DANIEL DEBONA

PHYSIOLOGICAL, BIOCHEMCIAL AND MICROSCOPIC ASPECTS OF THE RICE-*Bipolaris oryzae* INTERACTION MEDIATED BY A STROBILURIN FUNGICIDE

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
2016

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T

Debona, Daniel, 1987-

D287p
2016

Physiological, biochemical and microscopic aspects of the rice-*Bipolaris oryzae* interaction mediated by a strobilurin fungicide / Daniel Debona. – Viçosa, MG, 2016.

xii, 81f. : il. (algumas color.) ; 29 cm.

Orientador: Fabrício de Ávila Rodrigues.

Tese (doutorado) - Universidade Federal de Viçosa.

Inclui bibliografia.

1. Arroz - Efeito do fungicida. 2. *Bipolaris oryzae*. I. Universidade Federal de Viçosa. Departamento de Fitopatologia. Programa de Pós-graduação em Fitopatologia. II. Título.

CDD 22. ed. 633.18

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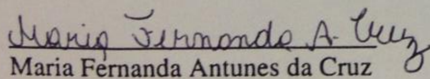
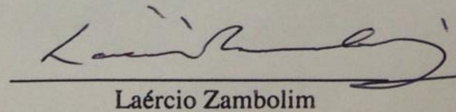
Aprovada: 17 de fevereiro de 2016.

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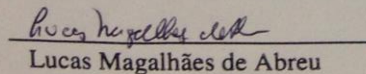
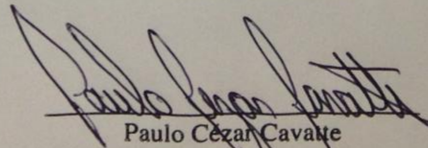

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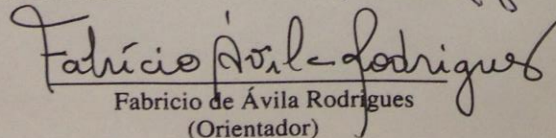

Fabricio de Ávila Rodrigues
(Orientador)

"Deus não escolhe os capacitados, capacita os escolhidos. Fazer ou não fazer algo só depende de nossa vontade e perseverança".

Albert Einstein

Aos meus amados pais Milton e Imelda,
ao meu irmão Darci,
a minha cunhada Luciana,
aos meus sobrinhos Diego e Rafael
e com carinho a Kelly:
OFEREÇO e DEDICO.

AGRADECIMENTOS

Agradeço primariamente ao meu grandioso Deus por mais essa conquista.

À Universidade Federal de Viçosa, ao Departamento de Fitopatologia e ao Programas de Pós-graduação em Fitopatologia por me proporcionarem condições para o desenvolvimento deste trabalho.

À Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) pelo apoio financeiro.

Ao Orientador Fabricio de Ávila Rodrigues pela amizade e compreensão nos momentos de dificuldades, além da orientação e apoio incondicional na execução deste trabalho.

À pesquisadora Marta Cristina Corsi de Filippi pelo fornecimento das sementes de arroz utilizadas neste trabalho.

Ao Professor Ricardo Silveiro Balardin pela orientação durante minha graduação, que me motivou a seguir a carreira de Fitopalogista.

A todos os professores do Programa de Pós-graduação em Fitopatologia que contribuíram para o meu aprendizado e formação profissional.

A todos os colegas do curso de Pós-Graduação em Fitopatologia, em especial ao Jonas Rios, Carlos E. Aucique-Pérez, Alessandro Fortunato, Alessandro Nicoli e Leonardo Araújo.

A todos os amigos do Laboratório da Interação Planta-Patógeno pela amizade e apoio na execução dos trabalhos.

A Kelly Juliane Telles Nascimento, pela ajuda incondicional em todas as etapas desse trabalho e, sobretudo, pela convivência maravilhosa.

Aos amigos Yasser, Dr. Han, Yenjit, Dongfang, Josielle, Natasha e Olanike pela ótima convivência e em especial ao Prof. Zhi-Yuan Chen pela orientação e ao Prof. Lawrence Datnoff pela viabilização da realização de meu Doutorado sanduíche na Louisiana State University.

Aos funcionários do Departamento de Fitopatologia: Braz, Bruno, Camilo, Sara, Nize, Suely e Sara.

Aos amigos de república Alessandro, Thiago e Gonimar pelos momentos de descontração.

A todos da minha família que sempre estiveram torcendo por mim.

A todas as pessoas que contribuíram direta e indiretamente para a realização deste trabalho.

BIOGRAFIA

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Em março de 2012, ingressou no curso de Doutorado em Fitopatologia na Universidade Federal Viçosa, sob orientação do Prof. Fabrício Ávila Rodrigues, defendendo a tese em fevereiro de 2016.

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RESUMO

DEBONA, Daniel, D.Sc. Universidade Federal de Viçosa, fevereiro de 2016. **Aspectos fisiológicos, bioquímicos e microscópicos da interação arroz-*Bipolaris oryzae* mediados por um fungicida do grupo das estrobilurinas.** Orientador: Fabrício de Ávila Rodrigues. Coorientador: Fábio Murilo da Matta.

As estrobilurinas estão entre os principais fungicidas usados no controle de doenças ao redor do mundo. Em adição ao seu efeito fungicida, elas também podem afetar aspectos fisiológicos e bioquímicos de plantas tratadas. No entanto, as alterações fisiológicas, bioquímicas e microscópicas decorrentes da aplicação de azoxistrobina (Az), a principal estrobilurina do mercado, na cultura do arroz permanecem elusivas. Para responder essas questões, foram realizadas análises detalhadas de trocas gasosas, da fluorescência da clorofila *a*, dos sistemas antioxidativo e de defesa, bem como análises de microscopia de luz em plantas de arroz (cultivar Metica-1) as quais receberam ou não a aplicação de Az (plantas +Fu e -Fu, respectivamente) e foram inoculadas ou não com *Bipolaris oryzae*, agente causal da mancha parda. Plantas +Fu não inoculadas mostraram menor fixação de carbono (C) do que plantas -Fu, sendo que tal redução não esteve relacionada a limitações fotoquímicas ou bioquímicas, mas ao decréscimo na condutância estomática, o qual limitou o influxo de CO_2 às células do mesófilo. A fotossíntese das plantas -Fu decresceu com a infeção por *B. oryzae*, principalmente por limitações fotoquímicas e bioquímicas. O excesso de energia decorrente da limitada fixação de C nas plantas +Fu inoculadas foi termicamente e efetivamente dissipado até 72 horas após a inoculação (hai). Para as plantas -Fu, entretanto, esse mecanismo não foi suficiente para prevenir uma crônica fotoinibição da fotossíntese. As plantas inoculadas não foram capazes de capturar e explorar completamente a energia solar coletada, mas tais restrições foram grandemente limitadas na presença de Az. Em geral, plantas +Fu não inoculadas não apresentaram alterações na atividade de enzimas antioxidativas, mas exibiram maiores concentrações de glutationa reduzida (GSH) do que plantas -Fu. A atividade da superóxido dismutase, peroxidase, peroxidase do ascorbato, peroxidase da glutationa, redutase da glutationa e glutationa-*S*-transferase foram aumentadas em decorrência da infecção por *B. oryzae*, mas tais aumentos foram menores nas plantas +Fu. A atividade da catalase foi diminuída nas plantas inoculadas comparadas às não inoculadas, independentemente do tratamento com fungicida. A concentração de GSH aumentou em resposta à infecção por *B. oryzae*, e as plantas +Fu sustentaram maiores níveis de GSH em estágios avançados da infecção fúngica do que

as plantas -Fu. As plantas inoculadas exibiram extensivo estresse oxidativo, como evidenciado pelas maiores concentrações de peróxido de hidrogênio e aldeído malônico comparado às plantas não inoculadas, porém aumentos menos pronunciados e mais tardios foram reportados nas plantas +Fu do que nas plantas -Fu. Plantas +Fu apresentaram maior atividade da β-1,3-glucanase, peroxidase, polifenol oxidase (PFO) e lipoxigenase (LOX) na ausência de inoculação com *B. oryzae* e de fenilalanina amônia liase (FAL), PFO e LOX 24 hai comparadas às plantas -Fu. A concentração de fenois solúveis totais (FST) foi transientemente reduzida pela infecção fúngica nas plantas +Fu, mas tais plantas apresentaram maiores níveis de FST 144 hai comparadas às plantas -Fu. Independentemente do tratamento com fungicida, a infecção por *B. oryzae* aumentou a atividade de todas as enzimas de defesa estudadas, mas tais aumentos foram usualmente maiores para as plantas -Fu do que para as plantas +Fu. A concentração de derivados da lignina e ácido tioglicólico foram aumentadas pela infecção fúngica nas plantas -Fu. Análises microscópicas revelaram que as hifas de *B. oryzae* colonizaram as células buliformes, da bainha do feixe, epidérmicas, guarda, do mesófilo e do feixe vascular, bem como espaçoes intercelulares, porém menos e menores células fúngicas foram observadas nas plantas +Fu do que nas plantas -Fu. No entanto, a limitada invasão fúngica verificada nas plantas +Fu não foi acompanhada por amplificadas reções de defesa, visto que as células das plantas -Fu reagiram através do acúmulo de material semelhante a fenois, considerando que tal reação foi limitada nas células de plantas +Fu. Portanto, nossos resultados mostraram que Az prejudicou a performance fotossintética de plantas não infectadas através de limitações difusivas, mas preveniu, numa grande extensão, o dano ao aparato fotossintético durante o processo infecioso de *B. oryzae*. Em adição, Az reduziu o estresse oxidative induzido por *B. oryzae* por limitar o desenvolvimento da mancha parda ao invés da ativação do sistema antioxidativo. Embora Az tenha reprogramado a atividade de enzimas de defesa, fato que pode ter contribuído para explicar a reduzida severidade da mancha parda observada em plantas tratadas +Fu, seu efeito fungicida desempenhou um papel fundamental em restringir a infecção por *B. oryzae*.

ABSTRACT

DEBONA, Daniel, D.Sc. Universidade Federal de Viçosa, February, 2016. **Physiological, biochemical and microscopic aspects of the rice-*Bipolaris oryzae* interaction mediated by a strobilurin fungicide.** Adviser: Fabrício de Ávila Rodrigues. Co-adviser: Fábio Murilo da Matta.

