual disease is hidden. Interestingly, this occurred for both control plants and those sprayed with epoxiconazole and pyraclostrobin. Evidently, both fungicides delayed CLR progress, but the proportion of virtual disease is a possible sign that the pathogen was alive inside the host tissues.

Several studies have shown that infections before any visible symptoms by using F_v/F_m can be detected. Bonfig et al. (2006) and Berger et al. (2007) reported that Chl*a*F images of *Arabidopsis thaliana* leaves in an incompatible interaction with *Pseudomonas syringae* pv. *tomato* showed changes in F_v/F_m from 18 to 21 h before visible symptoms, while in a compatible interaction with the same bacteria, changes were detected 24 h before visible symptoms. The detection of infection before visible symptoms was suggested to result from physiological effects of the pathogen on host cells such as eliciting defense responses or affecting carbon and nitrogen metabolism (Bonfig et al., 2006; Berger et al., 2007). Other examples are *Cercospora beticola* infecting *Beta vulgaris*, where F_s' (fluorescence level for a light adapted leaf) increased 2 h before

lesions appeared (Chaerle et al., 2004) and *Botrytis cinerea* infecting *Phaseolus vulgaris*, where F_s' increased 36 h before lesions were noticed (Chaerle et al., 2007). In all cases, areas where F_s' increased or F_v/F_m decreased were co-localized with subsequent necrosis.

Many studies showed that the incubation period of *H. vastatrix* varies during the year. In Brazil, the incubation period can range from 16 to 61 days depending on the time of year (Moraes et al., 1976; Kushalappa and Martins, 1980; Ghini et al., 2011). The results of this study allowed visualizing the first pustule at 26 dai. However, the same leaf exhibited altered levels of Chl*a*F from 10 dai, for all analyzed leaves. This suggests that photosynthetic damage starts earlier in the *H. vastatrix*-*C. arabica* interaction. In turn, the CLR incubation period was delayed on leaves sprayed with epoxiconazole and pyraclostrobin when compared with the non-sprayed leaves. This was expected and reflects the importance of these fungicides to slow CLR progress.

H. vastatrix had caused damage during tissue colonization to affect the PSII reaction centers before any visible damage. During the first 10 dai, it appears that the pathogen is not able to alter F_v/F_m, but eventually after 26 dai, the biotrophic phase creates sufficient stresses to reduce F_v/F_m even with non-visual symptoms. Biotrophic growth ends near 66 hai, when necrotrophic narrow secondary hyphae start growing directly through host cells and necrosis starts to appear at 72 hai (Shen et al., 2001).

Taken together, this study showed that *H. vastatrix* affected the photosynthetic capacity of *C. arabica* and the F_v/F_m parameter was able to show this effect before the appearance of visual symptoms. This approach could be a useful tool to accelerate the phenotyping of plant-pathogen interaction in the selection programs for resistant cultivars.

5-Acknowledgements

Profs. L. Zambolim and F.A. Rodrigues thank the “Conselho Nacional de Desenvolvimento Científico e Tecnológico” (CNPq) for their fellowship. This study was partially financed by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG). The authors thank Mr. Alessandro A. Fortunato for technical assistance.

6-References

Akers A, Koèhle HH, Gold RE. (1990) Uptake, transport and mode of action of BAS 480 F, a new triazole fungicide. Proceedings of the Brighton Crop Protection Conference. Farnham, UK: British Crop Protection Council, 837-845.

Baker NR. (2008) Chlorophyll fluorescence: a probe of photosynthesis *in vivo*. Annual Review of Plant Biology 59:89-113.

Baker NR, Oxborough K. (2004) Chlorophyll fluorescence as a probe of photosynthetic productivity, p. 65-82. *In* G. C. Papageorgiou and Govindjee [eds.], Chlorophyll *a* fluorescence: A signature of photosynthesis. Springer.

Baker NR, Oxborough K, Lawson T, Morison JI. (2001) High resolution imaging of photosynthetic activities of tissues, cell and chloroplast in leaves. Journal of Experimental Botany 52:615-21.

Balachandran S, Osmond CB, Daley PF. (1994) Diagnosis of earliest strain-specific interactions between tobacco mosaic virus and chloroplasts of tobacco leaves *in vivo* by means of chlorophyll fluorescence imaging. Plant Physiology 104:1059-1065.

Bartlett DW, Clough JM, Godwin JR, Hall AA, Hamer M, Parr-Dobrzanski B. (2002) The strobilurin fungicides. Pest Management Science 58:649-662.

Behr M, Humbeck K, Hause G, Deising HB, Wirsel SGR. (2010) The hemibiotroph *Colletotrichum graminicola* locally induces photosynthetically active green islands but globally accelerates senescence on aging maize leaves. Molecular Plant-Microbe Interactions 23:879-892.

Berger S, Benediktyova Z, Matous K, Bonfig K, Mueller MJ, Nedbal L, Roitsch T. (2007) Visualization of dynamics of plant-pathogen interaction by novel combination of chlorophyll fluorescence imaging and statistical analysis:

differential effects of virulent and avirulent strains of *Pseudomonas syringae* and of oxylipins on *Arabidopsis thaliana*. Journal of Experimental Botany 58:797-806.

Berger S, Papadopolous M, Schreiber U, Kaiser W, Roitsch T. (2004) Complex regulation of gene expression, photosynthesis and sugar levels by pathogen infection in tomato. Physiologia Plantarum 122:419-428.

Bock CH, Poole G, Parker PE, Gottwald TR. (2010) Plant disease severity estimated visually, by digital photography and image analysis, and by hyperspectral imaging. Critical Reviews in Plant Sciences 29:59-107.

Bonfig KB, Schreiber U, Gabler A, Roitsch T, Berger S. (2006) Infection with virulent and avirulent *Pseudomonas syringae* strains differentially affects photosynthesis and sink metabolism in Arabidopsis leaves. Planta 225:1-12.

Buchenauer H, Rohner E. (1981) Effects of triadimefon and triadimenol on growth of various plant species as well as on gibberellin content and sterol metabolism in shoot of barley seedlings. Pesticide Biochemistry and Physiology 15:58-70.

Capucho AS, Caixeta ET, Zambolim EM, Zambolim L. (2009) Herança da resistência do Híbrido de Timor UFV 443-03 à ferrugem-do-cafeeiro. Pesquisa Agropecuária Brasileira 44:276-282.

Capucho AS, Zambolim L, Duarte HSS, Vaz GRO. (2011) Development and validation of a standard area diagram set to estimate severity of leaf rust in *Coffea arabica* and *Coffea canephora*. Plant Pathology 60:1144-1150.

Chaerle L, Hagenbeek D, De Bruyne E, Valcke R, Van Der Straeten D. (2004) Thermal and chlorophyll-fluorescence imaging distinguish plant-pathogen interactions at an early stage. Plant and Cell Physiology 45:887-896.

Chaerle L, Hagenbeek D, Vanrobaeys X, Van Der Straeten D. (2007) Early detection of nutrient and biotic stress in *Phaseolus vulgaris*. International Journal of Remote Sensing 28:3479-3492.

Chou HM, Bundock N, Rolfe SA, Scholes JD. (2000) Infection of *Arabidopsis thaliana* leaves with *Albugo candida* (white blister rust) causes a reprogramming of host metabolism. Molecular Plant Pathology 1:99-113.

Conab (2013) Acompanhamento da Safra Brasileira de Café. Brasília, Brasil: Companhia Nacional de Abastecimento: Conab, survey, December 2013.

Crumley ML. (2013) The price of liberalization: smallholder coffee producers in the era of globalization. Mediterranean Quarterly 24:102-128.

de Ribou SB, Douam F, Hamant O, Frohlich MW, Negrutiu I. (2013) Plant science and agricultural productivity: Why are we hitting the yield ceiling? Plant Science 210:159-176.

Duraes FOM, Gama EEG, Magalhães PC, Marriel IE, Casela CR, Oliveira AC, Luchiari Junior A, Shanahan JF. (2001) The usefulness of chlorophyll fluorescence in screening for disease resistance, water stress tolerance, aluminium toxicity tolerance, and N use efficiency in maize. Seventh Eastern and Southern Africa Regional Maize Conference, pp. 356-360.

Fiorani F, Rascher U, Jahnke S, Schurr U. (2012) Imaging plant dynamics in heterogeneous environments. Current Opinion in Biotechnology 23:227-235.

Gao J, Hofstra G, Fletcher RA. (1988) Anatomical changes induced by triazoles in wheat seedlings. Canadian Journal of Botany 66:178-1185.

Ghini R, Hamada E, Pedro Júnior MJ, Gonçalves RRV. (2011) Incubation period of *Hemileia vastatrix* in coffee plants in Brazil simulated under climate change Summa Phytopathologica 37:85-93.

Gopi R, Sridharan R, Somasundaram R, Lakshmanan GMA, Panneerselvam R. (2005) Growth and photosynthetic characteristics as affected by triazoles in *Amorphophallus campanulatus* Blume. General and Applied Plant Physiology 31:171-180.

Gopi R, Sujatha BM, Rajan SN, Karikalan L, Panneerselvam R. (1999) Effect of triadimefon in the NaCl stressed cowpea (*Vigna ungiculata*) seedlings. Indian Journal of Agricultural Sciences 69:743-745.

Grossmann K, Kwiatkowski J, Caspar G. (1999) Regulation of phytohormone levels, leaf senescence, and transpiration by the strobilurin kresoxim-methyl in wheat (*Triticum aestivum*). Journal of Plant Physiology 154:805-808.

Hong Y, Zhimim Y, Jingyl Y. (1995) Biological response of *Hosta plantaginea* to mepiquat chloride and paclobutrazole. Journal of Nanjing Agricultural University 18:18-22.

Iqbal MJ, Goodwin PH, Leonardos ED, Grodzinski B. (2012) Spatial and temporal changes in chlorophyll fluorescence images of *Nicotiana benthamiana* leaves following inoculation with *Pseudomonas syringae* pv. *tabaci*. Plant Pathology 61:1052-1062.

Kasele IN, Shannahan JF, Nielsel DC. (1995) Impact of growth retardants on corn leaf morphology and gas exchange traits. Crop Science 35:190-194.

Kishorekumar A, Jaleel CA, Manivannan P, Sankar B, Sridharan R, Somasundaram R, Panneerselvam R. (2006) Differential effects of hexaconazole and paclobutrazol on the foliage characteristics of Chinese potato (*Solenostemon rotundifolius* Poir., J.K. Morton). Acta Biologica Szegediensis 50:127-129.

Kushalappa AC, Eskes AB. (1989) Coffee rust: epidemiology, resistance and management. Boca Raton: CRC Press. 345 pp.

Kushalappa AC, Martins CP. (1980) Incubation periods for *Hemileia vastatrix* on coffee in Viçosa, Minas Gerais. Fitopatologia Brasileira 6:177-183.

Madden LV, Hughes G, van den Bosch F. (2007) The Study of Plant Disease Epidemics. St. Paul, MN:APS Press.

Mahlein AM, Oerke EC, Steiner U, Dehne HW. (2012) Recent advances for sensing plant disease for precision crop protection. European Journal of Plant Pathology 133:197-209.

Maxwell K, Johnson N. (2000) Chlorophyll fluorescence – a practical guide. Journal of Experimental Botany 51:659-668.

Meyer S, Saccardy-Adji K, Rizza F, Genty B. (2001) Inhibition of photosynthesis by *Colletotrichum lindemuthanium* in bean leaves determined by chlorophyll fluorescence imaging. Plant, Cell and Environment 24:947-955.

Moraes AS, Sugimori MH, Ribeiro IJA, Ortolani AA, Pedro Jr M.J. (1976) Período de incubação de *Hemileia vastatrix* Berk et Br. em três regiões do estado de São Paulo. Summa Phytopathologica 2:32-38.

Muller P, Li XP, Niyogi KK. (2001) Nonphotochemical quenching. A response to excess light energy. Plant Physiology 125:1558-1566.

Muthukumarasamy M, Panneerselvam R. (1997) Triazole induced protein metabolism in the salt stressed *Raphanus sativus* seedlings. Journal of the Indian Botanical Society 76:39-42.

Novais RF, Neves JCL, Barros NF. (1991) Ensaio em ambiente controlado. In: Oliveira AL, Garrido WE, Araújo JD, Lourenço S (Eds.) Métodos de pesquisa em fertilidade do solo. EMBRAPA SEA. Brasília. pp. 153-189.

Nutter FW, Gleason ML, Jenco JH, Christinas NC. (1993) Assessing the accuracy, intra-rater repeatability, and inter-rater reliability of disease assessment systems. Phytopathology 83:806-812.

Panneerselvam R, Muthukumarasamy M, Karikalan L. (1997) Triadimefon enhances growth and net photosynthetic rate in NaCl stressed plants of *Raphanus sativus* L. Photosynthetica 34:605-609.

Perez-Bueno ML, Ciscato M, van de Ven M, Garcia-Luque I, Valcke R, Baron M. (2006) Imaging viral infection: studies on *Nicotiana benthamiana* plants infected with the pepper mild mottle tobamovirus. Photosynthesis Research 90:111-123.

Rodriguez-Moreno L, Pineda M, Soukupova J, Macho AP, Beuzon CR, Baron M, Ramos C. (2008) Early detection of bean infection by *Pseudomonas syringae* in asymptomatic leaf areas using chlorophyll fluorescence imaging. Photosynthesis Research 96:27-35.

Rolfe SA, Scholes JD. (2010) Chlorophyll fluorescence imaging of plant-pathogen interactions. Protoplasma 247:163-175.

Schreiber U, Schliwa U, Bilger W. (1986) Continuous recording of photochemical and nonphotochemical fluorescence quenching with a new type of modulation fluorometer. Photosynthesis Research 10:51-62.

Shaner G, Finney RE. (1977) The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat. Phytopathology 67:1051-1056.

Shen S, Goodwin PH, Hsiang T. (2001) Infection of Nicotiana species by the anthracnose fungus *Colletotrichum orbiculare*. European Journal of Plant Pathology 107:767-773.

Silva MC, Nicole M, Guimarães Junior L, Rodrigues CJ. (2002) Hypersensitive cell death and post-haustorial defense responses arrest the orange rust (*Hemileia*

vastatrix-race II) growth in resistant coffee leaves. Physiological and Molecular Plant Pathology 60:169-183.

Swarbrick PJ, Schultze-Lefert P, Scholes JD. (2006) Metabolic consequences of susceptibility and resistance (race-specific and broad-spectrum) in barley leaves challenged with powdery mildew. Plant, Cell and Environment 29:1061-1076.

Tung J, Goodwin PH, Hsiang T. (2013) Chlorophyll fluorescence for quantification of fungal foliar infection and assessment of the effectiveness of an induced systemic resistance activator. European Journal of Plant Pathology 136:301-315.

Vale FXR, Fernandes Filho EI, Liberato JR. (2003) QUANT: A software plant disease severity assessment. In: Close R, BraithwaiteM, Havery I, eds. Proceedings of the 8th International Congress of Plant Pathology, 2003. Sydney, Australia: Horticulture Australia, 105.

Vale FXR, Jesus Junior WC, Zambolim L. (2004) Epidemiologia Aplicada ao Manejo de Doenças de Plantas. Belo Horizonte, Brazil: Perffil Editora. 532 p.

Vega FE, Rosenquist E, Collins W. (2003) Global project needed to tackle coffee crisis. Nature 425:343.

Venâncio WS, Rodrigues MAT, Begliomini E, Souza NL. (2003) Physiological effects of strobilurin fungicides on plants. Publicatio UEPG, Ciências Exatas e da Terra, Ciências Agrárias e Engenharias 9:59-68.

Waller JM, Bigger M, Hillocks RJ. (2007) Coffee pests, diseases and their management. Oxfordshire: CAB International. 400 p.

Wiggins TE, Jager BJ. (1993) Mode of action of the new methoxyacrylate antifungal agent ICIA5504. Biochemical Society Transactions 22:68S.

Zambolim L, Acuña RS, Vale FXR, Chaves GM. (1992) Influência da produção do cafeeiro sobre o desenvolvimento da ferrugem (*Hemileia vastatrix*). Fitopatologia Brasileira 17:32-35.

Zambolim L, Vale FXR, Costa H, Pereira AA, Chaves GM. (2002) Epidemiologia e Controle Integrado da Ferrugem do Cafeeiro. In: Zambolim L (Ed.). O Estado da Arte de Tecnologias de Produção de Café. Viçosa. UFV. p. 369-450.

Zambolim L, Vale FXR, Pereira AA, Chaves GM. (1999) Manejo Integrado das Doenças do Cafeeiro. In: Zambolim, (Ed.). Produção de Café com Qualidade. Viçosa: UFV. p.134-215.

7-List of figures

Fig. 1. Real severity versus visual severity of coffee leaf rust on coffee leaves. Data are from 75 leaves containing different visual severity levels. * = significant by the Student's *t*-test ($P \leq 0.05$).

Fig. 2. Virtual severity (real severity minus visual severity) versus visual severity based on estimates of coffee leaf rust severity.

Fig. 3. Development of coffee leaf rust symptoms (visual) and the estimates of F_v/F_m (maximal photosystem II [PS II] quantum yield) on leaves of coffee plants non-sprayed (control) (A) and sprayed with epoxiconazole (B) and pyraclostrobin (C) and inoculated with *Hemileia vastatrix*. Bar = 1 cm.

Fig. 4. Coffee leaf rust progress curves for plants non-sprayed (A) and sprayed with epoxiconazole (B) and pyraclostrobin (C). The virtual (F_v/F_m - visual) curves were the difference between disease percentage from F_v/F_m images analysis and disease percentage from visual symptoms images. Visual curves were based on visual symptoms from photographs taken from 2 to 40 days after inoculation from 30 whole leaves for each treatment. The F_v/F_m (maximal photosystem II [PS II] quantum yield) curves were obtained from images of whole coffee leaves generated by Imaging PAM (Pulse-Amplitude-Modulation). Bars represent the standard error.

Fig. 5. Area under rust progress curve (AURPC) for coffee plants non-sprayed (control) (1) and sprayed with epoxiconazole (2) and pyraclostrobin (3). Means of real severity,

virtual severity and visual severity followed by the same letter among treatments are not significantly different by Tukey's test at $P \leq 0.05$. $n = 6$.

Fig. 6. Variations on the F_v/F_m parameter on pustules obtained from leaves of coffee plants non-sprayed (control) or sprayed with pyraclostrobin and epoxiconazole. One centimeter transect was performed at each pustule represented in the photographs for the pyraclostrobin (a), epoxiconazole (b) and control (c). $n = 50$. Bars = 1 cm.

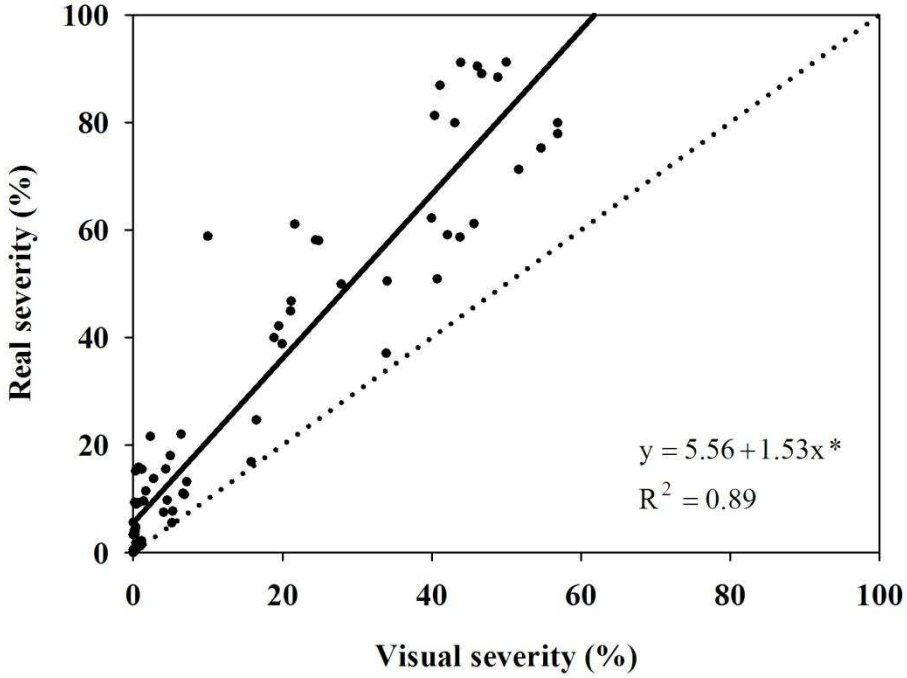

Figure 1

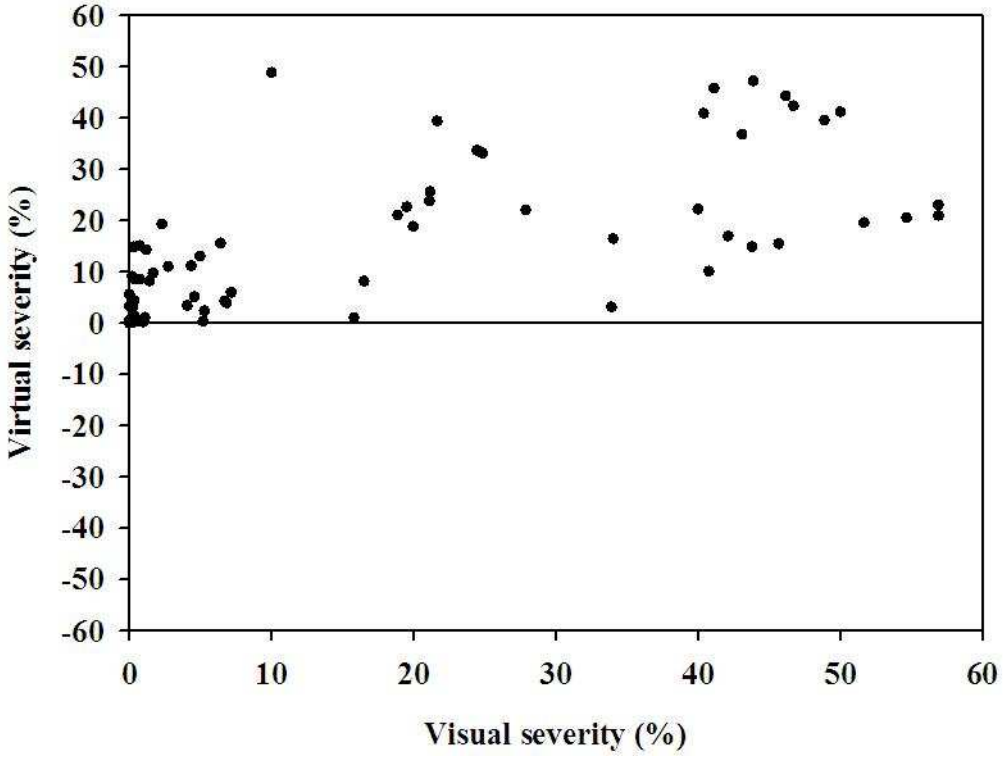

Figure 2

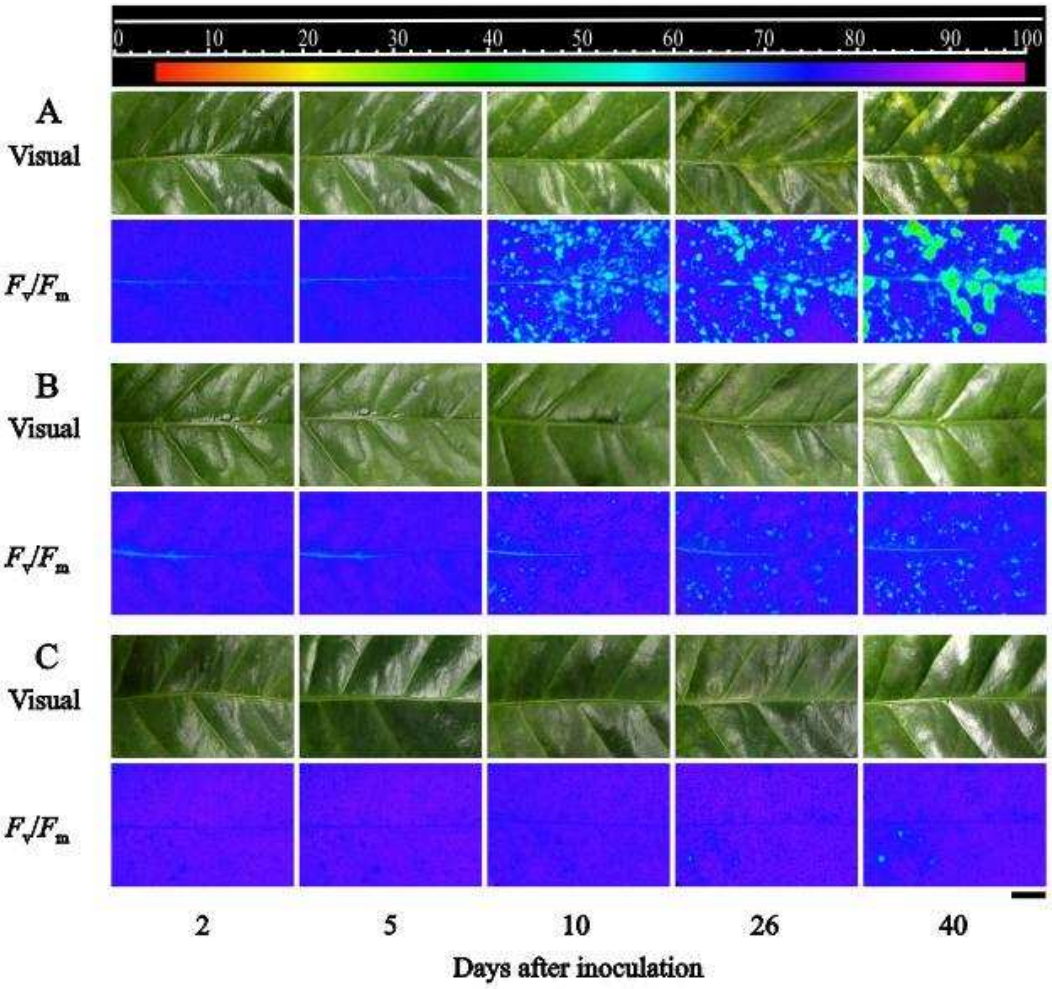
0
10
20
30
40
50
60
70
80
90
100
A
Visual
F_v/F_m
B
Visual
F_v/F_m
C
Visual
F_v/F_m
2
5
10
26
40
Days after inoculation