Strobilurins are among the most important fungicides that are used for plant disease control worldwide. In addition to their fungicide effect, strobilurins can also affect some physiological and biochemical aspects of treated plants. Nonetheless physiological, biochemical and microscopic alterations caused by azoxystrobin (Az), the main marketed strobilurin, in rice crop remain elusive. To address this issue, detailed gas exchange measurements and chlorophyll *a* fluorescence analysis, antioxidant and defense systems assays as well light microscopy analysis were performed on rice plants (cultivar Metica-1) that were sprayed or non-sprayed with Az (+Fu and -Fu plants) and either challenged or not with *Bipolaris oryzae*, the causal agent of brown spot. The +Fu non-inoculated plants displayed lower carbon (C) fixation than -Fu plants, and such decrease was not related to photochemical or biochemical limitations but rather to decreased stomatal conductance that limited the CO_2 influx into the mesophyll cells. The photosynthesis of -Fu plants decreased upon *B. oryzae* infection, which was chiefly governed by photochemical and biochemical limitations. The energy surplus that was caused by limited C fixation in the +Fu inoculated plants was thermally and effectively dissipated until 72 hours after inoculation (hai). For the -Fu plants, however, this mechanism was not sufficient to prevent chronic photoinhibition to photosynthesis. The inoculated plants were not able to fully capture and exploit the collected light energy, but these constraints were greatly limited in the presence of Az. In general, the +Fu plants did not show changes in the activities of antioxidant enzymes, but displayed higher concentrations of reduced glutathione (GSH) than -Fu plants. The activities of superoxide dismutase, peroxidase, ascorbate peroxidase, glutathione peroxidase, glutathione reductase, and glutathione-*S*-transferase were increased upon *B. oryzae* infection, but such increases were lower in the +Fu plants. Catalase activity decreased in the inoculated plants compared to the non-inoculated plants regardless of fungicide treatment. The GSH concentration increased in response to the *B. oryzae* infection, and the +Fu plants sustained higher levels of GSH at advanced stages of fungal infection than did the -Fu plants. The inoculated plants exhibited an extensive oxidative stress as evidenced by higher concentrations of hydrogen peroxide and malondialdehyde

compared to the non-inoculated plants, but lower and later increases were recorded in the +Fu plants than in the -Fu plants. The +Fu plants displayed higher activities of *β*-1,3-glucanase, peroxidase, polyphenol oxidase (PPO) and lipoxygenase (LOX) in the absence of *B. oryzae* inoculation and of phenylalanine ammonia lyase, PPO and LOX at 24 hai compared to the -Fu plants. Concentrations of total soluble phenols (TSP) were transiently reduced by fungal infection in the +Fu plants, but such plants presented higher TSP levels at 144 hai relative to their -Fu counterparts. Irrespective of the fungicide treatment, *B. oryzae* infection increased activities of all defense enzymes studied, but such increases usually were higher for the -Fu plants than for the +Fu plants. Concentrations of lignin thioglycolic acid derivatives were increased following fungal infection in the -Fu plants. Microscopic analyses revealed that hyphae from *B. oryzae* colonized bulliform, bundle sheath, epidermal, guard, mesophyll and vascular bundle cells besides intercellular spaces, but fewer and smaller fungal cells were noticed in the +Fu than in the -Fu plants. However, the constrained fungal invasion verified in the +Fu plants was not accompanied by amplified defense reactions since cells of the -Fu plants reacted by accumulating phenol-like material, whereas such reaction was only limited in cells of the +Fu plants. Overall, our results showed that Az impaired the photosynthetic performance of non-infected plants by diffusive constraints but prevented, to a greater extent, the damage to the photosynthetic apparatus during the infection process of *B. oryzae*. In addition, Az reduced *B. oryzae*-induced oxidative stress by limiting brown spot development rather than by activating the antioxidant system. Although Az had transiently reprogrammed activities of defense enzymes, which may have contributed for explaining the reduced brown spot severity observed in the +Fu plants, its fungicidal activity played a major role in reducing *B. oryzae* infection.

GENERAL INTRODUCTION

Brown spot, caused by the necrotrophic fungus *Bipolaris oryzae* (Breda de Haan) Shoemaker [teleomorph: *Cochliobolus miyabeanus* (Ito and Kuribayashi) Drechs. ex Dastur.], is a serious threat to rice production (Ou, 1985) because it can cause great yield losses (up to 74%) besides negatively affecting grain quality (Kohls et al., 1987). Germinating conidia secrete the non-host-selective toxins ophiobolins A and B (Xiao et al., 1991), which leads to membrane disruption, increasing electrolyte leakage and malondialdehyde concentration (Dallagnol *et al.*, 2011b). Fungal penetration occurs through stomata or directly through the cuticle, and symptoms are visible as early as 18 hours after inoculation (Tullis, 1935; Dallagnol et al., 2009). Brown spot symptoms include light reddish-brown lesions with a gray center surrounded by a dark to reddish-brown margin with a bright yellow halo, and disease severity is greatly influenced by the environmental conditions and light intensity (Ou, 1985; Dallagnol et al., 2011a)

Because cultivars with vertical resistance are not available to growers (Ou, 1985; Dallagnol et al., 2011b), fungicide spraying has been the primary strategy for brown spot control (Ou, 1985; Dallagnol et al., 2011b). Strobilurins are a major fungicide class that are used for plant disease control because of their (meso)systemic and broad-spectrum activities, controlling rusts, powdery mildews, downy mildews, leaf spots and anthracnoses (Bartlett et al., 2002). The fungicide effect of strobilurins occurs because they inhibit fungal energy production by blocking the *bc1* cytochrome complex III of the electron transport chain of mitochondria (Ypema and Gold, 1999; Bartlett et al., 2002; Köehle et al., 2003; Venancio et al., 2003).

In addition to their fungicide effect, strobilurins can also improve the biochemical and physiological aspects of plants under pathogen absence (Bartlett et al., 2002; Köehle et al., 2003; Venancio et al., 2003). Fagan et al. (2010) found that pyraclostrobin increased the phostosynthetic performance and nitrate reductase activity of soybean plants. Azoxystrobin-treated wheat plants showed higher activities of SOD, POX and CAT, which were coupled to decreased O_2^- and MDA concentrations, chlorophyll degradation and leaf senescence compared to non-sprayed plants (Wu and Von-Tiedemann 2001; Zhang et al. 2010). Strobilurins fungicides also can prime plants for enhanced defense (Conrath *et al.*, 2015). Pyraclostrobin primed tobacco plants for accelerated *PR1* induction after *Tobacco mosaic virus* (TMV) attack (Herms et al., 2002), whereas azoxystrobin enhanced the production of secondary metabolites

(phenolics and lignin) and enzymes activities (POX, PPO and PAL) in rice plants infected by *Pyricularia oryzae* (Sundravadana et al., 2007). Despite these resports, the effect of azoxystrobin, the main marketed strobilurin, in physiological, biochemical and microscopic aspects of rice plants remains largely elusive. Here, therefore, we investigated the photosynthetic and photochemical performance, concentrations of photosynthetic pigments, activities of antioxidant enzymes, concentrations of antioxidant compounds, cellular damage, activities of defense enzymes, concentrations of defense compounds and histological responses in rice plants that were either treated or non-treated with azoxystrobin and challeged or non-challenged with *B. oryzae*.

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Chapter 1

Physiological changes promoted by a strobilurin fungicide in the rice-*Bipolaris oryzae* interaction

ABSTRACT

Strobilurins are among the most important fungicides that are used for plant disease control worldwide. In addition to their fungicide effect, strobilurins can also improve crop physiology. Nonetheless, the impact of azoxystrobin (Az), the main marketed strobilurin, on physiological aspects of rice-*Bipolaris oryzae* interaction remains elusive. Detailed gas exchange measurements and chlorophyll *a* fluorescence analysis were used to examine the photosynthetic performance of rice plants (cultivar Metica-1) that were non-sprayed or sprayed with Az (-Fu and +Fu plants, respectively) and either challenged or not with *B. oryzae*, the causal agent of brown spot. The +Fu non-inoculated plants showed lower carbon (C) fixation than -Fu plants and such decrease was not related to photochemical or biochemical limitations but rather to decreased stomatal conductance that limited the CO_2 influx into the mesophyll cells. The photosynthesis of the -Fu plants decreased upon *B. oryzae* infection, which was chiefly governed by photochemical and biochemical limitations. The energy surplus that was caused by limited C fixation in the +Fu inoculated plants was thermally and effectively dissipated until 72 hours after inoculation. For the -Fu plants, however, this mechanism was not sufficient to prevent chronic photoinhibition to photosynthesis. The inoculated plants were not able to fully capture and exploit the collected light energy, but these constraints were greatly limited in the +Fu plants. In conclusion, Az impaired the photosynthetic performance of non-infected plants by diffusive constraints but prevented, to a greater extent, the damage to the photosynthetic apparatus during the infection process of *B. oryzae*.

Keywords: *Oryza sativa*, azoxystrobin, brown spot, chlorophyll *a* fluorescence, gas exchange variables.

INTRODUCTION

Brown spot, caused by the necrotrophic fungus *Bipolaris oryzae* (Breda de Haan) Shoemaker [teleomorph: *Cochliobolus miyabeanus* (Ito and Kuribayashi) Drechs. ex Dastur.], is the second most important disease in the rice crop, only surpassed by blast (*Pyricularia oryzae*) (Ou, 1985). Brown spot negatively affects grain quality and can cause yield losses of up to 74% (Kohls et al., 1987). After their deposition on a wet rice leaf, conidia germinate and secrete the non-host-selective toxins ophiobolins A and B, which contribute largely to symptom development (Xiao et al., 1991) and suppress host defense responses (Tullis, 1935; Vidhyasekaran et al., 1992). The fungus can penetrate through stomata or enter directly through the cuticle, and the incubation period is as short as 18 hours (Tullis, 1935; Dallagnol et al., 2009). Brown spot symptoms include light reddish-brown lesions with a gray center surrounded by a dark to reddish-brown margin with a bright yellow halo, and disease severity is greatly influenced by the environmental conditions and light intensity (Ou, 1985; Dallagnol et al., 2011a).

Strategies for brown spot control are limited because cultivars with high levels of resistance are not available to growers (Ou, 1985; Dallagnol et al., 2011b). Consequently, fungicide spraying has been the primary strategy to limit the yield losses that are caused by brown spot (Ou, 1985). Strobilurins constitute one of the major fungicide classes that are used for plant disease control because of their (meso)systemic and broad-spectrum activities (Bartlett et al., 2002). The fungicide effect of strobilurins occurs because they inhibit fungal energy production by blocking the *bc1* cytochrome complex III of the electron transport chain of mitochondria (Ypema and Gold, 1999; Bartlett et al., 2002; Köehle et al., 2003; Venancio et al., 2003). Because spore germination is the more energy-demanding stage, strobilurins are highly effective when applied preventatively, and their efficiency drastically decreases after fungal penetration (Ypema and Gold, 1999; Bartlett et al., 2002).

In addition to their fungicide effect, strobilurins can also affect the biochemical and physiological aspects of plants under pathogen absence (Bartlett et al., 2002; Köehle et al., 2003; Venancio et al., 2003). Such changes include an increase in the activity of nitrate reductase, a key enzyme that is involved in nitrogen assimilation (Köehle et al., 2003; Fagan et al., 2010); an increased activity of antioxidant enzymes, thereby preventing oxidative damage that is caused by stressful conditions (Wu and Tiedemann, 2001; Köehle et al., 2003; Zhang et al., 2010); reduced ethylene synthesis, which may prevent

chlorophyll degradation (Grossmann and Retzlaff, 1997; Köehle et al., 2003); and increased abscisic acid concentration, thereby improving the water-use efficiency (Grossmann et al., 1999; Köehle et al., 2003). Despite these reports, few studies have assessed the strobilurin impact on plant photosynthesis (Nason et al., 2007; Fagan et al., 2010; Diaz-Espejo et al., 2012). Furthermore, the effect of azoxystrobin, the main marketed strobilurin, in rice physiology remains elusive. Therefore, the hypotheses of the present study were as follows: i) azoxystrobin can improve the photosynthetic performance of rice plants, and ii) azoxystrobin can limit the damage to photosynthetic apparatus that is caused by *B. oryzae* infection. To address these issues, detailed gas exchange measurements and chlorophyll *a* fluorescence analysis were combined to examine the effects of azoxystrobin on the photosynthetic performance of rice plants that were either challenged or not with *B. oryzae*.

MATERIAL AND METHODS

Plant growth and fungicide spraying. Rice seeds from the cultivar Metica-1were surface sterilized in 10% (v v^{-1}) NaOCl for 2 min, rinsed in sterilized water for 3 min, and sown in plastic pots (20 cm in diameter) (Ecovaso, Jaguariúna, SP, Brazil) that were filled with 2 kg of a substrate made from a 1:1:1 mixture of pine bark, peat, and expanded vermiculite (Tropstrato®, Vida Verde, Mogi Mirim, SP, Brazil). A total of ten seeds were sown per pot, and five days after seedling emergence, each pot was thinned to four seedlings. The substrate in each pot was fertilized with a nutrient solution that is based on Clark (1975) and contained 1.04 mM $Ca(NO_3)_2.4H_2O$, 1 mM NH_4NO_3, 0.8 mM KNO_3, 0.069 mM KH_2PO_4, 0.931 mM KCl and 0.6 mM $MgSO4.7H_2O$, 19 µM H_3BO_3, 2 µM $ZnSO_4.7H_2O$, 0.5 µM $CuSO_4.5H_2O$, 7 µM $MnCl_2.4H_2O$, 0.6 µM $Na_2MoO_4.4H_2O$, 90 µM $FeSO_4.7H_2O$, and 90 µM EDTA bisodic. The nutrient solution was prepared using deionized water and applied weekly. The plants were watered as needed with deionized water. The treatments consisted of plants that were sprayed or not with azoxystrobin. The fungicide was sprayed 24 hours before *B. oryzae* inoculation on rice plants at the V_8 growth stage (Counce et al., 2000). Azoxystrobin (Priori®, Syngenta, Basel, Switzerland, 2 ml c. p. L^{-1}) was prepared on the morning of spraying in deionized water + mineral oil (Nimbus®, Syngenta, Basel, Switzerland, 0.5% v v^{-1}). The control plants were sprayed with deionized water + mineral oil. The fungicide treatments were sprayed using a CO_2 pressurized backpack sprayer that was equipped with a flat fan nozzle (XR 110 02®, Teejet, Glendale Heights, IL, USA) at a 200,000-Pa pressure to give a spray volume of 200 L ha^{-1}.