Figure 3

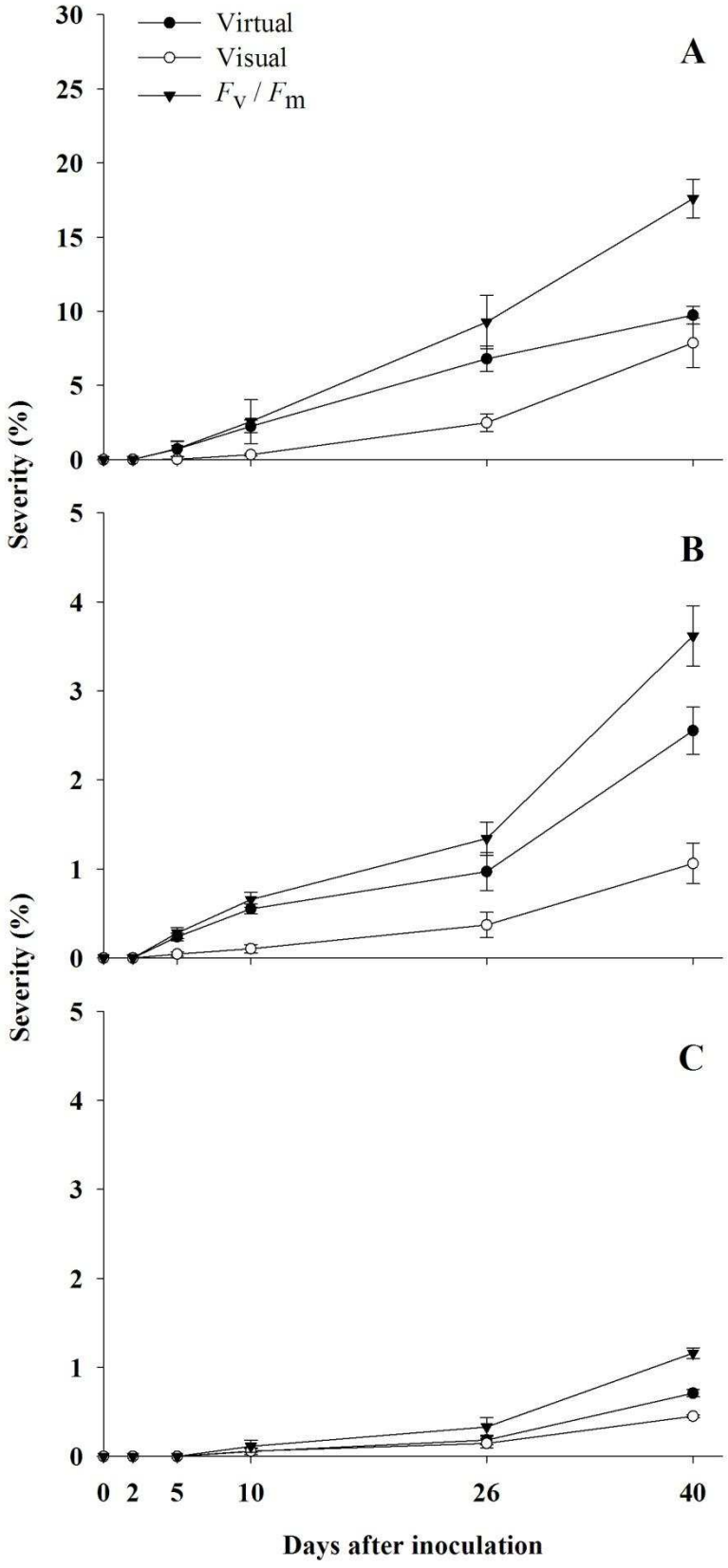
Virtual
Visual
F_v / F_m
A
B
C
Severity (%)
Severity (%)
0
5
10
15
20
25
30
0
1
2
3
4
5
0 2 5 10 26 40
Days after inoculation

Figure 4

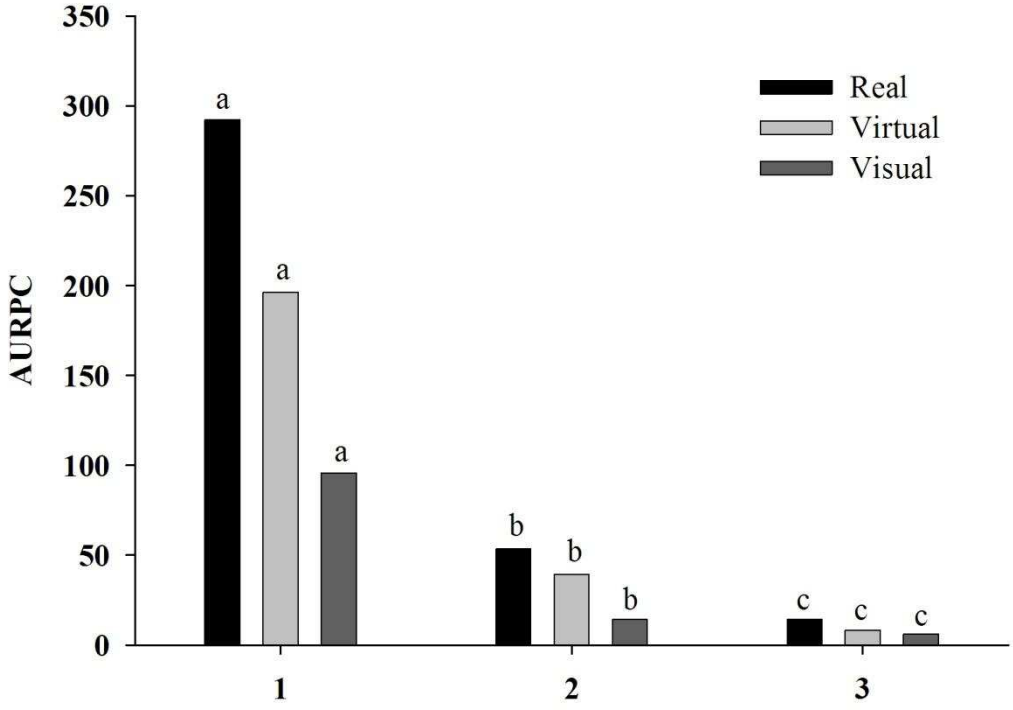
350
300
250
200
150
100
50
0
AURPC
a
a
a
b
b
b
c
c
c
1
2
3
Real
Virtual
Visual

Figure 5

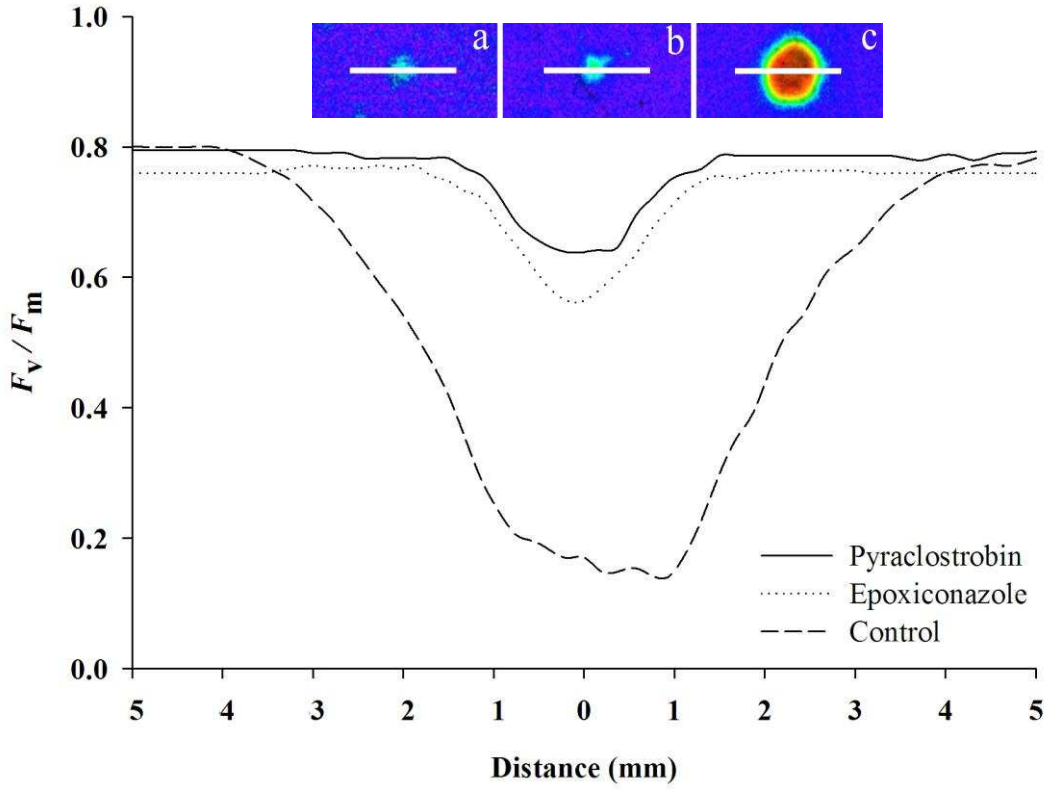
a
b
c
1.0
0.8
0.6
0.4
0.2
0.0
F_v / F_m
5
4
3
2
1
0
1
2
3
4
5
Distance (mm)
Pyraclostrobin
Epoxiconazole
Control

Figure 6

CHAPTER 2 - *Hemileia vastatrix* IMPACTS PHOTOSYNTHESIS ON COFFEE LEAVES AS DETERMINED BY CHLOROPHYLL *a* FLUORESCENCE IMAGING

Jaime Honorato Júnior[1], Laércio Zambolim[1], Carlos Eduardo Aucique-Pérez[1], Renata Sousa Resende[1], Fabrício Ávila Rodrigues[1]

Author's addresses: [1]Universidade Federal de Viçosa, Departamento de Fitopatologia, Viçosa, Minas Gerais State, Zip Code 36570-000, Brazil.

(correspondence to L. Zambolim. E-mail: zambolim@ufv.br) and F. A. Rodrigues. E-mail: fabricio@ufv.br)

Abstract

This study investigated the relationships between photosynthetic pigments, defense and anti-oxidative stress enzymes and some chlorophyll *a* fluorescence parameters on leaves of coffee plants sprayed with epoxiconazole and pyraclostrobin and inoculated or non-inoculated with *Hemileia vastatrix*. Coffee plants were grown in greenhouse and urediniospores were inoculated. Fungicides were sprayed and coffee leaf rust severity, catalase, peroxidases, β-1,3-glucanases and chitinases activities, chlorophyll a, b and carotenoids concentration and chlorophyll *a* fluorescence parameters (F_0, F_v/F_m, Y(II) and ETR) were determined. Coffee leaf rust severity decreased until 40 days after inoculation on plants sprayed with epoxiconazole and pyraclostrobin. Catalase, peroxidases, *β*-1,3-glucanases and chitinases activities were different among treatments. There was a significant reduction in pigments concentration between non-inoculated and inoculated plants but not, in plants sprayed with epoxiconazole and pyraclostrobin. All chlorophyll *a* fluorescence parameters were different between non-inoculated and inoculated plants and non-sprayed and fungicides sprayed plants. Changes on chlorophyll *a* fluorescence parameters were detected in non-inoculated and inoculated plants sprayed with epoxiconazole and pyraclostrobin. Also, the effect of fungicides on photosynthesis should be studied to provide more opportunities to understanding the impacts on plant defense mechanisms that lead to reduced coffee leaf rust symptoms.

Keywords: *Coffea arabica* L., coffee leaf rust, plant pigments, strobilurin, triazole.

1-Introduction

Coffee is the most important commodity in the international agricultural trade, generating over 90 billion dollars each year and involving about 500 million people from cultivation to consumption (Batista et al., 2012). Coffee Leaf Rust (CLR), caused by the biotrophic fungus *Hemileia vastatrix* Berkeley & Broome, is considered the major disease of *Coffee arabica* cultivars and is responsible for 15 to 50% decreases in coffee yield when control measures are not used (Zambolim et al., 1992, 1999; van der Vossen, 2001; Waller et al., 2007). In conditions of severe CLR epidemics, plant defoliation and, eventually, plant death can occur (Kushalappa and Eskes, 1989; Silva et al., 2006).

The reduction in pigments concentration, structural damage to the chloroplasts, impairments in energy dissipation via chlorophyll *a* fluorescence and increases in leaf temperature are the most notable negative effects that result from pathogens infection (Petit et al., 2006; Zhao et al., 2011; Resende et al., 2012). Indeed, there are decrease in stomatal conductance, carbon metabolism and transport processes that affected the photosystem II (PSII) efficiency even though the specific mechanisms by which a restriction in metabolic turnover can result in decreases in PSII operating efficiency are not fully understood (Baker, 2008). For some coffee trees cultivars, the expression of the resistance to pathogens has also been associated with the involvement of enzymes related to oxidative stress such as lipoxygenases and peroxidases, in the phenylpropanoid pathway such as phenylalanine ammonia-lyases (PAL) and some PR proteins such as β-1,3-glucanases and chitinases (Maxemiuc-Naccache et al., 1992; Rojas et al., 1993; Silva et al., 2002, 2006, 2008; Guerra-Guimarães et al., 2009). Plant infection by pathogens causing rust has likewise been shown to affect photosynthesis and carbohydrate metabolism (Voegele et al., 2009; Major et al., 2010).