Inoculation procedure. The rice plants were inoculated with *B. oryzae* at the V_8 growth stage (Counce et al., 2000). Pathogen preservation and inoculum preparation were performed according to Dallagnol et al. (2011a). A conidial suspension of *B. oryzae* (5×10^3 conidia mL^{-1}) was applied as a fine mist to the leaves of each plant until runoff using a VL Airbrush atomizer (Paasche Airbrush Co., Chicago, IL, USA). Gelatin (1%, w v^{-1}) was added to the sterile water to aid conidial adhesion to the leaves. Immediately after inoculation, the plants were transferred to a growth chamber with a temperature of 25 ± 2°C and a relative humidity of 90 ± 5% and were subjected to an initial 24-hours dark period. After this period, the plants were transferred to a plastic mist growth chamber (MGC) inside a greenhouse for the duration of the experiment. The MGC was made of wood (2m wide, 1.5 m high, and 5 m long) and covered with 100-µm-thick transparent plastic. The temperature inside the MGC ranged from 25 ± 2°C (day) to 20 ± 2°C (night). The relative humidity was maintained at 92 ± 3% using a misting system in which nozzles (model NEB-100; KGF Company, São Paulo, Brazil) sprayed mist every 30 min above the plant canopy. The relative humidity and temperature were measured with a thermohygrograph (TH-508, Impac, São Paulo, Brazil). The maximum natural photon flux density at the plant canopy height was approximately 900 µmol m^{-2} s^{-1}. Non-inoculated plants were kept in separate chambers but were exposed to the same conditions as the inoculated plants during the experiments.

Experimental design. A 2 × 2 factorial experiment, consisting of two fungicide treatments (with or without azoxystrobin spraying, referred to as +Fu or -Fu plants, respectively) and inoculated or non-inoculated plants was arranged in a completely randomized design with four replications. Each experimental unit consisted of one plastic pot containing four plants. The experiment was repeated once.

Quantification of epidemiological variables. The following epidemiological variables were evaluated: brown spot severity (BSS), final lesion size (FLS) and number of lesions per square centimeter (NL). The sixth leaf from the base to the apex of each plant was marked and used to evaluate the epidemiological variables that were mentioned above. The BSS on the marked leaf of each plant was scored at 24, 48, 72, 96, 120 and 144 hours after inoculation (hai) using a scale based on the percentage of diseased leaf area (Lenz et al., 2010). The area under the brown spot progress curve (AUBSPC) for each leaf in each plant was computed using the trapezoidal integration of the brown spot progress curve over time using the formula that was proposed by Shaner and Finney (1979). The disease progress rate (r) was determined based on the linear model $Y = a + rt$, where Y is the disease

severity expressed as a proportion, a is the constant, *r* is the disease progress rate, and t is the time (in hours), using the software SAS (v. 6.12; SAS Institute, Inc.).The FLS (mm) of ten random lesions on the marked leaf of each plant was measured at 144 hai using an electronic digital caliper. The NL of ten random areas on the marked leaf of each plant was determined at 144 hai. For the statistical analysis, the average of FLS and NL for each leaf was calculated. In addition, the leaves were also collected for scanning at 144 hai when the experiment was completed.

Evaluation of the leaf gas exchange variables. The gas exchange variables were measured on the sixth leaf (a total of four leaves per treatment) from the base to the apex 12, 24, 72, 120 and 168 hours after fungicide spraying (hafs). The net carbon assimilation rate (A), stomatal conductance to water vapor (g_s), internal CO_2 concentration (C_i), and transpiration rate (E) were estimated from 09:00 to 12:00 a.m., with exception of the evaluation made at 12 hafs which was performed at 09:00 p.m., under artificial and saturating photon irradiance (1,200 µmol m^{-2} s^{-1}) and an external CO_2 concentration of 400 µmol mol^{-1} using a portable open-system infrared gas analyzer (LI-6400, LI-COR Inc., Lincoln, NE, USA). All measurements were performed by fixing the block temperature at 25°C. The response curve of A versus the photosynthetic photon flux density ($PPFD$) was obtained using nine levels of $PPFD$: 0, 100, 500, 700, 1000, 1200 and 2000 µmol m^{-2} s^{-1}. Once a steady state was reached, $PPFD$ was gradually decreased to 0 µmol m^{-2} s^{-1}. The asymptote of photosynthesis under high light (A_{max}), apparent quantum yield (k) and light compensating point (LCP) were calculated using the Mitscherlich model equation as proposed by Potvin et al. (1990) and described below:

$$A = Amax\left[1 - e^{-k(PPFD - \mathrm{LCP})}\right]$$

The light saturating point (LSP) was estimated when the $PPFD$ level was approximately 90% of the A_{max} (Thomas and Bazzaz, 1999).

Chlorophyll *a* fluorescence imaging. The images and parameters of chlorophyll *a* fluorescence were obtained on the sixth leaf from the base to the apex at 12, 24, 72, 120 and 168 hafs using the MAXI version of the Imaging-PAM fluorometer and the Imaging Win software (Heinz Walz GmbH, Effeltrich, Germany). The chlorophyll *a* fluorescence emission transients were captured by a CCD ("charge-coupled device") camera with a resolution of 640 × 480 pixels in a visible sample area of 24 × 32 mm on each leaf. Initially, the leaves were dark-adapted for 30 min, after which they were carefully and individually fixed in a support at a distance of 18.5 cm from the CCD camera. The leaf

tissues were then exposed to a weak modulated measuring beam (0.5 µmol m^{-2} s^{-1}, 100 µs, 1 Hz) to determine the initial fluorescence (F_0) when all of the photosystem (PS) II reaction centers were "open". Next, a saturating white light pulse of 2,400 µmol m^{-2} s^{-1} (10 Hz) was applied for 0.8 s to ensure the maximum fluorescence emission (F_m) when all of the PS II reaction centers were "closed". From these initial measurements, the maximum PS II photochemical efficiency of the dark-adapted leaves was estimated using the variable-to-maximum chlorophyll *a* fluorescence ratio, $F_v/F_m = [(F_m - F_0)/F_m)]$. The leaf tissues were subsequently exposed to actinic photon irradiance (531 µmol m^{-2} s^{-1}) for 120 s to obtain the steady-state fluorescence yield (F_s), after which a saturating white light pulse (2,400 µmol m^{-2} s^{-1}; 0.8 s) was applied to achieve the light-adapted maximum fluorescence (F_m'). The light-adapted initial fluorescence (F_0') was estimated according to Oxborough and Baker (1997). Following Kramer *et al.* (2004), the energy that was absorbed by PS II for the following three yield components for dissipative processes was calculated as follows: the photochemical yield $[Y(II) = (F_m' - F_s)/ F_m')]$ and the yield for dissipation by down-regulation $[Y(NPQ) = (F_s /F_m') - (F_s /F_m)]$. Additionally, the photochemical quenching coefficient (q_P) was calculated as $q_P = (F_m' - F_s)/ (F_m' - F_0')$ (Krause and Weis, 1991). To quantitatively estimate the values of the chlorophyll *a* fluorescence imaging parameters, random circular leaf areas of 0.78 cm^2 each were defined on each leaf.

Photosynthetic pigments. Twenty-two foliar discs (8 mm in diameter), corresponding to the leaf areas where leaf gas exchange parameters were determined, were sampled at 168 hafs to determine the chlorophyll *a+b* (CHL *a+b*) and carotenoid (CAR) concentrations. Pigments from the foliar discs were extracted using acetone 85% (v v^{-1}), at 4ºC and quantified as described by Lichthenthaler (1987).

Data analysis. The experiment was repeated once, and the data from all of the variables were subjected to the analysis of variance (ANOVA). Within each sampling time, the means from the -Fu and +Fu treatments (for the non-inoculated or inoculated plants) and the means from the inoculated and non-inoculated plants (for the -Fu and +Fu treatments) were compared by the *t*-test ($P \leq 0.05$) using SAS (v. 6.12; SAS Institute, Inc.). For the ANOVA, the design was considered a 2 × 2 × 5 factorial experiment consisting of two fungicide treatments (-Fu and +Fu), non-inoculated or inoculated plants and five evaluation times (12, 24, 72, 120 and 168 hafs). The Pearson linear correlation technique was used to determine the association among the following variables: A, g_s, C_i, E and BSS. Only the data from inoculated plants at 48, 96 and 144 hai were used to determine these correlations

because these were the only times at which the leaf gas exchange parameters and BSS were determined.

RESULTS

Analysis of variance. The factor fungicide spraying (FS) was significant for all of the epidemiological variables; in addition, the factor evaluation time (ET) and the FS × ET interaction were significant for BSS (Table 1). The factors FS and plant inoculation (PI) were significant for the CHL *a*+*b* concentration and the FS × PI interaction was significant for both CHL *a*+*b* and CAR concentrations. At least one of the factors, as well as some double interactions, was significant for the gas exchange and chlorophyll *a* parameters. The FS × PI × ET interaction was significant for all of the gas exchange and chlorophyll *a* parameters, except for C_i, *E*, *Y*(*NPQ*) and q_P.

Epidemiological variables. As expected, azoxystrobin remarkably reduced BS development (Fig. 1A). The BSS increased from 3% to 23% for the-Fu plants and from 0.6% to 3% for the +Fu plants from 24 to 144 hai (Fig. 1B). The AUBSPC, *r*, NL and FLS were 87, 36, 28 and 82% lower, respectively, in the +Fu than in the -Fu plants (Fig. 2).

Leaf gas exchange parameters. Comparison between -Fu and +Fu plants in the absence of *B. oryzae* inoculation showed that azoxystrobin significantly reduced *A* (9-19%) and A_{max} (13-19%) from 12 to 168 hafs, g_s (21-36%) from 12 to 72 hafs, C_i (6-7%) and LSP (14-17%) from 12 to 24 hafs and *E* (28%) at 12 hafs, indicating that the fungicide spraying impaired rice photosynthesis under unstressed conditions (Figs. 3 and 4). The deleterious effect of *B. oryzae* infection in rice leaf gas exchange was confirmed by significant reductions in *A* (61-80%), g_s (68-78%), *E* (44-64%) and A_{max} (58-80%) from 72 to 168 hafs and LSP (31-69%) from 120 to 168 hafs observed in inoculated plants from the -Fu treatment compared to non-inoculated plants. By contrast, C_i was 5% higher at 168 hafs and LCP was 184, 195 and 292% higher at 72, 120 and 168 hafs, respectively, in inoculated than in non-inoculated plants. Although a similar trend was recorded among the +Fu plants, the *B. oryzae*-induced physiological constraints were greatly limited by azoxystrobin spraying since lower (34-38% for *A*, 43-47% for g_s, 28-30% for *E* and 33% for A_{max}) and later (120 hafs onwards) decreases were recorded relative to their non-sprayed counterparts. C_i was significantly decreased by 5% at 168 hafs and LSP by 24% at 72 hafs in inoculated plants compared to non-inoculated plants (Figs. 3 and 4).

Chlorophyll *a* fluorescence parameters. Images of chlorophyll *a* fluorescence did not show great differences in the color pattern for any parameter between the -Fu and +Fu

treatments for non-inoculated plants, indicating that biochemical constraints were not a prominent factor to explain decreases in *A* observed for the +Fu plants (Fig. 5a1-2, b1-2, c1-2 and d1-2). In a sharp contrast, a progressive loss of photosynthetic capacity was evident for the -Fu plants inoculated with *B. oryzae*, as indicated by the black areas in the F_v/F_m, *Y*(*II*) and q_P images (Fig. 5a3, a5, a7; b3, b5, b7; and c3, c5, c7). Interestingly, changes in *Y*(*II*) and q_P were more evident than those in F_v/F_m, indicating that the former parameters are more useful to assess the photosynthetic constraints posed by *B. oryzae* infection. The loss of photosynthetic capacity induced by *B. oryzae* infection was coupled to increased *Y*(*NPQ*) in order to alleviate the energy excess in the -Fu plants caused by a reduced CO_2 assimilation (Fig. 5d3, d5 and d7). Although changes in chlorophyll *a* images were also noticeable for the +Fu plants, such alterations were greatly limited when compared to the -Fu plants (Fig. 5a4, a6, a8; b4, b6, b8; c4, c6, c8; and d4, d6, d8).

There were no significant differences among the -Fu and +Fu plants in the absence of *B. oryzae* inoculation for the chlorophyll *a* fluorescence parameters regardless of the evaluation time (Fig. 6). Regardless of the fungicide treatment, the inoculated plants showed significant reductions in F_v/F_m (4-5%), *Y*(*II*) (13-46%) and q_P (10-25%) and increases in *Y*(*NPQ*) (17-41%) from 72 to 168 hafs compared to non-inoculated plants. However, significantly higher values of F_v/F_m (2-4%), *Y*(*II*) (22-25%) and q_P (12-21%) and lower values of *Y*(*NPQ*) (15-20%) were verified in +Fu plants relative to their -Fu counterparts, confirming that azoxystrobin spraying constrained *B. oryzae* infection and colonization, which explained the lesser damage to the photosynthetic apparatus observed in the +Fu plants.

Photosynthetic pigments. There were no significant differences for the concentration of photosynthetic pigments between -Fu and +Fu treatments for non-inoculated plants (Fig. 7). The *B. oryzae* infection significantly reduced the concentrations of CHL *a*+*b* (52%) and CAR (19%) for -Fu plants, but +Fu plants displayed significantly higher concentrations (80 and 20% for CHL *a*+*b* and CAR, respectively) than did -Fu plants, indicating that azoxystrobin spraying, by inhibiting the fungal growth, reduced the degradation of photosynthetic pigments.

Pearson correlation analysis. For the -Fu plants, *A* was positively correlated with g_s and *E* and negatively correlated with C_i (Table 2). Positive correlations occurred between *E* and g_s as well as between C_i and BSS. For the +Fu plants, *A* was positively correlated with g_s, C_i and *E* and negatively correlated with BSS. The g_s was positively correlated with C_i and *E*.

DISCUSSION

In addition to the fungicide effect, strobilurins can improve plant physiology, thereby increasing the crop yield in the absence of diseases through increased nitrate reductase (Köehle et al., 2003; Fagan et al., 2010) and antioxidant enzymes activities (Wu and Tiedemann, 2001; Köehle et al., 2003; Zhang et al., 2010), reduced ethylene synthesis (Grossmann and Retzlaff, 1997) and increased abscisic acid concentration (Grossmann et al., 1999; Köehle et al., 2003). However, there is a paucity of studies examining the photosynthetic performance of strobilurin-treated plants. This study, to the best of author's knowledge, is the first to assess the effect of azoxystrobin, the most important strobilurin fungicide that is recommended for disease control in rice, on the leaf gas exchange and chlorophyll *a* fluorescence parameters and on the concentration of photosynthetic pigments in the leaves of rice plants that were either challenged or not with *B. oryzae*.

In contrast to the working hypothesis of this study, the rice plants that were sprayed with azoxystrobin exhibited lower A, g_s, C_i and E values than did the non-sprayed plants as early as 12 hafs. Concomitant decreases were observed for A_{max} and LSP. Initially (12 and 24 hafs), reductions in A due to fungicide spraying were related to stomatal closure (lower g_s values), which limited the CO_2 influx into foliar mesophyll as evidenced by the decreased C_i. Then (120 hafs onwards), the stomata from the azoxystrobin-sprayed plants recovered their openness to comparable levels to the non-sprayed plants. Nevertheless, the +Fu plants exhibited a lower A than their -Fu counterparts until the end of the experiment. Because there were no differences at either the photochemical level or in the concentration of photosynthetic pigments for both -Fu and +Fu non-inoculated plants regardless of the evaluation time, it can be concluded that the reduction in A caused by azoxystrobin is governed chiefly by diffusive constraints, mainly at the stomatal level. Additional support for this hypothesis is provided by unchanged C_i for both -Fu and +Fu plants from 72 hafs onwards; if biochemical limitations were the case, a lower CO_2 assimilation and consequently increased C_i would be expected (Dallagnol et al., 2012, 2013; Debona et al. 2014). Regardless, further studies to evaluate the mesophyll conductance of the azoxystrobin-sprayed plants will be important to examine whether the decreases in A observed from 120 to 168 hafs uncoupled to decreased g_s and C_i could also be a consequence of diffusive limitations at the mesophyll level. Similar to the findings of this study, reductions in the A observed for barley, soybean and wheat plants that were sprayed with picoxystrobin or pyraclostrobin (two strobilurins) were associated with decreases in g_s

and C_i Nason et al., 2007). Nason et al. (2007) postulated that stomata could respond to changes in mesophyll photosynthesis either by sensing alterations in C_i or in the *pool* size of a yet unidentified C-fixing substrate. Alternatively, strobilurins could cause stomatal closure by increasing abscisic acid concentrations concentrations (Grossmann et al., 1999; Köehle et al., 2003). Nonetheless, another simple hypothesis hafs been invoked: because ATP production in guard cells limits the osmotic gradient across guard cell membranes, the strobilurin-inhibited *bc1* cytochrome may have reduced the cell guard metabolism, thereby causing a loss of turgor (Nason et al., 2007).

The greater disease symptoms on the leaves of the -Fu plants resulted in sharp reductions in *A*, g_s and *E* already at 48 hai. In contrast, the +Fu plants exhibited reduced disease symptoms as evidenced by lower values of BSS (89%), AUBSPC (87%), *r* (90%), NL (28%) and FLS (82%) which, in turn, resulted in lower and later (72 hai onwards) leaf gas exchange impairments. The results of this study are consistent with those reported by Dallagnol *et al.* (2012, 2013), who found severe limitations in *A*, g_s and *E* on *B. oryzae*-infected rice leaves. These authors suggest that the massive fungal colonization on the rice leaves negatively impacted biochemical processes that are related to CO_2 fixation and decreased their capacity to use solar energy for photosynthesis. In another study, Debona et al. (2014) showed that the *Pyricularia oryzae* infection in wheat leaves resulted in decreased *A* and g_s but increased C_i. They also analyzed the *A* response by varying C_i and concluded that the Rubisco carboxylation capacity dramatically decreased during later stages of fungal infection. Accordingly, in this study, the -Fu plants exhibited decreases in *A* and g_s but increases in C_i during later stages of *B. oryzae* infection (144 hai) relative to the non-inoculated plants, suggesting that photosynthetic impairments resulted primarily from biochemical constraints. The negative correlation between *A* and C_i and the positive correlation between C_i and BSS corroborate that assumption. For the +Fu plants, a simultaneous reduction in *A*, g_s and C_i was observed in response to *B. oryzae* infection, while positive correlations were reported among these parameters, suggesting that diffusive limitations would play a major role in photosynthesis impairments. However, this hypothesis does not appear to be true because reductions in *A* (38%) and g_s (47%) greatly surpassed those that were obtained for C_i (only 5%). Therefore, for both -Fu and +Fu plants, biochemical constraints play a pivotal role in the photosynthetic impairments that are caused by *B. oryzae* infection, which were greatly limited for the +Fu plants.

For several foliar diseases, photosynthesis is reduced from the beginning of the pathogen infection, and many of these diseases impair photosynthesis in asymptomatic,

although colonized, tissues, i.e., the so-called virtual lesion (Bastiaans, 1991; Berger et al., 2007; Domiciano et al., 2009; Debona et al., 2014). However, other diseases showed that photosynthesis is only impaired in symptomatic tissues (Alves et al., 2011). In this study, the photosynthesis decreases in both -Fu and +Fu inoculated plants greatly exceeded those expected based on the BSS. At 144 hai, for example, while the BSS reached 23% for the -Fu plants and 3% for the +Fu ones, the reductions in *A* achieved 80 and 38%, respectively. The correlation analysis confirmed this assumption because there was no significant correlation between *A* and BSS for the -Fu plants, and a low, although significant, coefficient was obtained between these parameters for the +Fu plants. Decreases in the *A* values for the non-colonized leaf areas due to *B. oryzae* may result from the non-host-selective toxins ophiobolins A and B, which are secreted by the fungus and diffuse themselves in surrounding areas of foliar tissue (Xiao et al., 1991), leading to an increase in lipid peroxidation and malondialdehyde (MDA) concentration and thereby decreasing the chlorophyll content and photosynthetic activity (Munne-Bosch and Alegre, 2002) and corroborating the decrease in pigment content in the -Fu plants in this study. Therefore, the data from this study suggest that the extent of the diseased leaf area is not a proper indicator of the potential impact of *B. oryzae* infection in *A*.

The light curve analysis revealed that upon *B. oryzae* infection, the *A* for the -Fu plants was less respondent to the increases in *PPFD* than for the +Fu plants, which most likely was a consequence of the intensive chlorophyll degradation that was observed for the former plants. Concurrent decreases in A_{max} and LSP in addition to an increase in LCP were recorded for the -Fu plants in response to *B. oryzae* infection. Furthermore, the lower slope of the line that was observed for the -Fu plants indicates that *A* was limited by the lower electron transport rate and RuBP regeneration. These observations are consistent with previous findings for the sweet orange-*Xylella fastidiosa* interaction, in which a lower LCP and slope of the line were verified in the bacteria-infected plants compared to the healthy plants (Haberman et al., 2003).

Given that carbon fixation, which typically represents the main sink for absorbed light in chloroplasts, dramatically decreased in the -Fu inoculated plants, the energy surplus must be dissipated through alternative ways (e.g., thermally), otherwise oxidative damage could occur (Krause and Weis, 1991; Lima et al., 2002). The fraction of absorbed light that is thermally dissipated can be analyzed by *Y*(*NPQ*) (Lima et al., 2002). Increases in *Y*(*NPQ*) occurred as early as 48 hai in both the -Fu and +Fu plants but to a lesser extent for the latter, which could contribute to a reduction in energy surplus regardless of the

fungicide treatment. However, the increased $Y(NPQ)$ was not sufficient to alleviate the energy excess in the-Fu plants, and a chronic photoinhibition to photosynthesis, analyzed by F_v/F_m, was observed from 48 hai for those plants. In contrast, for the +Fu plants, the thermal dissipation was efficient to mitigate the damage to the photosynthetic apparatus until 72 hai. However, at 144 hai, a chronic photoinhibition of photosynthesis occurred but to a lesser degree relative to the -Fu plants. Concurrent decreases in $Y(II)$ and q_P corroborated that the -Fu plants, and to a lesser extent the +Fu plants, were prematurely (from 48 hai) affected in their capacity to fully capture and exploit the collected energy. Similar photochemical dysfunctions were found for the wheat-*P. oryzae* interaction in which an increase in blast severity was related to lowered F_v/F_m, $Y(II)$ and q_P values and increased $Y(NPQ)$ values (Aucique-Perez et al., 2014). These impairments resulted from reductions in the chlorophyll concentration (Aucique-Perez et al., 2014), which agrees with what was observed for the -Fu plants in this study. Similar to the findings of this study, Dallagnol *et al.* (2012) reported that *B. oryzae* infection decreased the chlorophyll concentration, whereas the carotenoid concentration decreased less. According to these authors, adjustments in the pigment concentration and composition could result in an improved capacity for photoprotection, which assumes increased importance when A decreases. Nonetheless, it seems more likely that changes in pigment are largely associated with the action of non-host-selective toxins that are produced by *B. oryzae* (Dallagnol et al., 2012), which was particularly evident for the -Fu plants in this study. Therefore, the results of this study provide evidence that insufficient adjustments in the capture, use and dissipation of light play a pivotal role in explaining the damage to the photosynthetic apparatus that was observed in the -Fu plants because of *B. oryzae* infection. In contrast, the +Fu plants, which showed reduced disease symptoms, were more able to address the energy surplus that is caused by the reduction in CO_2 fixation, consequently leading to lower and later photoinhibition to photosynthesis and lower decreases in the capacity to capture and exploit the absorbed light energy.