Chlorophyll *a* fluorescence is based on light energy absorbed by chlorophylls in PSII that can either be re-emitted as fluorescence, used for photosynthesis (photochemical quenching) or lost as heat (non-photochemical quenching) (Duyens and Sweers, 1963; Baker, 2008). Increased energy used in one of these processes leads to less energy available to be used in the others (Maxwell and Johnson, 2000). The pulse amplitude modulated (PAM) technique detects the chlorophyll *a* fluorescence in a defined spectral band (usually 680–690 nm) (Maxwell and Johnson, 2000; Baker, 2008). Based on the ground and the maximum fluorescence of dark-adapted leaves as well as the fluorescence intensity during kinetic measurements, specific parameters allow conclusions about the current photosynthetic activity and the physiological state of the host tissues (Lichtenthaler et al., 2005; Baker, 2008).

Among the most widely used chlorophyll *a* fluorescence parameters is the ratio of variable and maximum fluorescence of dark-adapted tissue ($F_v/F_m = F_m\text{-}F_0/F_m$). A lower F_v/F_m value indicates that a proportion of PSII reaction centers are damaged, which is known as photoinhibition, and is often observed in plants under stress (Butler, 1978; Baker and Oxborough, 2004). In addition, effective PSII quantum yield (Y(II)) explains the changes in PSII operating efficiency, whether attributable to changes in non-photochemical quenching or to the ability of an excited PSII reaction center, to drive electron transport (Baker, 2008). Direct measurements of chlorophyll *a* fluorescence include F_0 (minimum fluorescence for a dark adapted leaf measured with a weak light in the absence of actinic light or a saturating pulse), F_s and F_s' (fluorescence levels for a dark and light adapted leaf, respectively, measured in actinic light with no saturating pulse) and F_m and F_m' (maximum fluorescence levels for a dark or light adapted leaf, respectively, after saturating pulses of intense light overloads the electron

transport chain between PSII and photosystem I (PSI)) (Maxwell and Johnson, 2000; Baker, 2008).

Other chlorophyll fluorescence parameters can be calculated from the above mentioned parameters (Baker, 2008) such as F_v (ability of PSII to perform photochemistry) (Bjorkman and Demmig, 1987) and NPQ (energy lost as heat and photoinhibitory processes in PSII and PSI) (Müller et al., 2001; Kramer et al., 2004) indicates the ability of the plant to dissipate the extra light in the form of harmless heat. The electron transfer rate (ETR) measures the amount of absorbed light energy used for photosynthesis, which can be directly compared to the photosynthetic CO_2 assimilation rate (Schreiber, 1994; Scholes and Rolfe, 1996; Maxwell and Johnson, 2000). Lower Y(II) of tissues treated with diuron, a PSII inhibitor, was related to its increased penetration (Habash et al., 1985). However, there are no reports about studies involving these chlorophyll parameters and, for example, the fungicides absorption regarding plant disease control.

Strobilurin (azoxystrobin) impact on photosynthesis did not affect the chlorophyll content in winter wheat, but its application delayed the increase of reactive oxygen species (ROS), thus delaying the leaf senescence and prolonging the duration of flag leaf photosynthesis (Zhang et al., 2010). On the other hand, the use of triazoles enhanced chlorophyll and carotenoid concentrations in rice seedlings (Guirong et al., 1995) and okra (Sujatha et al., 1999). Triadimefon increased chlorophyll concentration in tomato (Buchenauer and Rohner, 1981), radish (Muthukumarasamy and Panneerselvam, 1997), cowpea (Gopi et al., 1999) and wheat leaves (Gao et al., 1988). Triazole compounds increased the level of cytokinin, which might stimulate chlorophyll biosynthesis (Jaleel et al., 2008). Triazoles accelerated chloroplast differentiation and protected their integrity, increased chlorophyll production and enlarged chloroplasts

(Fletcher et al., 2000; Jaleel et al., 2008). By contrast, foliar application of epoxiconazole retarded cleavers growth (Benton and Cobb, 1997). In addition, seven days after treatment, epoxiconazole reduced oxygen evolution as determined by electron flow from water to ferricyanide and the associated electron transport capability of isolated thylakoids (Benton and Cobb, 1997). Considering fluorescence, Y(II) and F_v/F_m were reduced by some fungicides due to a decrease in photochemical quenching (q_p) (Krugh and Miles, 1996).

The aim of this study was to investigate the relationships between photosynthetic pigments, defense and anti-oxidative stress enzymes and some chlorophyll *a* fluorescence parameters on leaves of coffee plants sprayed with epoxiconazole and pyraclostrobin and inoculated or non-inoculated with *H. vastatrix*.

2-Materials and Methods

2.1-Coffee plants growth

Coffee seeds (cv. "Catuaí Vermelho IAC 144") were sowed in a moist sand bed for 60 days. After this period, coffee plants were transplanted to plastic pots containing 1kg of a mix of soil, manure and sand (2.5:1:0.5 proportions). Soil pH was corrected by adding 1.5 g of dolomitic limestone to each pot 30 days before seedlings transplant. Coffee plants were fertilized at three days after transplant with 25 mL of nutritive solution (Novais et al., 1991) and then at each 7 days until the end of the experiment.

Six sets of three months-old plants (each set with three plants) were transferred to a growth chamber (relative humidity 90 ± 5% at 22 ± 2ºC and 12 h light (32.13 µmoles/m^2/s)) before being inoculated with *H. vastatrix*.

2.2-Inoculum production, plant inoculation and fungicides spraying

Before inoculation, the viability of *H. vastatrix* urediniospores was tested by placing them to germinate in 2% water-agar medium (Zambolim and Chaves, 1974). Only urediniospores greater than 30% viability was used in the experiments (Capucho et al., 2009). The abaxial surface of first pair of expanded leaves of each coffee plant was inoculated with urediniospores (1 mg/leaf) with the aid of a camel hair brush (Silva et al., 2002). After inoculation, leaves of each plant were sprayed with distilled water and plants were kept in a dark moist chamber for 48 h at 22 ± 2ºC. Thereafter, plants were transferred to a growth chamber (relative humidity of 90%, temperature of 22°C and 12 h photoperiod with fluorescent light (32.13 µmoles/m^2/s)).

Four days after inoculation (dai), a set of inoculated and non-inoculated plants were sprayed with the fungicides pyraclostrobin (199.5 g a.i. ha^{-1}) and epoxiconazole (75 g a.i. ha^{-1}). These fungicides were applied to the plants canopies with a manual backpack sprayer (Jacto PJH, JD-12P spray nozzle) gauged for applying a spray volume

of 400 L/ha. Inoculated and non-inoculated plants sprayed with distilled water served as the control treatments.

2.3-Assessment of CLR severity

Actual CLR severity was determined after photographic analysis of leaves from plants from each treatment at 2, 5, 10, 26 and 40 dai. For this analysis, the QUANT software (Vale et al., 2003) was used to determine the proportion of diseased area with a discriminate function. Data from CLR severity was used to calculate the area under rust progress curve (AURPC) according to Shaner and Finney (1977).

2.4-Determination of enzymes activities

The first pair of expanded leaves from the apex to the base, of non-inoculated and inoculated plants, was collected at 2, 5, 10, 26 and 40 dai. Samples consisted of two-centimeter leaf disks that were kept in liquid nitrogen during sampling and stored at -80ºC until further analyses.

To determine the activities of chitinases (CHI, EC 3.2.1.14), β-1,3-glucanases (GLU, EC 3.2.1.39), peroxidases (POX, EC 1.11.1.7) and catalase (CAT, EC 1.11.1.6), a total of 300 mg of leaf tissue (mix of 10 leaves per replication of each treatment) was ground into a fine powder in a mortar and pestle with liquid nitrogen. The fine powder was homogenized in an ice bath in 2 mL of a solution containing 100 mM potassium phosphate buffer (pH 6.8), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% polyvinylpyrrolidone (PVP) (w/v) and 4% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 20000 *g* for 25 min at 4°C and the supernatant was used as a crude enzyme extract. For each enzyme, each reaction was performed three times.

The CHI activity was determined by the method of Roberts and Selitrennikoff (1988) as modified by Harmanet et al. (1993). The reaction was initiated by the addition

of 20 µL aliquots of the crude enzyme extract to a mixture of 470 µL of buffer 50 mM sodium acetate (pH 5.0) and 10 µL of the substrate *p*-nitrophenyl-*β*-D-N-N-diacetylchitobiose (Sigma-Aldrich, São Paulo, Brazil) at a concentration of 2 mg/mL. The reaction mixture was incubated in a water bath at 37ºC for 2 h. The reaction was interrupted by adding 500 µL of 0.2 M sodium carbonate. In the control samples, only the sodium carbonate was used after adding the crude enzyme extract to the reaction mixture and the samples were incubated in a water bath at 37ºC for 2 h. The absorbance of the final product released by CHI was determined at 410 nm. The molar extinction coefficient of 7×10^4 mM cm^{-1} was used to calculate CHI activity, which was expressed in µMol of *p*-nitrophenyl produced by $min^{-1}mg^{-1}$of protein.

The GLU activity was determined as described by Lever (1972). The reaction was initiated by the addition of 20 µL aliquots of the crude enzyme extract to a mixture of 230 µL of buffer 100 mM sodium acetate (pH 5.0) and 250 µL of the substrate laminarin (Sigma-Aldrich, São Paulo, Brazil) in a concentration of 4 mg/mL. The reaction mixture was incubated in a water bath for 30 min at 45ºC. After the incubation period, the amount of reducing sugars was determined by adding 500 µL of dinitrosalicylic acid to the mixture and then incubating the resulting mixture in a water bath for 15 min at 100ºC. The reaction was interrupted by cooling the samples in an ice bath to 30ºC. In the control samples, the reaction mixture was the same, except that the extract was added after heating the mixture at 100ºC. The absorbance of the product released by GLU was measured at 540 nm and the activity of GLU was expressed in absorbance units min^{-1} mg^{-1} of protein.

The POX activity was determined by the oxidation of pyrogallol according to the method of Kar and Miashra (1976). A mixture of 295 µL of distilled water, 250 µL of 100 mM potassium phosphate buffer (pH 6.8), 200 µL of 100 mM pyrogallol and 200

µL of 100 mM hydrogen peroxide was added to 55 µL of the crude enzyme extract. The absorbance was measured in spectrophotometer (Evolution 60, Thermo Scientific, Waltham, MA, USA) at 420 nm each 10 s for 1 min at 25ºC after the addition of crude enzyme extract to the mixture in a total of seven readings. The molar extinction coefficient of 2.47 mM^{-1} cm^{-1} was used to calculate POX activity (Chance and Maehley, 1955), which was expressed in mM purpurogallin produced min^{-1} mg^{-1} of protein. Throughout the process, the microcentrifuge tubes were covered with aluminium foil to protect the mixture from light oxidation.

The CAT activity was estimated by the rate of hydrogen peroxidation (H_2O_2) decomposition at 240 nm (Havir and McHale, 1989). The reaction was initiated after the addition of 40 µl of the crude enzyme extract to 960 µl of a mixture containing 100 mM potassium phosphate buffer (pH 6.8) and 12.5 mM H_2O_2. An extinction coefficient of 36 $M^{-1}cm^{-1}$ (Anderson et al., 1995) was used to calculate the CAT activity, which was expressed as mM min^{-1} mg^{-1} of protein.

The concentration of total soluble protein of the extracts was measured according to the Bradford's method (Bradford, 1976).

2.5-Determination of photosynthetic pigments concentration

The first pair of expanded leaves from the apex to the base, of non-inoculated and inoculated plants was collected at 2, 5, 10, 26 and 40 dai. Samples consisted of two-centimeter leaf disks that were kept in liquid nitrogen during sampling and stored at -80ºC until further analyses. The DMSO extraction technique of Hiscox and Israelstam (1979) was used for pigments extraction. Leaf disks were incubated at 60ºC until become completely colorless. Results were expressed in grams of pigments per square meter of leaf disk. Absorbance of the DMSO-chlorophyll extractions and blank (only DMSO) were measured at 480, 649.1 and 665.1 nm using a spectrophotometer and the

concentration of photosynthetic pigments (chlorophyll *a* [Chl*a*], chlorophyll *b* [Chl*b*] and carotenoids) was estimated according to Lichtenthaler (1987). The concentrations of Chl*a*, Chl*b* and carotenoids were expressed as grams per square meter of leaf disk. The Chl *a*/*b* ratio was calculated.