In conclusion, the results of this study showed that azoxystrobin decreased rice photosynthesis mainly by reducing the CO_2 influx at the stomata level with no apparent effect in the photosynthetic photochemistry and biochemistry. The *B. oryzae* infection dramatically decreased photosynthesis, which was governed chiefly by biochemical constraints. The decreases in photosynthesis greatly exceed the diseased leaf area, suggesting that disease severity is not a proper indicator to examine the photosynthetic damage that is caused by *B. oryzae* infection. In addition, azoxystrobin was able to

preserve, to a greater extent, the ability of rice leaves to capture, use and dissipate the light energy during the infection process of *B. oryzae*.

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TABLES AND FIGURES

Table 1. Analysis of variance of the effects of fungicide spraying (FS), plant inoculation (PI), evaluation time (ET), and their interactions in brown spot severity (BSS), the area under brown spot progress curve (AUBSPC), the progress rate (*r*), the number of lesions (NL) per cm^2, the final lesion size (FLS), the net CO_2 assimilation rate (*A*), the stomatal conductance to water vapor (g_s), the internal CO_2 concentration (C_i), the transpiration rate (*E*), the maximal net carbon assimilation rate (A_{max}), the light compensation point (LCP), the light saturation point (LSP), the variable-to-maximum chlorophyll *a* fluorescence ratio (F_v/F_m), the photochemical yield [*Y*(*II*)], the quenching coefficient (q_P), the yield for dissipation by down-regulation [*Y*(*NPQ*)], and the concentrations of chlorophyll *a*+*b* (CHL *a*+*b*) and carotenoids (CAR).

Variables[a]	FS	PI	ET	FS × PI	FS × ET	PI × ET	FS × PI × ET
BSS	**	-	**	-	**	-	-
AUBSPC	**	-	-	-	-	-	-
r	**	-	-	-	-	-	-
NL	**	-	-	-	-	-	-
FLS	**	-	-	-	-	-	-
A	ns	**	**	**	**	**	**
g_s	**	**	**	**	**	**	**
C_i	**	ns	**	**	**	ns	ns
E	ns	**	**	**	**	**	ns
A_{max}	ns	**	**	**	**	**	**
LCP	**	**	**	**	**	**	**
LSP	**	ns	**	ns	**	ns	**
F_v/F_m	**	**	**	**	ns	**	*
Y(*II*)	**	**	**	**	**	**	*
q_P	**	**	**	*	ns	**	ns
Y(*NPQ*)	**	**	**	**	**	**	ns
CHL *a*+*b*	*	**	-	**	-	-	-
CAR	ns	ns	-	*	-	-	-

[a]Levels of probability: [ns] = nonsignificant, [*] = 0.05 and [**] = 0.01.

Table 2. Pearson correlation coefficients among the leaf gas exchange variables net carbon assimilation rate (*A*), stomatal conductance to water vapor (g_s), internal CO_2 concentration (C_i), transpiration rate (*E*), and brown spot severity (BSS) in the leaves of plants that were not sprayed (above diagonal) or sprayed (below diagonal) plants with the fungicide azoxystrobin and inoculated with *Bipolaris oryzae*.

Variables[a]	*A*	g_s	C_i	*E*	BSS
A	-	**0.93**	**-0.63**	**0.78**	-0.47
g_s	**0.97**	-	-0.31	**0.92**	-0.19
C_i	**0.68**	**0.83**	-	-0.18	**0.70**
E	**0.64**	**0.65**	0.49	-	0.12
BSS	**-0.52**	-0.49	-0.29	-0.02	-

[a]Values in bold are significant ($P \leq 0.05$) based on the *t*-test.

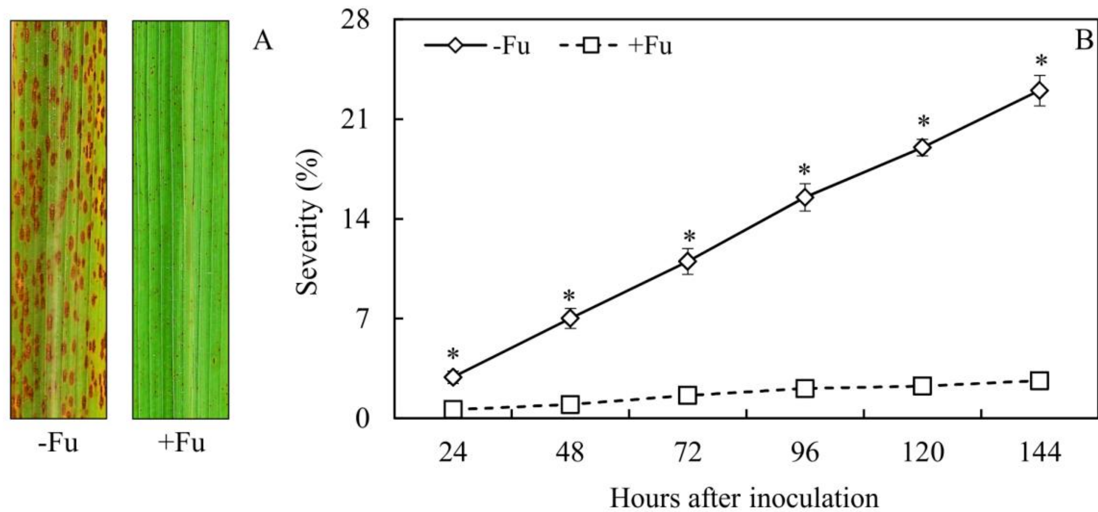

Figure 1. Brown spot (BS) symptoms 144 hours after inoculation (A) and the progress of BS (B) determined on the leaves of plants that were not sprayed (-Fu) or sprayed (+Fu) with the fungicide azoxystrobin and inoculated with *Bipolaris oryzae*. The means from the -Fu and +Fu treatments within each evaluation time that are followed by an asterisk (*) in (B) are significantly different ($P \leq 0.05$) by *t*-test. The bars represent the standard error of the means ($n = 4$).

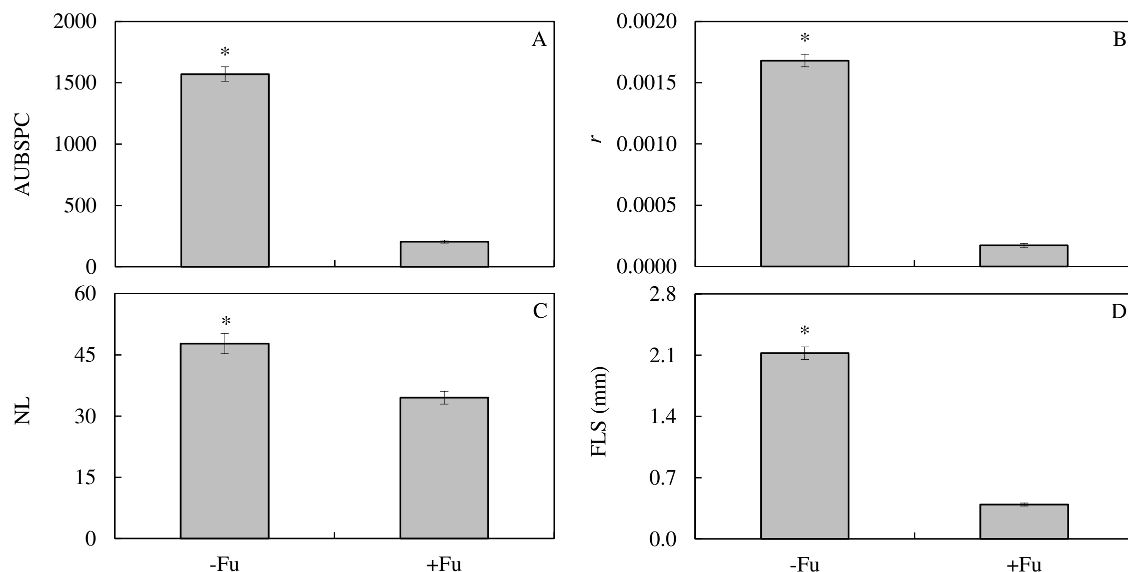

Figure 2. Area under brown spot progress curve (AUBSPC) (A), progress rate (*r*) (B), number of lesions (NL) per cm^2 (c), and final lesion size (FLS) (d) determined on the leaves of plants that were not sprayed (-Fu) or sprayed (+Fu) with the fungicide azoxystrobin and inoculated with *Bipolaris oryzae*. Means from the -Fu and +Fu treatments within each evaluation time that are followed by an asterisk (*) are significantly different ($P \leq 0.05$) by *t*-test. The bars represent the standard error of the means ($n = 4$).

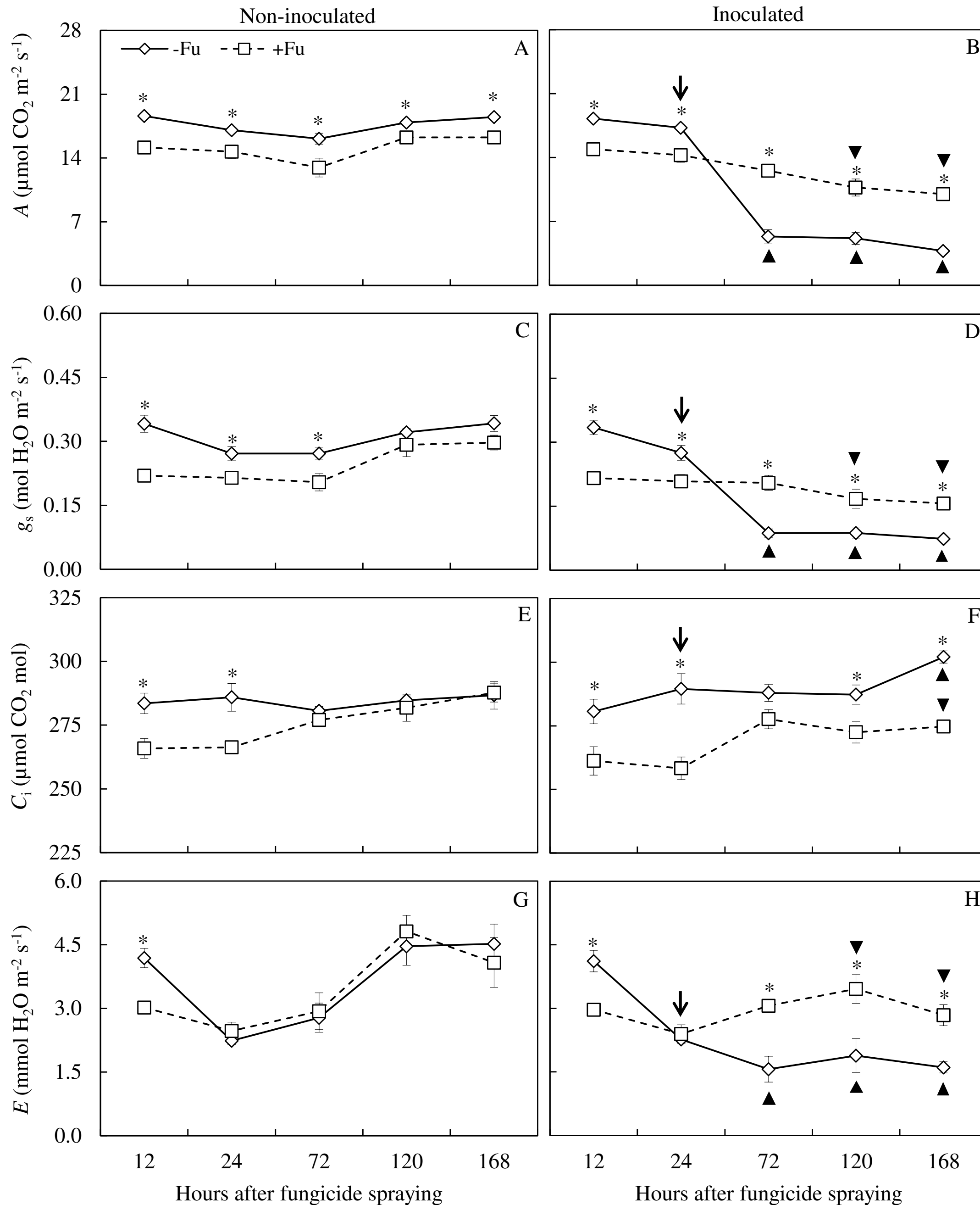

Figure 3. Leaf gas exchange parameters net carbon assimilation rate (*A*) (A and B), stomatal conductance to water vapor (g_s) (C and D), internal CO_2 concentration (C_i) (E and F), and transpiration rate (*E*) (G and H) determined on the leaves of plants that were not sprayed (-Fu) or sprayed (+Fu) with the fungicide azoxystrobin and not inoculated (A, C, E, and G) or inoculated (B, D, F, and H) with *Bipolaris oryzae*. Means from the -Fu and +Fu treatments within each evaluation time that are followed by an asterisk (*) are significantly different ($P \leq 0.05$) by *t*-test. Means from the non-inoculated and inoculated treatments within each evaluation time that are followed by the symbols ▲ or ▼ for the -Fu and +Fu treatments, respectively, are significantly different ($P \leq 0.05$) by *t*-test. Arrows indicate the time of *B. oryzae* inoculation. The bars represent the standard error of the means ($n = 4$).

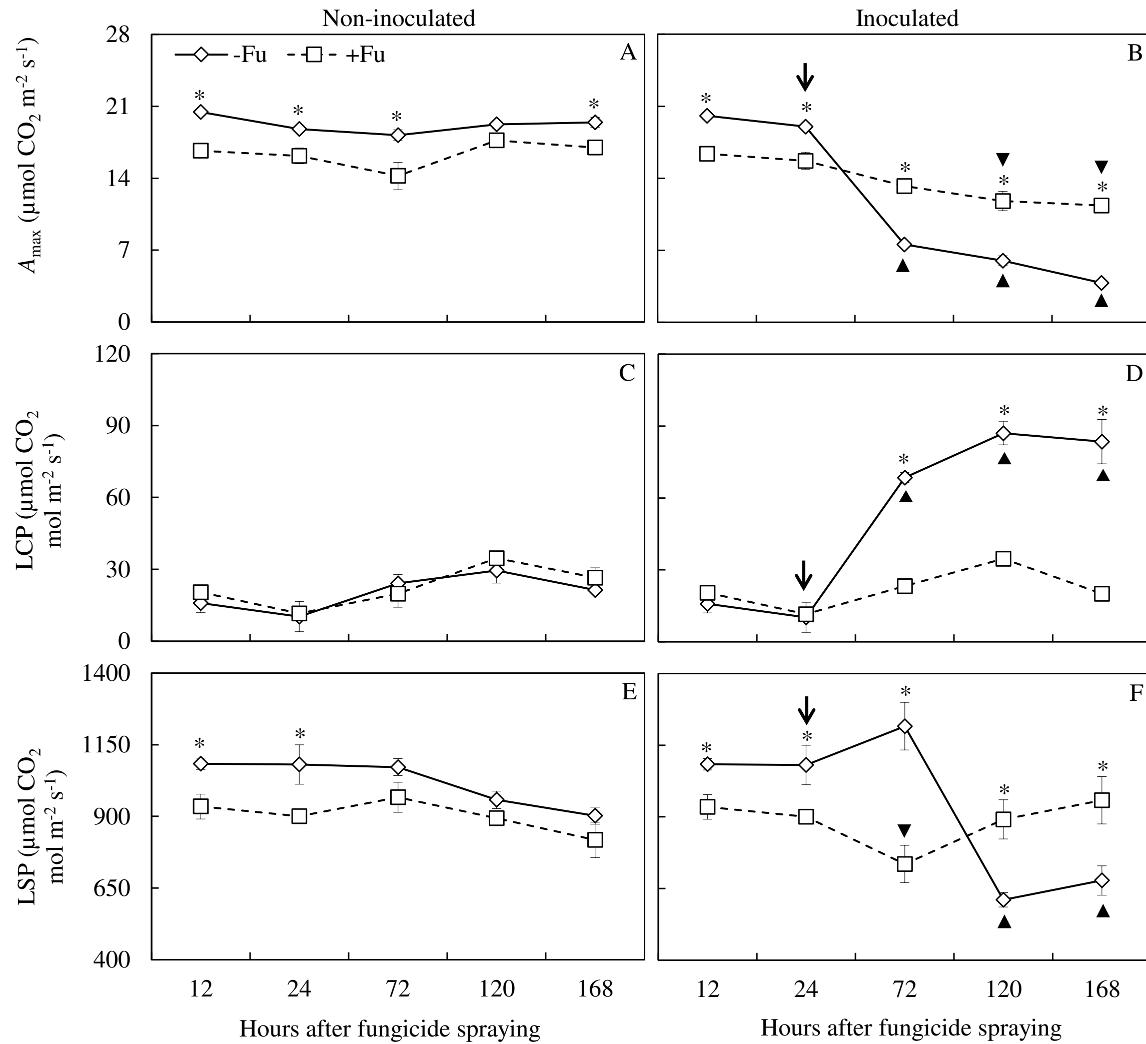

Figure 4. Maximal net carbon assimilation rate (A_{max}) (A and B), light compensation point (LCP) (C and D), and light saturation point (LSP) (E and F) determined on the leaves of plants that were not sprayed (-Fu) or sprayed (+Fu) with the fungicide azoxystrobin and not inoculated (A, C and E) or inoculated (B, D and F) with *Bipolaris oryzae*. Means from the -Fu and +Fu treatments within each evaluation time that are followed by an asterisk (*) are significantly different ($P \leq 0.05$) by *t*-test. Means from the non-inoculated and inoculated treatments within each evaluation time that are followed by the symbols ▲ or ▼ for the -Fu and +Fu treatments, respectively, are significantly different ($P \leq 0.05$) by *t*-test. Arrows indicate the time of *B. oryzae* inoculation. The bars represent the standard error of the means ($n = 4$).

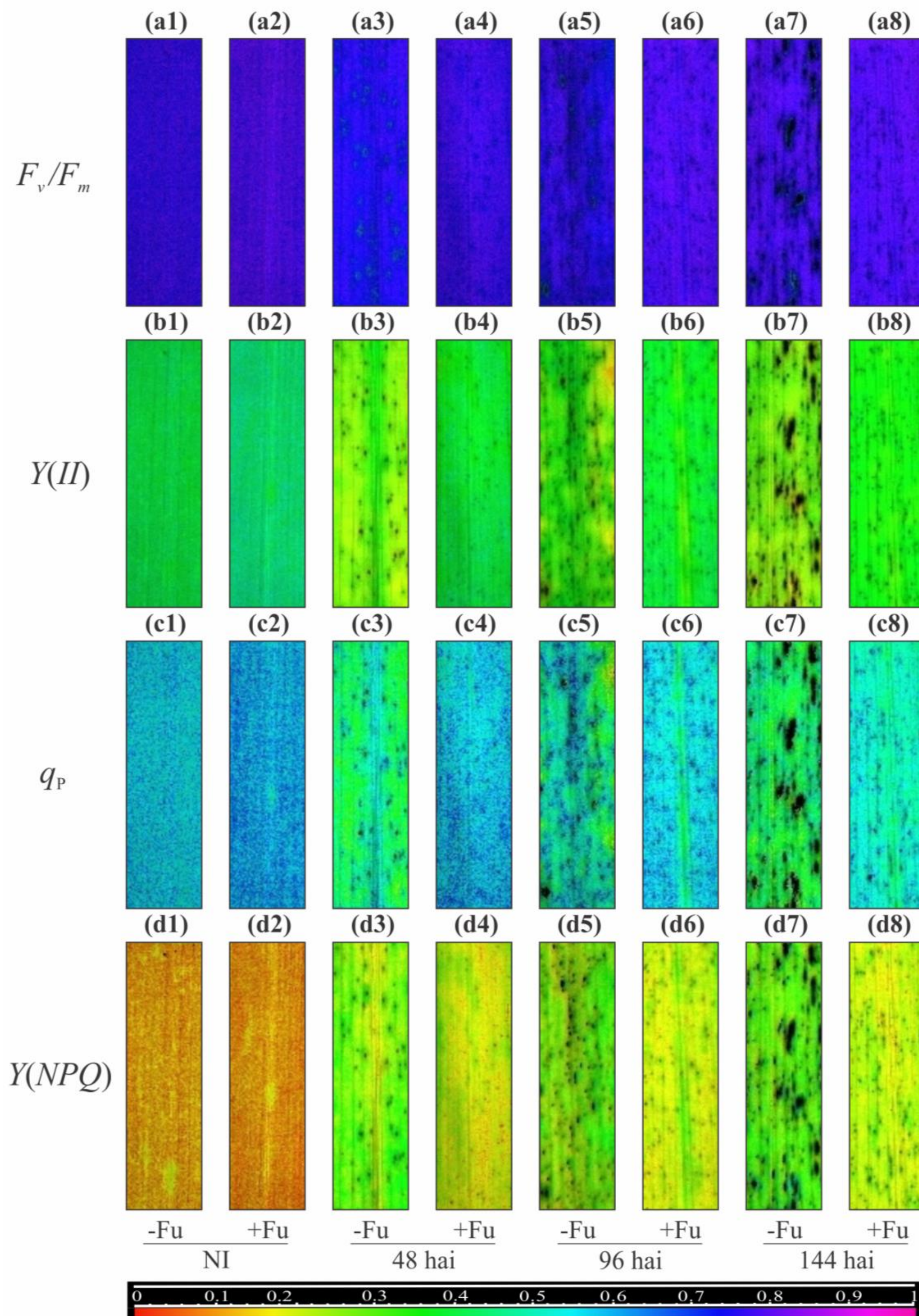

Figure 5. Images of chlorophyll *a* fluorescence of the variable-to-maximum chlorophyll *a* fluorescence ratio (F_v/F_m) (a1-a8), photochemical yield [$Y(II)$] (b1-b8) quenching coefficient (q_P) (c1-c8) and yield for dissipation by down-regulation [$Y(NPQ)$] (d1-d8) determined on the leaves of plants that were not sprayed (-Fu) (a1, a3, a5, a7, b1, b3, b5, b7, c1, c3, c5, c7, d1, d3, d5 and d7) or sprayed (+Fu) (a2, a4, a6, a8, b2, b4, b6, b8, c2, c4, c6, c8, d2, d4, d6 and d8) with the fungicide azoxystrobin and not inoculated (NI) or inoculated with *Bipolaris oryzae* 48, 96 and 144 hours after inoculation (hai).