2.6-Determination of chlorophyll *a* fluorescence (Chl*a*F) parameters

The fluorescence imaging parameters were determined by the use of Imaging-PAM M-Series chlorophyll fluorometer and the software version 2.32 ImagingWIN (both Heinz Walz GmbH, Effeltrich, Germany) between 2:00h and 6:00h am. The sensor system consists of 44 LED's-lamp high power (450 nm) required to apply fluorescence excitation, actinic illumination and saturation pulses. These LEDs are arranged in pairs, with each pair featuring a red (660 nm) and a near-infrared (780 nm) LED. A CCD camera with 640 × 480 resolution pixels was located above the plants canopies at a distance of 18.5 cm, giving an image area of 10 × 13 cm. Plants were adapted to darkness for 45 min (Baker, 2008), then the sprayed leaves were exposed to a light pulse intensity of 0.5 µmol m^{-2} s^{-1} (1Hz), establishing the minimum fluorescence image (F_0). Next, a saturating pulse of blue light (470 nm) of 2400 µmol m^{-2} s^{-1} intensity (10 Hz) was delivered at 0.8 s in order to obtain the maximum fluorescence image (F_m). The software performed the calculation and image of the fluorescent quantum efficiency ($F_v/F_m = (F_m - F_0)/F_m$) (Baker, 2008). A pulse of actinic light beginning with 250 µmol m^{-2} s^{-1} for 2 min and ending with a pulse of saturating light determined the fluorescence yield (F), the maximum fluorescence yield (F_m') and the effective PSII quantum yield [($YII = (F_m' – F)/ F_m'$)]. In order to measure changes in photosynthetic electron transport in the leaves, the relative ETR was determined (ETR = Y(II) × 0.5 × PAR × Abs). This parameter includes the value for the known flux of photosynthetically active radiation (PAR) and absorptivity [Abs = 1 – (R/NIR)], where R is the signal intensity for

reflected red light (660 nm) and NIR the intensity for reflected near-infrared light (780 nm), describing the absorption of PAR by YII (0.84) (Schreiber, 1994). Plants were removed from the growth chamber for Chl*a*F imaging at 5, 10, 26 and 40 dai.

For quantitative analyses of whole leaves, the software used six areas of interest (AOI) of 2 cm^2 each that were randomly distributed over each analyzed leaf. For each variant, three individual plants were used and the respective results were averaged. For quantitative analyses of individual symptoms, 150 AOI of 10 mm^2 each were used to determine the photosynthetic parameters in chlorotic areas and the corresponding surrounding areas. For each variant, three plants were used and the results were averaged.

2.7-Experimental design and data analysis

For all experiments, a 2 × 2 × 5 factorial experiment, consisting of two fungicides (pyraclostrobin and epoxiconazole), non-inoculated and inoculated plants and five evaluations time was arranged in a completely randomized design with five replications. The experiment was repeated once. Each experimental unit corresponded to a plastic pot containing one plant. For chlorophyll and enzymes determinations, a total of 150 plants were used in each experiment (30 plants per each treatment and evaluation time). For the determination of Chl*a*F parameters, a total of 18 plants were used in each experiment (3 plants per each treatment and evaluation time). The measurements were made on the same leaf (one per pot) at five evaluation times (2, 5, 10, 26 and 40 dai). Data for all variables were subjected to an analysis of variance (ANOVA) and the means from the treatments were compared by either *t*-test or Tukey's test ($P \leq 0.05$) using the SAS software (SAS Institute Inc., Cary, NC).

3-Results

The factors fungicides, plant inoculation and evaluation times as well as their interactions, were significant for CLR severity (Table 1). At least one of the factors fungicides, plant inoculation or evaluation times as well as some of their interactions were significant for Sev, F_0, F_v/F_m, Y(II), ETR, Chl*a*, Chl*b*, Chl*a*/*b* ratio, Car, CAT, POX, GLU and CHI. The interaction fungicide × plant inoculation × evaluation time was not significant only for F_0 (Table 1).

3.1-CLR severity and AURPC

The CLR severity decreased until 40 dai on plants sprayed with epoxiconazole and pyraclostrobin compared with the control treatment (Fig. 1A). The AURPC was significantly lower for epoxiconazole and pyraclostrobin treatments (Fig. 1B). The CLR severity and the AURPC showed a similar trend across treatments based on a preliminary experiment (data not shown).

3.2-Enzymes activities

Fungicides, plant inoculation and evaluation time were significant for the CAT and POX activities (Table 1). Plant inoculation and evaluation time were significant for GLU activity. None of the factors were significant for CHI. Most of the double interactions were significant for the enzymes studied, except the fungicide × plant inoculation interection for CAT and CHI activities. The fungicide × plant inoculation × evaluation time interaction was significant for all enzymes studied.

CAT activity increased for inoculated plants of all treatments compared to the non-inoculated plants (Fig. 2A). CAT activity increased for non-inoculated plants sprayed with epoxiconazole and pyraclostrobin at 5 and 10 dai (Fig. 2B). POX activity was higher for inoculated plants compared with the non-inoculated plants (Figs. 2C and

D). CHI and GLU activities were similar between non-inoculated plants and inoculated sprayed or non-sprayed with epoxiconazole and pyraclostrobin (Figs. 2E-H).

3.3-Leaf pigments concentration

Fungicides, plant inoculation and evaluation time were significant for the concentration of Chl*a* and the Chl*a*/*b* ratio (Table 1). Plant inoculation and evaluation time were significant for the concentration of Chl*b*. Fungicides and evaluation time were significant for the concentration of carotenoids. Most of the double interactions were significant for the pigments concentration, except the fungicide × plant inoculation interaction for concentration of Chl*b*. The fungicide × plant inoculation × evaluation time interaction was significant for Chl*a*, Chl*b* and carotenoids.

There was a significant reduction in pigments concentration between non-inoculated and inoculated treatments (Fig. 3). The concentration of Chl*a* (Fig. 3B), carotenoids (Fig. 3H) and the Chl*a*/*b* ratio (Fig. 3F) increased at 40 dai for inoculated plants sprayed with epoxiconazole and pyraclostrobin.

3.4-Chl*a*F parameters

The parameter F_0 for inoculated plants at 40 dai, were 44.1, 42.65 and 21.32% higher in comparison to the non-inoculated plants for control, epoxiconazole and pyraclostrobin treatments, respectively (Fig. 4A and B). F_v/F_m was significant only between non-inoculated and inoculated control plants. There was no significant difference between non-inoculated and inoculated plants sprayed with epoxiconazole and pyraclostrobin for F_v/F_m (Fig. 4C and D). Y(II) and ETR for non-sprayed inoculated plants decreased from 10 dai (Fig. 4F and H), but increased from 10 dai for the piraclostrobin treatment in comparison to non-inoculated plants sprayed with pyraclostrobin (Fig. 4E-H).

4-Discussion

Previous findings showed that fungicides can improve the resistance of several plants against abiotic and biotic stress (Bartlett et al., 2002; Herms et al., 2002; Schreiber and Desveaux, 2008). However, the effects of fungicides on coffee plants infected by *H. vastatrix*, specifically at the level of photosynthesis, have never been investigated. There are some reports of the effect of triazole and strobilurin on the photosynthetic capacity of healthy coffee plants. Strobilurin fungicides can reduce the rate of stomatal conductance to water vapor (g_s) in leaves of wheat, barley and soybean plants (Nason et al., 2007). Coincidently, plants sprayed with strobilurin fungicides have a lower rate of transpiration (E), a lower CO_2 intercellular (C_i) and a lower CO_2 net assimilation (A) compared with control plants or plants sprayed with epoxiconazole (Nason et al., 2007). Decreases in A might be due to the low activities of photosynthetic enzymes such as the Rubisco (Guo et al., 2005), carbonic anhydrase, which converts CO_2 to HCO_3 or enzymes involved in the degradation of photoassimilates (Baker et al., 1997). There are few specific reports on the influence of these molecules on parameters of Chl*a*F. Paclobutrazol and hexaconazole (triazoles, like epoxiconazole) and Kresoxim-methyl (a strobilurin, like pyraclostrobin) increased chlorophyll levels on healthy Chinese potato and wheat, respectively (Grossmann et al., 1999; Kishorekumar et al., 2006; Petit et al., 2012). It is assumed that increases in chlorophyll levels are directly correlated with increases in the levels of Chl*a*F, however, there was no correlation of F_v/F_m and F_0 parameters with chlorophyll *a* and *b* for healthy plants sprayed with both pyraclostrobin and epoxiconazole.

In the present study, plants sprayed with pyraclostrobin and inoculated with *H. vastatrix* showed great values for Y(II) and ETR as compared to plants sprayed with epoxiconazole. In turn, non-inoculated plants sprayed with pyraclostrobin had Y(II) and

ETR values very similar to non-sprayed plants or plants sprayed with epoxiconazole. It is known that pyraclostrobin increases concentration of endogenous levels of abcisic acid (ABA) up to a maximum of 269% on the control (Grossmann et al., 1999). ABA inhibits growth and stomatic opening, especially when plants were under environmental stress, thus improving the utilization of water under conditions of water stress and the adaptation to low temperatures (Grossmann et al., 1999). ABA was originally isolated as a causing factor of abscission and also promotes leaf senescence only at high concentration (Grossmann, 2000). Certainly, infection with *H. vastatrix* was the cause of major stress in inoculated coffee plants. Higher values of Y (II) and ETR on leaves of plants sprayed with pyraclostrobin and infected by *H. vastatrix* may be an indirect evidence of great levels of ABA.

As plant pathogen can affects also the photosynthetic apparatus of plants (pigments, electron transport chain, enzymes of the Calvin cycle catalyzing CO_2 fixation), Chl*a*F is greatly modified in many plant species (Kuckenberg et al., 2009; Bürling et al., 2011; Mahlein et al., 2012). F_v/F_m parameter was severely reduced for inoculated plants from 26 to 40 dai. The F_v/F_m parameter was kept stable from 2 to 40 dai, for the non-inoculated leaves. These observations agree with several reports where F_v/F_m parameter is almost constant for many different plant species measured under the non-stressed conditions and equals to 0.832 ± 0.004 (Bjorkman and Demmig, 1987). For stressed plants, F_v/F_m parameter is markedly reduced. For this reason, the F_v/F_m parameter is frequently used as an indicator of the photoinhibition or other kind of injury caused to the PSII complexes (Rohacek, 2002). F_v/F_m values can be lowered due to contribution of the chlorophyll fluorescence emission from PSI to the F_0 level (Lavergne and Trissl, 1995). This effect might be significant especially in the case of

stressed plants and has to be taken into account when analyzing chlorophyll fluorescence data (Pfündel, 1998).

There are few examples involving defense response to biotrophic fungi and photosynthesis. When source leaves of tobacco were infected with *Phytophthora nicotianae*, full activation of defense responses and the HR was preceded by interruption of photosynthetic electron transfer and down-regulation of photosynthetic activity during the first hours after the inoculation (Scharte et al., 2005). By contrast, the infection of bean (*Phaseolus vulgaris*) leaves with *Colletotrichum lindemuthianum* led to necrosis and successive down-regulation of photosynthesis at later stages of fungal infection (Meyer et al., 2001). Collapse of photosynthetic activity inevitably leads to a metabolic transition from source to sink in infected tissues (Essmann et al., 2008). The resulting demand for carbohydrates and energy becomes compensated through increased activities of cell wall invertases, hexose transporters, the oxidative pentose phosphate pathway and respiratory metabolism (Essmann et al., 2008; Scharte et al., 2005). Such reprogramming of primary carbon metabolism may further enhance the expression of defense-related genes and favor the production of secondary compounds with antimicrobial activity (Bolton, 2009). In the present study, plants inoculated with *H. vastatrix* showed a reduction in Chl*a*F parameters levels related with reduced levels of chlorophyll *a*, the more abundant chlorophyll in the leaf tissue (Suzuki et al., 1997), in non-sprayed inoculated plants. To corroborate with this information, these same plants showed lower F_v/F_m, Y (II) and ETR values, which indicates a strong impairment in the electron transport during photosynthetic process (Baker, 2008). In the same direction, there was a decrease in the activity of catalase, peroxidases and β-1,3-glucanases during the infection process of *H. vastatrix* at 26 and 40 dai. Plants sprayed

with pyraclostrobin and epoxiconazole showed similar levels of activity of these enzymes in comparison to non-sprayed plants.

Also, the green islands induced by *H. vastatrix* on coffee leaves resemble green bionissia as defined by Walters et al. (2008), because there was formation of necrosis only nearly 40 dai. This is evident in the analysis of photosynthetic parameters because neither F_0 or F_v/F_m reached 0, the point indicating the occurrence of necrotic cells. Likewise, Y(II) and ETR values were not close to 0, the indicative point of necrotic tissues.

Changes on Chl*a*F parameters were detected in non-inoculated and inoculated plants sprayed with epoxiconazole and pyraclostrobin. Also, the effect of fungicides on photosynthesis should be studied to provide more opportunities to understanding the impacts on plant defense mechanisms that lead to reduced CLR symptoms. Thus, the determination of changes and relationships among the chlorophyll *a* parameters, disease severity, plant enzymes activities and pigments in response to *H. vastatrix* infection will be essential to assess the importance of resource and energy allocation for proper activation of host mechanisms of resistance. In conclusion, the results of the present study clearly demonstrated that sprayed plants with epoxiconazole and pyraclostrobin had lower CLR severity in association with changes in Chl*a*F parameters, pigments and enzymes activities performance.

5-Acknowledgements

Profs. L. Zambolim and FA. Rodrigues thank the “Conselho Nacional de Desenvolvimento Científico e Tecnológico” (CNPq) for their fellowship. The authors thank Mr. Alessandro A. Fortunato for technical assistance. This study was supported by CAPES, CNPq and FAPEMIG.

6-References

Anderson D, Prasad K, Stewart R. (1995) Changes in isozyme profiles of 11 catalase, peroxidase and glutathione reductase during acclimation to chilling in 12 mesocotyls of maize seedlings. Plant Physiology 109:1247-1257.

Baker NR. (2008) Chlorophyll fluorescence: a probe of photosynthesis *in vivo*. Annual Review of Plant Biology 59:89-113.