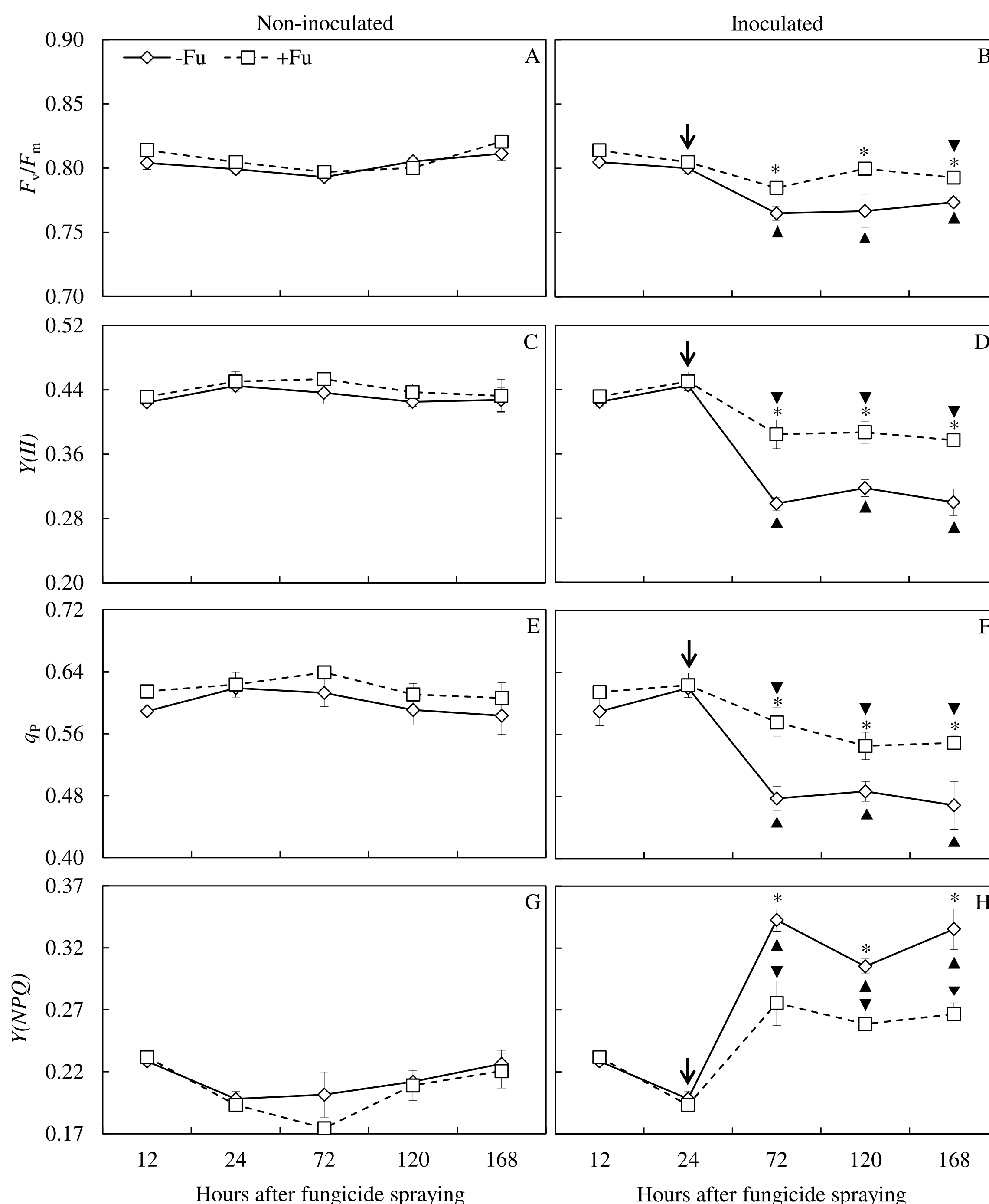

Figure 6. Chlorophyll *a* parameters variable-to-maximum chlorophyll *a* fluorescence ratio (F_v/F_m) (A and B), photochemical yield [$Y(II)$] (C and D), quenching coefficient (q_P) (E and F) and yield for dissipation by down-regulation [$Y(NPQ)$] (G and H), and determined on the leaves of plants that were not sprayed (-Fu) or sprayed (+Fu) with the fungicide azoxystrobin and not inoculated (A, C, E, and G) or inoculated (B, D, F, and H) with *Bipolaris oryzae*. Means from the -Fu and +Fu treatments within each evaluation time that are followed by an asterisk (*) are significantly different ($P \leq 0.05$) by *t*-test. Means from the non-inoculated and inoculated treatments within each evaluation time that are followed by the symbols ▲ or ▼ for the -Fu and the +Fu treatments, respectively, are significantly different ($P \leq 005$) by *t*-test. Arrows indicate the time of *B. oryzae* inoculation. The bars represent the standard error of the means ($n = 4$).

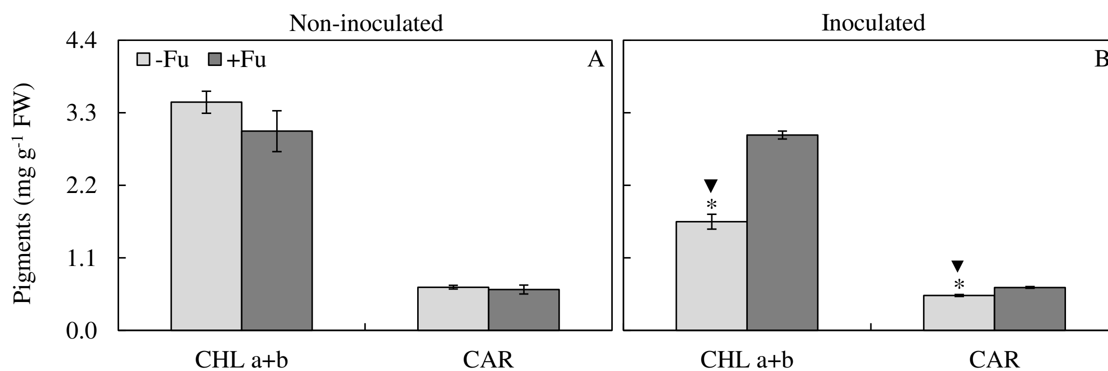

Figure 7. Concentrations of the photosynthetic pigments chlorophyll *a+b* (CHL *a+b*) and carotenoids (CAR) determined on the leaves of plants that were not sprayed (-Fu) or sprayed (+Fu) with the fungicide azoxystrobin and not inoculated (A) or inoculated (B) with *Bipolaris oryzae*. Means from the -Fu and +Fu treatments within each evaluation time that are followed by an asterisk (*) are significantly different ($P \leq 0.05$) by *t*-test. Means from the non-inoculated and inoculated treatments within each evaluation time that are followed by the symbol ▼ are significantly different ($P \leq 005$) by *t*-test. The bars represent the standard error of the means ($n = 4$). FW = fresh weight.

Chapter 2

A strobilurin fungicide relieves *Bipolaris oryzae*-induced oxidative stress in rice

ABSTRACT

Although strobilurins are one of the most effective and broad spectrum classes of systemic fungicides, they may also increase plant stress tolerance by modulating the activity of antioxidant enzymes. To address this issue, activities of antioxidant enzymes and concentrations of antioxidant metabolites and oxidative stress-related compounds was investigated in rice plants (cv. Metica-1) that were non-sprayed or sprayed with azoxystrobin (Az) (-Fu and +Fu plants, respectively) and either inoculated or not with *Bipolaris oryzae*, the causal agent of brown spot. The +Fu non-inoculated plants did not show alterations in enzyme activities, but displayed higher glutathione reduced (GSH) concentrations than -Fu plants. The activities of superoxide dismutase, peroxidase, ascorbate peroxidase, glutathione peroxidase, glutathione reductase, and glutathione-*S*-transferase were increased upon *B. oryzae* infection, but such increases were lower in the +Fu plants. Catalase activity decreased in the inoculated plants compared to the non-inoculated plants regardless of fungicide treatment. The GSH concentration increased in response to the *B. oryzae* infection, and the +Fu plants sustained higher levels of GSH at advanced stages of fungal infection than did the -Fu plants. The inoculated plants exhibited an extensive oxidative stress as evidenced by higher concentrations of hydrogen peroxide and malondialdehyde compared to the non-inoculated plants, but lower and later increases were recorded in the +Fu plants than in the -Fu plants. Therefore, Az greatly reduces *B. oryzae*-induced oxidative stress by limiting brown spot development rather than by activating antioxidant enzymes. The GSH, however, was Az-modulated, and this may partially explain the constrained oxidative stress observed in the +Fu plants.

Keywords: *Oryza sativa*, antioxidant metabolism, azoxystrobin, brown spot, reactive oxygen species.

INTRODUCTION

Brown spot (BS), caused by *Bipolaris oryzae* (Breda de Haan) Shoemaker [teleomorph: *Cochliobolus miyabeanus* (Ito and Kuribayashi) Drechs. ex Dastur.], is one of the most constraining factors to worldwide rice production, reducing, on average, 10% of the attainable yield and negatively affecting grain quality (Barnwal et al., 2013). BS development is favored by high temperatures (25-30°C) and high rainfall or dew, but abiotic stresses such as drought and nutrient deficiency are also associated with BS outbreaks (Ou, 1985; Barnwal et al., 2013). Typical BS symptoms on leaf blades appear approximately 18 hours after inoculation and are characterized by light reddish-brown or gray-centered lesions surrounded by a dark reddish-brown margin with a bright yellow halo (Ou, 1985; Dallagnol et al., 2011).

An increase in the concentration of reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl (OH^-) is a remarkable feature of plants infected by pathogens (Magbanua et al., 2007; Debona et al., 2012). Although the ROS accumulation can initially contribute to plant disease resistance because of their importance as antimicrobial, cell wall strengthening, and signaling molecules (Hammond-Kosack and Kones, 1996), the imbalance between production and removal of ROS can result in oxidative damage (Debona et al., 2012; Fortunato et al., 2015; Magbanua et al., 2007). Necrotrophic pathogens are known to cause extensive oxidative stress by producing or inducing plants to produce ROS to kill the host cells for nutrient withdrawal and preventing the induction of defense responses (Mengiste, 2012). Germinating conidia of *B. oryzae*, for example, produce the non-host selective toxins ophiobolins A and B (Xiao et al., 1991), which in addition to suppressing rice defenses (Vidhyasekaran et al., 1992) are thought to lead to increased concentrations of malondialdehyde (MDA), considered to be a cellular damage indicator metabolite, and degradation of photosynthetic pigments in rice leaves (Dallagnol et al., 2011).

To cope with pathogen-induced ROS generation, plants possess an enzymatic and non-enzymatic antioxidant system that has been shown to play a key role in plant disease resistance. For instance, Debona et al. (2012) found that although *Pyricularia oryzae* infection increased ROS production in a resistant cultivar of wheat leaves by showing higher activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione-*S*-transferase (GST), and glutathione reductase (GR), the plants displayed less oxidative stress than a susceptible

counterpart. Similarly, in the soybean-*Corynespora cassiicola* interaction, the higher activities of some antioxidant enzymes and concentrations of ascorbate (AsA), a key antioxidant compound, were associated with soybean resistance to target spot and limited fungal-induced oxidative stress (Fortunato et al., 2015). The lowered severity of anthracnose and cellular damage observed in silicon-supplied sorghum plants also were related to increased activities of SOD, CAT, APX and GR (Resende et al., 2012).

The unavailability of resistant cultivars of commercial rice to growers makes BS control difficult; hence, in addition to a balanced mineral nutrition, BS has mostly been controlled through fungicide sprays (Ou, 1985; Barnwal et al., 2013). Strobilurins are one of the most important classes of fungicides used currently because of their broad-spectrum (meso)systemicity and high efficiency of control (Ypema and Gold, 1999; Bartlett et al., 2002). Strobilurins block the *bc1* cytochrome complex III of the electron transport chain of mitochondria and inhibit energy production by fungal cells (Ypema and Gold, 1999; Bartlett et al., 2002). Soon after their launch, however, it was recognized that strobilurins, in addition to their fungicidal effect, could increase plant stress tolerance, presumably through an increased activity of antioxidant enzymes (Wu and Von-Tiedemann, 2001). Accordingly, azoxystrobin-treated wheat plants showed higher activities of SOD, POX and CAT, which were coupled to decreased O_2^- and MDA concentrations, chlorophyll degradation and leaf senescence compared to non-sprayed plants (Wu and Von-Tiedemann, 2001; Zhang et al., 2010).