Baker NR, Oxborough K. (2004) Chlorophyll fluorescence as a probe of photosynthetic productivity, p. 65-82. *In* G. C. Papageorgiou and Govindjee [eds.], Chlorophyll *a* fluorescence: A signature of photosynthesis. Springer.

Baker NR, Nogués S, Allen DJ. (1997) Photosynthesis and photoinhibition. In: Lumsden PJ (ed) Plants and UV-B: Responses to environmental change. Cambridge University Press, Cambridge, pp 95-111.

Bartlett DW, Clough JM, Godwin JR, Hall AA, Hamer M, Parr-Dobrzanski B. (2002) The strobilurin fungicides. Pest Management Science 58:649-662.

Batista KD, Araújo WL, Antunes WC, Cavatte PC, Moraes GABK, Martins SCV, DaMatta FM (2012) Photosynthetic limitations in coffee plants are chiefly governed by diffusive factors. Trees 26:459-468.

Benton JM, Cobb AH. (1997) The modification of phytosterol profiles and *in vitro* photosynthetic electron transport of *Galium aparine* L. (cleavers) treated with the fungicide epoxiconazole. Plant Growth Regulation 22:93-100.

Bjorkman O, Demmig B. (1987) Photon yield of O_2 evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origins. Planta 170:489-504.

Bolton MD. (2009) Primary metabolism and plant defense-fuel for the fire. Molecular Plant-Microbe Interactions 22:487-947.

Bradford MN. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Annals of Biochemistry 72:248-254.

Buchenauer H, Rohner E. (1981) Effects of triadimefon and triadimenol on growth of various plant species as well as on gibberellin content and sterol metabolism in shoot of barley seedlings. Pesticide Biochemistry Physiology 15:58-70.

Bürling K, Hunsche M, Noga G. (2011) Use of blue-green and chlorophyll fluorescence measurements for differentiation between nitrogen deficiency and pathogen infection in wheat. Journal of Plant Physiology 168:1641-1648

Butler W. (1978) Energy distribution in the photochemical apparatus of photosynthesis. Annual Review of Plant Physiology 29:345-378.

Capucho AS, Caixeta ET, Maciel-Zambolim E, Zambolim L. (2009) Herança da resistência do Híbrido de Timor UFV 443-03 à ferrugem-do-cafeeiro. Pesquisa Agropecuária Brasileira 44:276-282.

Chance B, Maehley AC. (1955) Assay of catalases and peroxidases. Methods in Enzymology 2:764-775.

Duyens LNM, Sweers HE. (1963) Mechanisms of two photochemical reactions in algae as studied by means of fluorescence. In H. Tamiya (Ed.), Studies on microalgae and photosynthetic bacteria (pp. 353-372). Tokyo: University of Tokyo Press.

Essmann J, Schmitz-Thom I, Schön H, Sonnewald S, Weis E, Scharte J. (2008) RNA interference-mediated repression of cell wall invertase impairs defense in source leaves of tobacco. Plant Physiology 147:1288-1299.

Fletcher RA, Gilley A, Davis TD, Sankhla N (2000) Triazoles as plant growth regulators and stress protectants. Horticulture Review 24:55-138.

Gao J, Hofstra G, Fletcher RA. (1988) Anatomical changes induced by triazoles in wheat seedlings. Canadian Journal of Botany 66:1178-1185.

Gopi R, Sujatha BM, Rajan SN, Karikalan L, Panneerselvam R. (1999) Effect of triadimefon in the NaCl stressed cowpea (*Vigna ungiculata*) seedlings. Indian Journal of Agricultural Science 69:743-745.

Grasso V, Sierotzki H, Garibaldi A, Gisi U. (2006) Relatedness among agronomically important rusts based on mitochondrial cytochrome b gene and ribosomal ITS sequences. Journal of Phytopathology 154:110-118.

Grossmann K. (2000) The mode of action of auxin herbicides: a new ending to a long, drawn out story. Trends in Plant Science 5:506-508.

Grossmann K, Kwiatkowski J, Retzlaff G. (1999) Regulation of phytohormone levels, leaf senescence and transpiration by the strobilurin kresoxim-methyl in wheat (*Triticum aestivum*). Journal of Plant Physiology 154:805-808.

Guerra-Guimarães L, Silva MC, Struck C, Loureiro A, Nicole M, Rodrigues Jr. CJ, Ricardo CPP. (2009) Chitinases of *Coffea arabica* genotypes resistant to orange rust *Hemileia vastatrix*. Biologia Plantarum 53:702-706.

Guirong C, Lin X, Kaijiun W, Yan ZA, Yijiu P, Yuming I, Xiang WM. (1995) The physiological and yield increasing effects of multi-effect triazole treatment of green house rice seedling. Henan Nongye Kexue, No.2, 2-4. (ch, 5 ref). Xinyang Higher Agriculture Training School, China.

Guo DP, Guo YP, Zhao JP, Liu H, Peng Y, Wang QM, Chen JS, Rao GZ. (2005) Photosynthetic rate and chlorophyll fluorescence in leaves of stem mustard (*Brassica juncea* var. *tsatsai*) after turnip mosaic virus infection. Plant Science 168:57-63.

Habash D, Pervcival M, Baker N. (1985) Rapid chlorophyll fluorescence technique for the study of penetration of photosynthetically active herbicides into leaf tissue. Weed Research 25:389-395.

Hahn M, Mendgen K. (2001) Signal and nutrient exchange at biotrophic plant–fungus interfaces. Current Opinion in Plant Biology 4:322-327.

Harman GE, Hayes CK, Lorito M, Broadway RM, Pietro A, Peterbauer C, Tronsmo A. (1993) Chitinolytic enzymes of *Trichoderma harzianum*: purification of chitobiosidase and endochitinase. Phytopathology 83:313-318.

Havir EA, McHale NA. (1989) Enhanced-peroxidatic activity in specific catalase isozymes of tobacco, barley, and maize. Plant Physiology 91:812-815.

Herms S, Seehaus K, Koehle H, Conrath U. (2002) A strobilurin fungicide enhances the resistance of tobacco against Tobacco mosaic virus and *Pseudomonas syringae* pv. *tabaci*. Plant Physiology 130:120-127.

Hiscox JD, Israelstam GF. (1979) A method for the extraction of chlorophyll from leaf tissue without maceration. Canadian Journal of Botany 57:1332-1334.

Jaleel CA, Gopi R, Panneerselvam R. (2008) Growth and photosynthetic pigments responses of two varieties of *Catharanthus roseus* to triadimefon treatment. Comptes Rendus Biologie 331:272-277.

Kangasjärvi S, Neukermans J, Li S, Aro E-M, Noctor G. (2012) Photosynthesis, photorespiration, and light signalling in defence responses. Journal of Experimental Botany 63:1619-1636.

Kar M, Miashra D. (1976) Catalase, peroxidase and polyphenoloxidase activities during rice leaf senescence. Plant Physiology 57:315-319.

Kishorekumar A, Jaleel CA, Manivannan P, Sankar B, Sridharan R, Somasundaram R, Panneerselvam R. (2006) Differential effects of hexaconazole and paclobutrazol on

the foliage characteristics of Chinese potato (*Solenostemon rotundifolius* Poir., J.K. Morton). Acta Biologica Szegediensis 50:127-129.

Kramer D, Johnson G, Kiirats O, Edwards G. (2004) New fluorescence parameters for the determination of QA redox state and excitation. Photosynthesis Research 79:209-218.

Krugh BW, Miles D. (1996) Monitoring the effects of five ‘‘nonherbicidal’’ pesticide chemicals of terrestrial plants using chlorophyll fluorescence. Environmental Toxicology Chemistry 15:495-500.

Kuckenberg J, Tartachnyk I, Noga G. (2009) Temporal and spatial changes of chlorophyll fluorescence as a basis for early and precise detection of leaf rust and powdery mildew infections in wheat leaves. Precision Agriculture 10:34-44.

Kushalappa AC, Eskes AB. (1989) Coffee rust: epidemiology, resistance and management. Boca Raton: CRC Press. 345 pp.

Lavergne J, Trissl HW. (1995) Theory of fluorescence induction in photosystem II: Derivation of analytical expressions in a model including exciton-radical-pair equilibrium and restricted energy transfer between photosynthetic units. Biophysics Journal 68:2474-2492.

Lever M. (1972). A new reaction for colorimetric determination of carbohydrates. Analytical Biochemistry 47:273-279.

Lichtenthaler HK. (1987) Chlorophylls and carotenoids, the pigments of photosynthetic biomembranes. Pages 350-382 in: Methods in Enzymology. R. Douce and L. Packer, eds. Academic Press Inc., New York.

Lichtenthaler HK, Buschmann C, Knapp M. (2005) How to correctly determine the different chlorophyll fluorescence parameters and the chlorophyll fluorescence

decrease ratio RFd of leaves with the PAM fluorometer. Photosynthetica 43:379-393.

Mahlein AK, Oerke EC, Steiner U, Dehne HW. (2012) Recent advances in sensing plant diseases for precision crop protection. European Journal of Plant Pathology 133:197-209.

Maxemiuc-Naccache V, Braga MR, Dietrich SMC. (1992) Chitinase and β-1,3-glucanase changes in compatible and incompatible combinations between coffee leaf disks and coffee rust (*Hemileia vastatrix*). Revista Brasileira de Botânica 15:145-150.

Maxwell K, Johnson N. (2000) Chlorophyll fluorescence – a practical guide. Journal of Experimental Botany 51:659-668.

Meyer S, Saccardy-Adji K, Rizza F, Genty B. (2001) Inhibition of photosynthesis by *Colletotrichum lindemuthianum* in bean leaves determined by chlorophyll fluorescence imaging. Plant, Cell and Environment 24:927-955.

Muller P, Li XP, Niyogi KK. (2001) Nonphotochemical quenching. A response to excess light energy. Plant Physiology 125:1558-1566.

Muthukumarasamy M, Panneerselvam R. (1997) Triazole induced protein metabolism in the salt stressed *Raphanus sativus* seedlings. Journal of the Indian Botanic Society 76:39-42.

Nason MA, Farrar J, Bartlett D. (2007) Strobilurin fungicides induce changes in photosynthetic gas exchange that do not improve water use efficiency of plants grown under conditions of water stress. Pest Management Science 63:1191-1200.

Novais RF, Neves JCL, Barros NF. (1991) Ensaio em ambiente controlado. In: Oliveira AL, Garrido WE, Araújo JD, Lourenço S (Eds.) Métodos de pesquisa em fertilidade do solo. EMBRAPA-SEA. Brasília. pp. 153-189.

Petit AN, Fontaine F, Vatsa P, Clément C, Vaillant-Gaveau N. (2012) Fungicide impacts on photosynthesis in crop plants. Photosynthesis Research 111:315-326.

Petit AN, Vaillant N, Boulay M, Clement C, Fontaine F. (2006) Alteration of photosynthesis in grapevines affected by esca. Phytopathology 96:1060-1066.

Pfündel E. (1998) Estimating the contribution of photosystem I to total leaf chlorophyll fluorescence. Photosynthesis Research 56:185-195.

Resende RS, Rodrigues FA, Cavatte PC, Martins SCV, Moreira WR, Chaves ARM, DaMatta FM. (2012) Leaf gas exchange and oxidative stress in sorghum plants supplied with silicon and infected by *Colletotrichum sublineolum*. Phytopathology 102:892-898.

Roberts WK, Selitrennikoff CP. (1988) Plant and bacterial chitinases differ in antifungal activity. Journal of General Microbiology 134:169-176.

Rohaček, K (2002) Chlorophyll fluorescence parameters: the definitions, photosynthetic meaning, and mutual relationships. Photosynthetica 40:13-29.

Rojas ML, Montes de Gómez V, Ocampo CA. (1993) Stimulation of lipoxygenase activity in cotyledonary leaves of coffee reacting hypersensitively to the coffee leaf rust. Physiological and Molecular Plant Pathology 43:209-219.

Scharte J, Schön H, Weis E. (2005) Photosynthesis and carbohydrate metabolism in tobacco leaves during an incompatible interaction with *Phytophthora nicotianae*. Plant, Cell and Environment 28:1421-1435.

Scholes JD, Rolfe SA. (1996) Photosynthesis in localized regions of oat leaves infected with crown rust (*Puccinia coronata*): quantitative imaging of chlorophyll fluorescence. Planta 199:573-582.

Schreiber U. (1994) New emitter-detector-cuvette assembly for measuring modulated chlorophyll fluorescence of highly diluted suspensions in conjunction with the standard PAM fluorometer. Zeitschrift für Naturforsch 49:646-656.

Schreiber K, Desveaux D. (2008) Message in a bottle: chemical biology of induced disease resistance in plants. The Plant Pathology Journal 24:245-268.

Shaner G, Finney RE. (1977) The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat. Phytopathology 67:1051-1056.

Silva MC, Guerra-Guimarães L, Loureiro A, Nicole MR. (2008) Involvement of peroxidases in the coffee resistance to orange rust (*Hemileia vastatrix*). Physiological and Molecular Plant Pathology 72:29-38.

Silva MC, Nicole M, Rijo L, Geiger JP, Rodrigues CJ. (1999) Cytochemistry of plant-rust fungus interface during the compatible interaction *Coffea arabica* (cv. Caturra)-*Hemileia vastatrix* (race III). International Journal of Plant Sciences 160:79-91.

Silva MC, Nicole M, Guerra-Guimarães L, Rodrigues Jr. CJ. (2002) Hypersensitive cell death and post-haustorial defense responses arrest the orange rust (*Hemileia vastatrix*) growth in resistant coffee leaves. Physiological and Molecular Plant Pathology 60:169-183.

Silva MDC, Várzea VMP, Guimarães LG, Azinheira HG, Fernandez D, Petitot AS, Bertrand B, Lashermes P, Nicole M. (2006) Coffee resistance to the main diseases: leaf rust and coffee berry disease. Brazilian Journal of Plant Physiology 18:119-147.

Sujatha BM, Gopi R, Karikalan L, Rajan SN, Panneerselvam R. (1999) Effect of triadimefon treatment on bhendi (*Abelmoschus esculantus* (L.) moench.) seedlings under salinity condition. Indian Journal of Plant Physiology 4:307-310.

Suzuki JY, Bollivar DW, Bauer CE. (1997) Genetic analysis of chlorophyll biosynthesis. Annual Review of Genetics 31:61-89

Vale FXR, Fernandes Filho EI, Liberato JR. (2003) QUANT: A software plant disease severity assessment. In: Close R, Braithwaite M, Havery I, eds. Proceedings of the 8th International Congress of Plant Pathology, 2003. Sydney, Australia: Horticulture Australia, 105.

Van der Vossen AM. (2001) Agronomy I: Coffee Breeding Practices. In: Coffee: recent developments. (Clarke R.J., Vitzhum O.G., Eds.). Blackwell Sciences. pp.184-201

Vieira A, Talhinhas P, Loureiro A, Thürich J, Duplessis S, Fernandez D, Silva MC, Paulo OS, Azinheira HG (2012) Expression profiling of genes involved in the biotrophic colonisation of *Coffea arabica* leaves by *Hemileia vastatrix*. European Journal of Plant Pathology 133:261-277.

Voegele RT, Hahn M, Mendgen K. (2009) The Uredinales: cytology, biochemistry, and molecular biology. In: Deising HB (ed) and Esser K (Ser ed) Plant relationships. The Mycota, vol. 5. 2nd ed.

Voegele RT, Mendgen K. (2010) Nutrient uptake in rust fungi: How sweet is parasitic life? In Borlaug Global Rust Initiative, Technical Workshop, 30-31 May 2010, St. Petersburg, Russia (pp. 119-136).

Waller JM, Bigger M, Hillocks RJ. (2007) Coffee pests, diseases and their management. Oxfordshire: CAB International. 400 p.

Walters DR, McRoberts N, Fitt BDL. (2008) Are green islands red herrings? Significance of green islands in plant interactions with pathogens and pests. Biological Reviews of the Cambridge Philosophical Society 83:79-102.

Zambolim L, Acuña RS, Vale FXR, Chaves GM. (1992) Influência da produção do cafeeiro sobre o desenvolvimento da ferrugem (*Hemileia vastatrix*). Fitopatologia Brasileira 17:32-35.

Zambolim L, Chaves GM. (1974) Efeito de baixas temperaturas e do binômio temperatura-umidade relativa sobre a viabilidade dos uredosporos de *Hemileia vastatrix* Berk. et Br. e *Uromyces phaseolityca* Arth. Experientiae 18:151-184.

Zambolim L, Vale FXR, Costa H, Pereiria AA. Chaves GM. (2002) Epidemiologia e controle integrado da ferrugem do cafeeiro. pp. 369-450 In: Zambolim L. (Ed.) O Estado da Arte de Tecnologias na Produção de Café. Viçosa-MG: Universidade Federal de Viçosa.

Zambolim L, Vale FXR, Pereira AA, Chaves GM. (1999) Manejo integrado das doenças do cafeeiro. In: Zambolim, (Ed.). Produção de café com qualidade. Viçosa: UFV, 1999. p. 134-215.

Zhang YJ, Zhang X, Chen CJ, Zhou MG, Wang HC. (2010) Effects of fungicides JS399-19, azoxystrobin, tebuconazole, and carbendazim on the physiological and biochemical indices and grain yield of winter wheat. Pesticide Biochemistry Physiology 98:151-157.

Zhao D, Glynn NC, Glaz B, Comstock JC, Sood S. (2011) Orange rust effects on leaf photosynthesis and related characters of sugarcane. Plant Disease 95:640-647.

7-List of tables and figures

Table 1. Probability values (*P*-values) of the effects of fungicides (F), plant inoculation (PI) and evaluation times (ET) for the variables coffee leaf rust severity (Sev), initial fluorescence (F_0), maximum PSII quantum yield (F_v/F_m), effective PSII quantum yield (Y(II)), electron transport rate (ETR), chlorophyll *a* (Chl*a*), chlorophyll *b* (Chl*b*), chlorophyll *a* and *b* ratio (Chl*a*/*b*), carotenoids (Car), catalase (CAT), peroxidases (POX), *β*-1,3-glucanases (GLU) and chitinases (CHI)

Fig. 1. Coffee leaf rust progress curves (A) and area under rust progress curve (AURPC) (B) for coffee plants sprayed with epoxiconazole (E) and pyraclostrobin (P) or non-sprayed (control) and inoculated with *Hemileia vastatrix*. For AURPC, means followed by the same letter in each column did not differ by Tukey's test at $P \leq 0.05$. The bars represent the standard error of the mean. $n = 30$.

Fig. 2. Activities of catalase (CAT) (A and B), peroxidases (POX) (C and D), *β*-1,3-glucanases (GLU) (E and F) and chitinases (CHI) (G and H) on leaves of coffee plants non-inoculated (A, C, E and G) or inoculated (B, D, F and H) with *Hemileia vastatrix*. Means for non-inoculated and inoculated treatments followed by an asterisk are significantly different by *t*-test ($P \leq 0.05$). Means followed by the same letter within each treatment do not differ by Tukey's test at $P \leq 0.05$. Two experiments were conducted with consistent results.

Fig. 3. Concentrations of chlorophyll *a* (A and B), chlorophyll *b* (C and D), chlorophyll *a*/*b* ratio (E and F) and carotenoids (G and H) on leaves of coffee plants non-inoculated

(A, C, E and G) or inoculated (B, D, F and H) with *Hemileia vastatrix*. Means for non-inoculated and inoculated treatments followed by an asterisk are significantly different by *t*-test ($P \leq 0.05$). Means followed by the same letter within each treatment do not differ by Tukey's test at $P \leq 0.05$. Two experiments were conducted with consistent results.

Fig. 4. Chlorophyll fluorescence *a* parameters minimum fluorescence for a dark adapted leaf (F_0) (A and B), maximum PSII quantum efficiency (F_v/F_m) (C and D), effective PSII quantum yield (Y(II)) (E and F) and electron transport rate (ETR) (G and H) on leaves of coffee plants non-inoculated (A, C, E and G) or inoculated (B, D, F and H) with *Hemileia vastatrix*. Means for inoculated and non-inoculated treatments followed by an asterisk are significantly different by *t*-test ($P \leq 0.05$). Means followed by the same letter within each treatment do not differ by Tukey's test at $P \leq 0.05$. $n = 6$. Two experiments were conducted with consistent results.

Fig. 5. Imaging of the maximum PSII quantum efficiency (F_v/F_m) and effective PSII quantum yield (Y(II)) obtained from leaves of coffee plants non-sprayed (A) or sprayed with epoxiconazole (B) and pyraclostrobin (C) and non-inoculated (NI) or inoculated (I) with *Hemileia vastatrix*. Bar = 1 cm.

Table

Variables	F	PI	ET	F × PI	ET × F	ET × PI	ET × F × PI
	F-based P values						
Sev	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
F_0	< 0.001	< 0.001	< 0.001	0.508	0.011	< 0.001	0.214
F_v/F_m	< 0.001	< 0.001	0.001	0.059	0.016	< 0.001	0.019
Y(II)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.366	< 0.001
ETR	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Chl*a*	< 0.001	0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Chl*b*	0.286	< 0.001	< 0.001	0.073	< 0.001	< 0.001	< 0.001
Chl*a/b*	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Car	< 0.001	0.108	< 0.001	< 0.001	< 0.001	0.006	< 0.001
CAT	< 0.001	< 0.001	< 0.001	0.312	< 0.001	< 0.001	< 0.001
POX	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
GLU	0.288	< 0.001	< 0.001	0.006	< 0.001	0.021	< 0.001
CHI	0.117	0.439	0.252	0.689	0.003	< 0.001	0.006

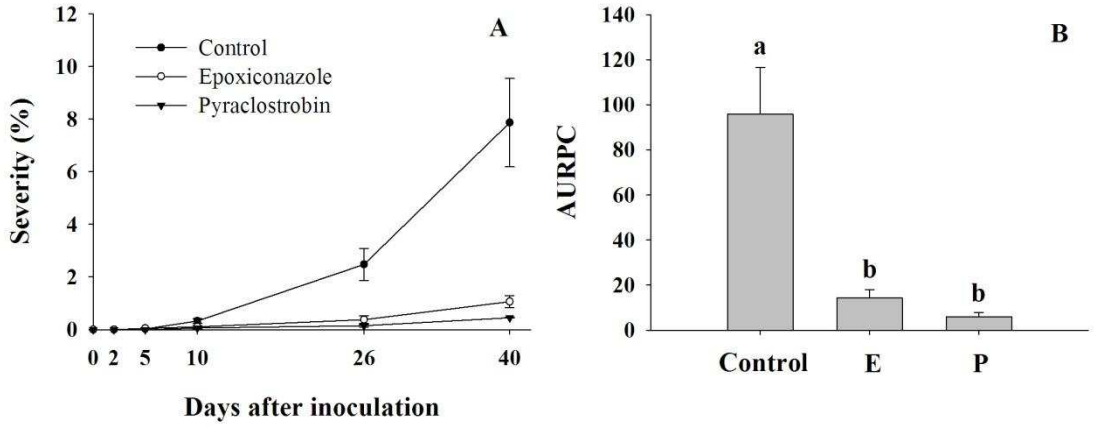

Figure 1

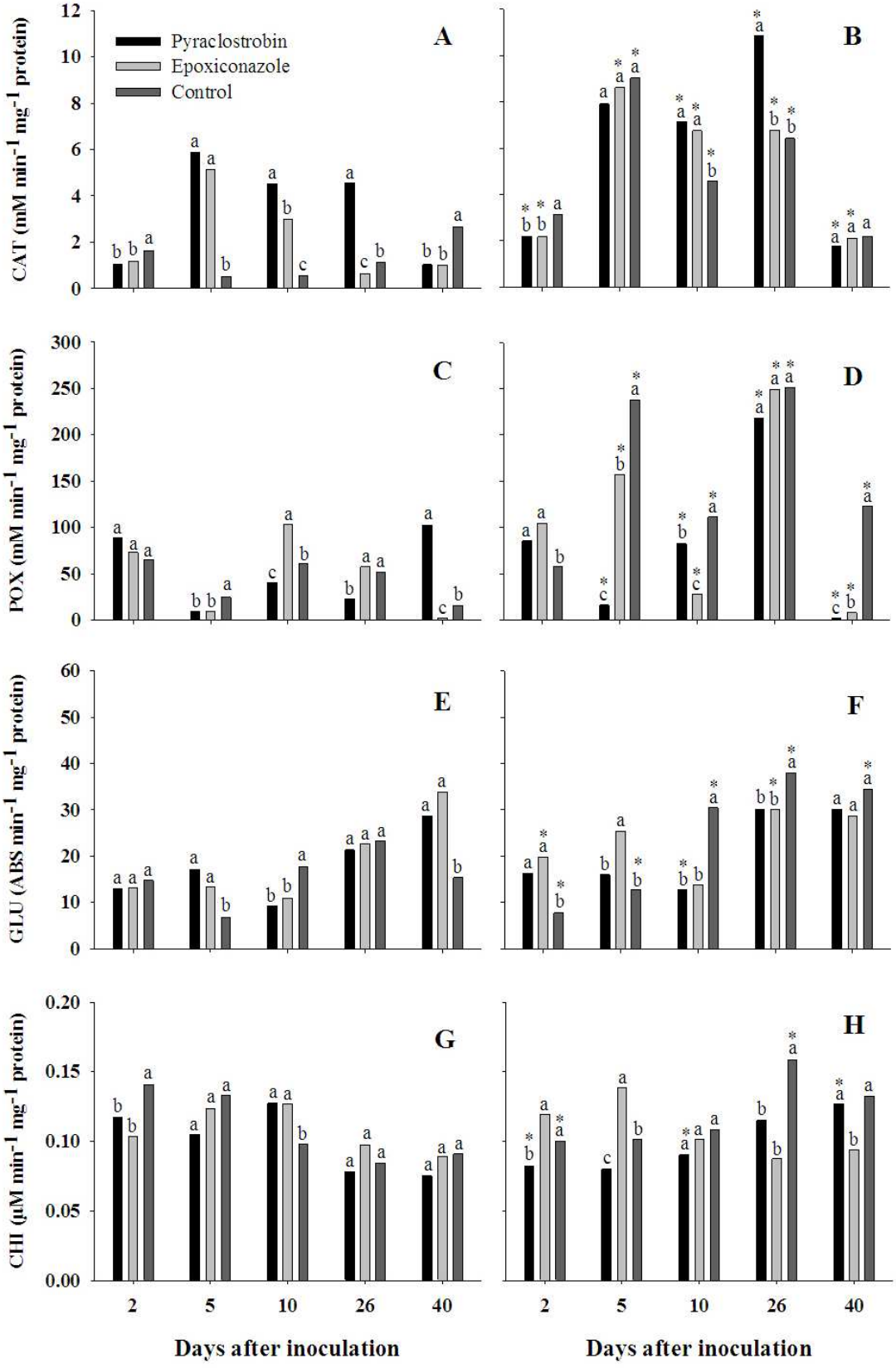
Pyraclostrobin
Epoxiconazole
Control
A
B
C
D
E
F
G
H
CAT (mM min-1 mg-1 protein)
POX (mM min-1 mg-1 protein)
GLU (ABS min-1 mg-1 protein)
CHI (µM min-1 mg-1 protein)
Days after inoculation
Days after inoculation

Figure 2

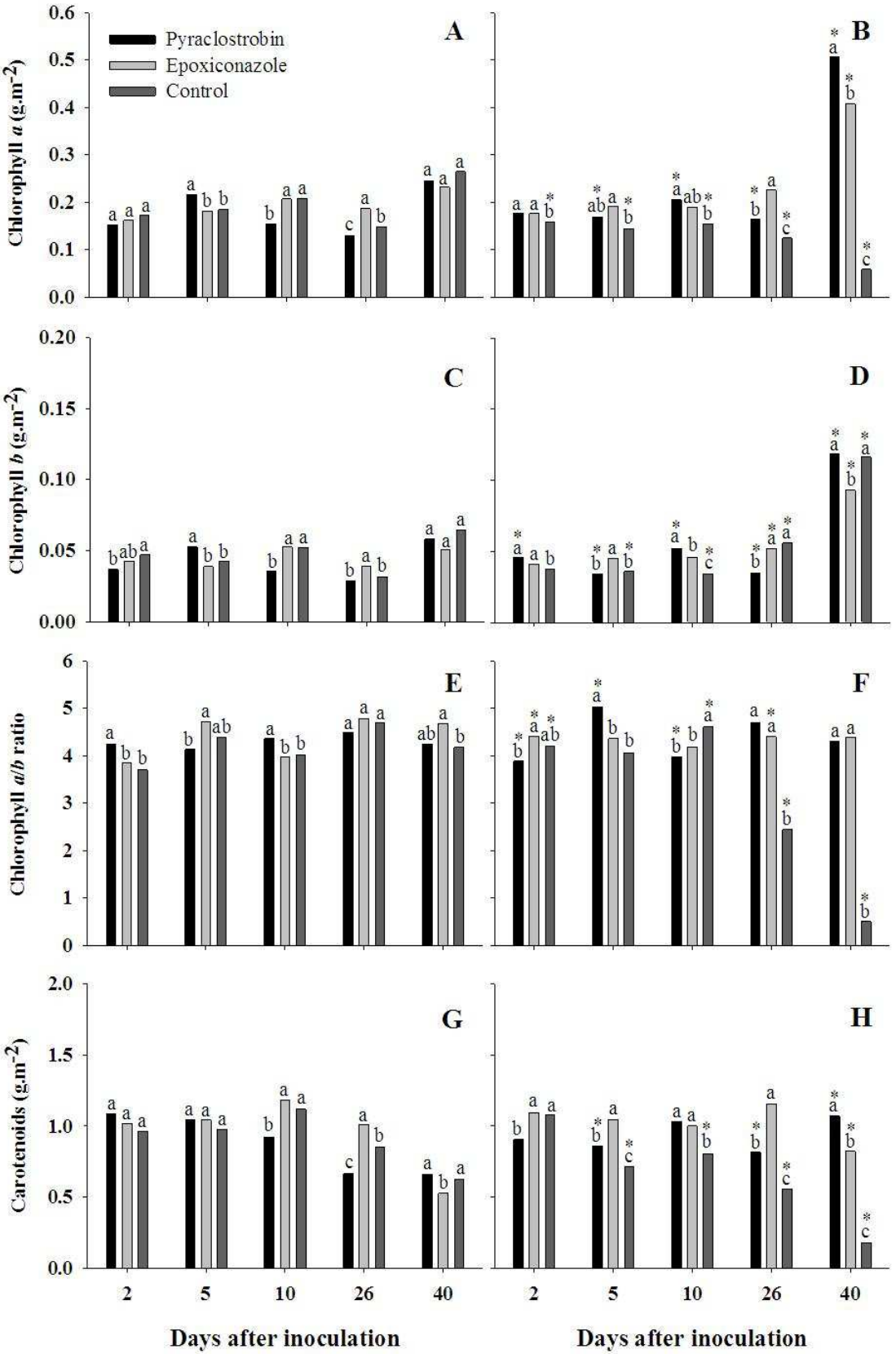
Pyraclostrobin
Epoxiconazole
Control
A
B
C
D
E
F
G
H
Chlorophyll a (g.m-2)
Chlorophyll b (g.m-2)
Chlorophyll a/b ratio
Carotenoids (g.m-2)
Days after inoculation
Days after inoculation

Figure 3

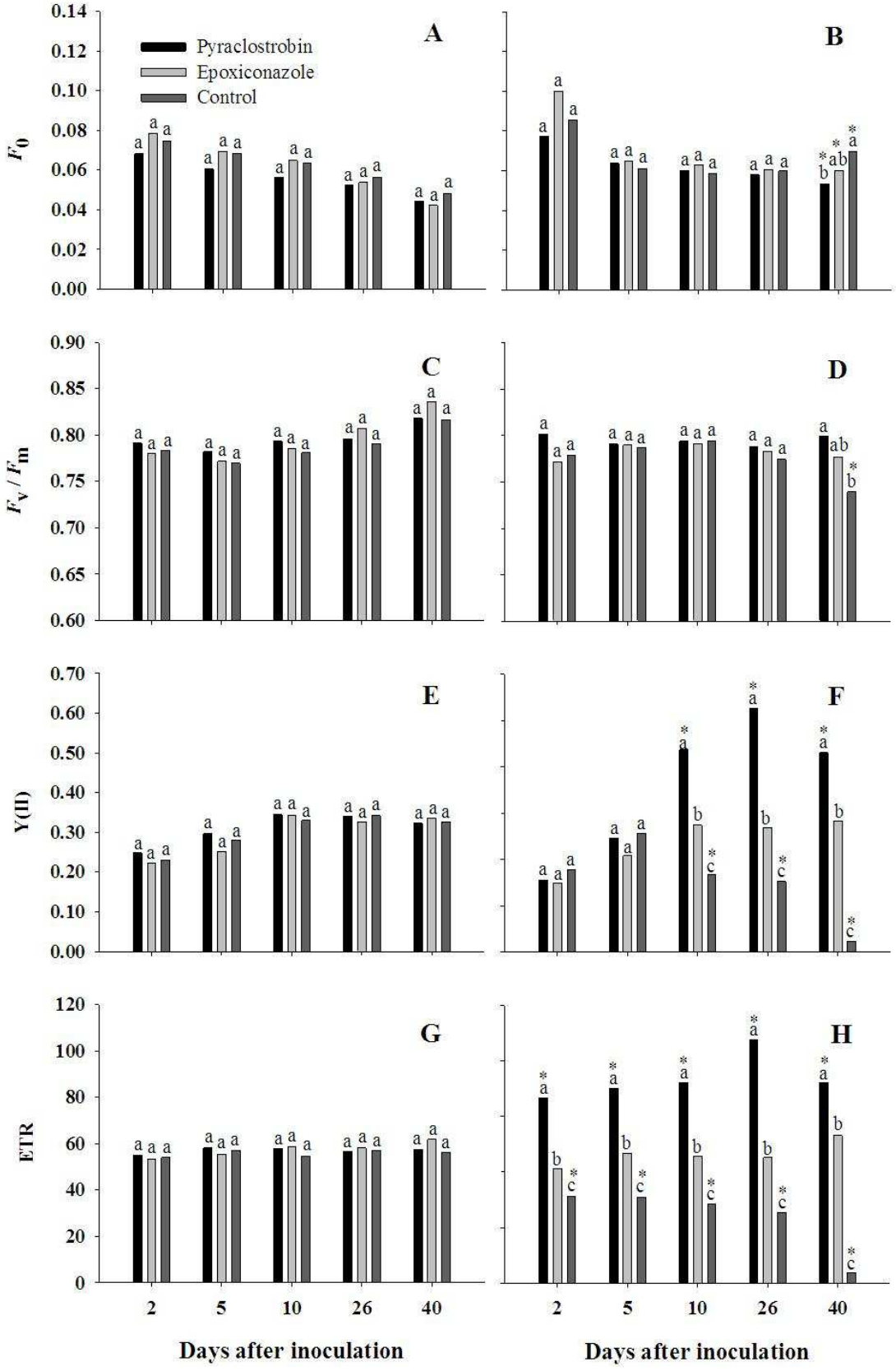
Pyraclostrobin
Epoxiconazole
Control
A
B
C
D
E
F
G
H
F_0
F_v / F_m
Y(II)
ETR
Days after inoculation
Days after inoculation

Figure 4

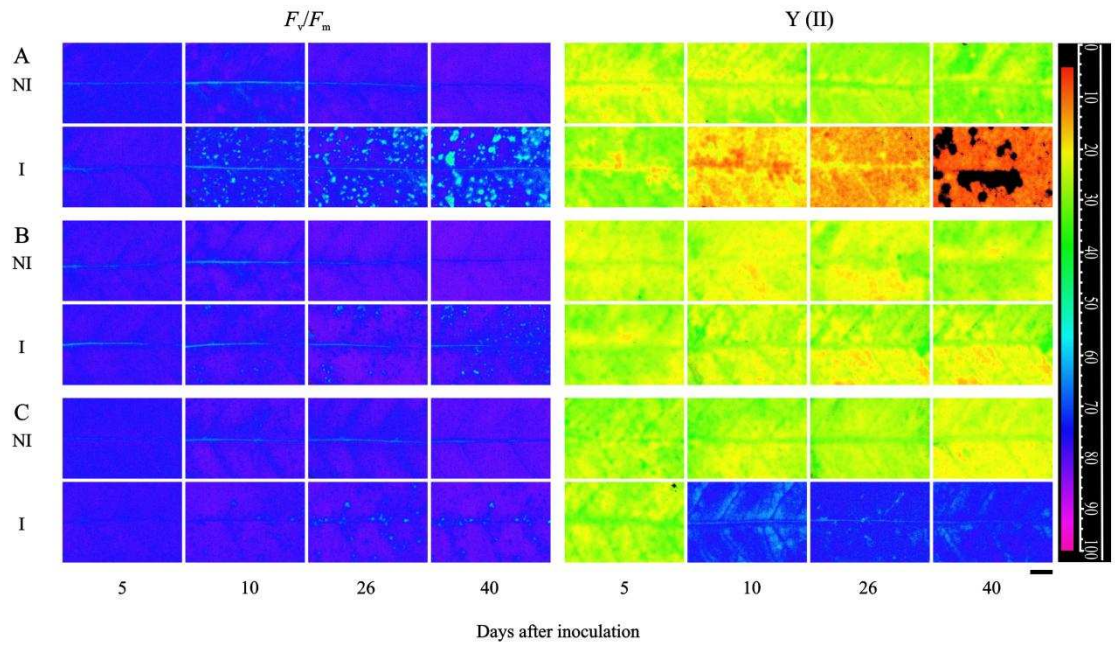
F_v/F_m
Y (II)
A
NI
I
B
NI
I
C
NI
I
0
10
20
30
40
50
60
70
80
90
100
5
10
26
40
5
10
26
40
Days after inoculation

Figure 5

CONCLUSÕES GERAIS

CAPÍTULO 1

1) A severidade da ferrugem do cafeeiro diminui até 40 dias após a inoculação nas plantas pulverizadas com epoxiconazol e piraclostrobina.

2) Existe uma relação linear significativa entre severidade real e visual das áreas foliares estimadas pelo parâmetro F_v/F_m e para cada unidade adicional na severidade visual, há um aumento de 1,53 unidades na severidade real.

3) Para os tratamentos com epoxiconazol e piraclostrobina, os sintomas da ferrugem do cafeeiro diminuem tomando como base as imagens visual e as geradas a partir do parâmetro F_v/F_m da fluorescência da clorofila *a*.

4) A redução nos valores do parâmetro F_v/F_m no epicentro das pústulas das folhas pulverizadas com epoxiconazol e piraclostrobina é maior do que no tratamento controle.

5) *Hemileia vastatrix* afeta a capacidade fotossintética de *Coffea arabica* e o parâmetro F_v/F_m de fluorescência da clorofila *a* é capaz de mostrar esse efeito antes do aparecimento dos sintomas visuais.

CAPÍTULO 2

1) As atividades de catalase, peroxidases, β-1,3-glucanases e quitinases são diferentes entre plantas inoculadas e não-inoculadas.

2) Há redução significativa na concentração de pigmentos entre as plantas não-inoculadas e inoculadas, mas não há redução significativa, entre as plantas pulverizadas com epoxiconazol e piraclostrobina.

3) Os parâmetros de fluorescência da clorofila *a* F_0, F_v/F_m, Y(II) e ETR são diferentes entre plantas não-inoculadas e inoculadas. No entanto, não são detectadas diferenças entre plantas não-pulverizadas e pulverizadas com fungicidas.