Despite the reports that azoxystrobin can increase plant antioxidant capacity, the activities of only a few enzymes have been studied so far, and they have not been investigated under biotic stress. In addition, the exact changes *B. oryzae* infection induces in the oxidative status of rice plants remain largely unknown. Therefore, two hypotheses were formulated and tested in the current study: i) *B. oryzae* infection can increase oxidative stress in rice, thereby requiring an activation of antioxidant machinery, and ii) azoxystrobin, in addition to its fungicidal effect, can potentiate an increase in the activities of antioxidant enzymes and increase the concentration of antioxidant compounds, thereby alleviating *B. oryzae*-induced oxidative stress. To address these issues, the activities of an array of antioxidant enzymes, the concentrations of two key antioxidant compounds [glutathione reduced (GSH) and AsA] and the concentrations of oxidative stress-related compounds (H_2O_2 and MDA) were examined to determine the effects of azoxystrobin and *B. oryzae* infection on the oxidative status of rice plants.

MATERIAL AND METHODS

Plant growth and fungicide spraying. Ten rice seeds (cv. Metica-1), previously surface sterilized in NaOCl (10 %, v v^{-1}) for 2 min and rinsed in sterilized water for 3 min, were sown in plastic pots (20-cm-diameter) (Ecovaso, Jaguariúna, São Paulo, Brazil) filled with 2 kg of substrate consisting of a mixture of pine bark, peat, and expanded vermiculite (1:1:1 ratio, Tropstrato®, Vida Verde, Mogi Mirim, São Paulo, Brazil). Each pot was thinned to four plants five days after seedling emergence. A nutrient solution prepared with deionized water and containing 1.04 mM$Ca(NO_3)_2.4H_2O$, 1 mM NH_4NO_3, 0.8 mM KNO_3, 0.069 mM KH_2PO_4, 0.931 mMKCl and 0.6 mM $MgSO_4.7H_2O$, 19 µM H_3BO_3, 2 µM $ZnSO_4.7H_2O$, 0.5 µM $CuSO_4.5H_2O$, 7 µM $MnCl_2.4H_2O$, 0.6 µM $Na_2MoO_4.4H_2O$, 90 µM $FeSO_4.7H_2O$, and 90 µM EDTA bisodic was applied weekly (Clark 1975). In addition, the plants were watered with deionized water as needed. The fungicide treatments consisted of spraying or not spraying the plants with azoxystrobin. The fungicide was sprayed 24 hours before *B. oryzae* inoculation on rice plants at the V_8 growth stage (Counce et al. 2000). Azoxystrobin (Priori®, Syngenta, Basel, Switzerland, 2 ml c. p. L^{-1}) was prepared on the morning of spraying using deionized water + mineral oil (Nimbus®, Syngenta, Basel, Switzerland, 0.5 % v v^{-1}). The control plants were sprayed with deionized water + mineral oil. The fungicide treatments were sprayed using a CO_2 pressurized backpack sprayer equipped with a flat fan nozzle (XR 110 02®, Teejet, Glendale Heights, IL, USA) working at a 200,000 Pa pressure to deliver a spray volume of 200 L ha^{-1}.

Inoculation procedure. The inoculation procedure followed that described by Dallagnol et al. (2011). Twenty milliliters of a conidial suspension of *B. oryzae* (5×10^3 conidia mL^{-1}) was applied as a fine mist to the leaves of each plant at the V_8 growth stage using a VL Airbrush atomizer (Paasche Airbrush Co., Chicago, IL, USA). Gelatin (1%, w v^{-1}) was added to the sterile water to aid conidial adhesion to the leaves. Then, the plants were kept in the dark in a growth chamber (25 ± 2°C temperature and 90 ± 5 % relative humidity) during the first 24 hours after inoculation. Subsequently, the plants were transferred to a wood-made mist growth chamber (MGC, 2 m wide, 1.5 m high, and 5 m long, covered with 100-µm-thick transparent plastic), which was built inside a greenhouse, where they remained until the termination of the experiment. The relative humidity and temperature were measured with a thermohygrograph (TH-508, Impac, São Paulo, Brazil). The temperature inside the MGC ranged from 25 ± 2°C (day) to 20 ± 2°C (night), and a misting system in which nozzles (model NEB-100; KGF Company, São Paulo, Brazil) sprayed

mist every 30 min above the plant canopy was used to keep a relative humidity of 92 ± 3 %. The maximum natural photon flux density at plant canopy height was approximately 900 μmol m^{-2} s^{-1}. The non-inoculated plants were kept in separate chambers, but exposed to the same conditions as the inoculated plants during the experiments.

Experimental design. The experiment was arranged in a 2 × 2 factorial, consisting of two fungicide treatments (plants sprayed and non-sprayed with azoxystrobin, hereafter referred to as +Fu or -Fu plants, respectively) and two inoculation conditions (inoculated with *B. oryzae* or mock-inoculated with water). The treatments were replicated four times, and each replication consisted of a single plastic pot with four plants. The experiment was repeated once.

Quantification of epidemiological variables. The following epidemiological variables were evaluated: brown spot severity (BSS), final lesion size (FLS), and the number of lesions (NL) by square centimeter of leaf. The sixth leaf, from the base to the apex, of the main culm of each plant (a total of 16 leaves per treatment) was marked and used to evaluate the epidemiological variables mentioned above. The BSS on the marked leaf of each plant was scored at 24, 48, 72, 96, 120, and 144 hours after inoculation (hai) using a scale based on the percentage of leaf area showing BS symptoms (Lenz et al., 2010). The area under the brown spot progress curve (AUBSPC) for each leaf in each plant was computed using the trapezoidal integration of the brown spot progress curve over time (Shaner and Finney, 1977). The disease progress rate (r) was determined based on the linear model $Y = a + r*t$, where Y is the disease severity expressed as a proportion, a is the constant, r is the disease progress rate, and t is the time (in hours), using the software SAS (v. 6.12; SAS Institute, Inc.). The FLS (mm) of ten random lesions on the marked leaf of each plant was measured at 144 hai using an electronic digital caliper. The NL of ten random areas on the marked leaf of each plant was determined at 144 hai. For statistical analysis, the average FLS and NL for each leaf was calculated.

Antioxidant enzymes assays. The enzyme assay methodology followed that described by Debona et al. (2012). Samples from the sixth leaf of the main culm of each plant (a total of 16 leaves per treatment) were collected at 12, 24, 48, 72, 120, and 168 hours after fungicide spraying (hafs). The leaf samples were kept in liquid nitrogen during sampling and subsequently stored at -80°C until further analysis. A total of 300 mg of leaf tissue (mix of four leaves collected per replication) was ground into a fine powder in a mortar and pestle with liquid nitrogen to determine the activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione peroxidase

(GPX), and glutathione-*S*-transferase (GST). The fine powder was homogenized in an ice bath in 2 ml of a solution containing 50 mM potassium phosphate buffer (pH 6.8), 0.1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), and 2% (w v^{-1}) polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 12,000 *g* for 15 min at 4°C and the supernatant was used as the crude enzyme extract. To determine the glutathione reductase (GR) activity, a total of 300 mg of leaf tissue was ground as described above. The fine powder was homogenized in an ice bath in 2 ml of a solution containing 100 mM potassium phosphate buffer (pH 7.5), 0.1 mM EDTA, 1 mM *DL*-dithiothreitol, 1 mM PMSF, and 2% (w v^{-1}) PVPP. The homogenate was centrifuged as described previously.

The ability of SOD to photochemically reduce nitrobluetetrazolium (NBT) was used to determine SOD activity (Del Longo et al., 1993). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 0.1 mM EDTA, and 2 µM riboflavin. The reaction was started after the addition of 50 µL of the crude enzyme extract to 1.95 mL of the reaction mixture. The reaction was allowed to proceed at 25°C under a 15 W lamp. After 10 min of light exposure, the light was blocked and the production of formazan blue, which resulted from the photoreduction of NBT, was monitored using a spectrophotometer (Evolution 60, Thermo Fisher Scientific Inc.) to determine the absorbance at 560 nm. The control reaction mixtures were kept in darkness for 10 min and the absorbance was measured at 560 nm and the experimental values (light) were subtracted from the control values. The amount of enzyme necessary to inhibit NBT photoreduction by 50% corresponded to one unit of SOD.

The rate of H_2O_2 decomposition at 240 nm was measured for 1 min at 25°C to determine CAT activity (Havir and McHale, 1987). The reaction was initiated by adding 60 µL of the crude enzyme extract to 1.94 mL of the reaction mixture containing 50 mM potassium phosphate buffer (pH 6.8) and 20 mM H_2O_2.

The POX activity was assayed based on the colorimetric determination of pyrogallol oxidation (Kar and Mishra 1976) after the addition of 20 µL of the crude enzyme extract to 1.98 ml of the reaction mixture containing 25 mM potassium phosphate (pH 6.8), 20 mM pyrogallol, and 20 mM H_2O_2. The absorbance of coloured purpurogallin was recorded at 420 nm for 1 min at 25°C and used for POX activity determination.

The methodology proposed by Nakano and Asada (1981) was used to determine APX activity. The reaction was started after the addition of 60 µL of the crude enzyme extract to 1.94 ml of the reaction mixture containing 50 mM potassium phosphate buffer

(pH 6.8), 1 mM H_2O_2, and 0.8 mM ascorbate. The rate of ascorbate oxidation was measured at 290 nm for 1 min at 25°C and used to determine APX activity.

One hundred microliters of the crude enzyme extract was added to a mixture containing 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 0.114 M NaCl, 1 mM GSH, 0.2 mM nicotinamide adenine dinucleotide phosphate-oxidase (NADPH), 0.25 mM H_2O_2, and 1 U of GR to determine the GPX activity (Nagalakshmi and Prasad, 2001). The absorbance was measured at 340 nm for 1 min at 30°C. The reaction mixture used to determine the GR activity contained 100 mM potassium phosphate (pH 7.5), 1 mM EDTA, 1 mM glutathione oxidized (GSSG), and 0.1 mM NADPH prepared in 0.5 mM Tris-HCl buffer (pH 7.5) (Carlberg and Mannervik, 1985). The reaction was started by the addition of 100 µL of the crude enzyme extract to 1.9 mL of the substrate mixture. The absorbance at 340 nm was recorded for 1 min at 30°C. The GST activity was determined using the methodology proposed by Habig et al. (1974). The reaction mixture contained 1.35 mL of 50 mM potassium phosphate buffer (pH 6.5), 50 mM reduced glutathione (GSH), and 150 µl of the crude enzyme extract. Then, 500 µl of 30 mM 1-chloro-2,4-dinitrobenzene was added to the mixture to start the reaction, and the absorbance was monitored at 340 nm for 4 min at 25°C.

The soluble protein concentrations of the extracts were measured by the method of Bradford (1976) using bovine serum albumin as the standard protein.

Antioxidant metabolites assays. The concentrations of two antioxidant metabolites (AsA and GSH) were determined. Samples from the sixth leaf of the main shoot of each plant (a total of 16 leaves per treatment) were collected at 12, 24, 48, 72, 120, and 168 hafs. The AsA concentration was determined through the homogenization of 300 mg of leaf tissue in 2 mL of 6% (w v^{-1}) acid trichloroacetic (TCA), centrifuged at 15,000 *g* for 5 min at 4°C, following the methodology of Kampfenkel et al. (1995). Two hundred microliters of the crude extract was placed to react with 800 µL of 0.2 M sodium phosphate buffer (pH 7.4) at 42°C for 15 min. Subsequently, 1.0 mL of 10% (w v^{-1}) TCA, 800 µL of 42% (v v^{-1}) H_3PO_4, 800 µL of 4 % (w v^{-1}) 2,2'-dipyridyl (dissolved in 70 % ethanol), and 400 µL of 3 % (w v^{-1}) $FeCl_3$ were added to the mixture. After vigorous stirring, the mixture was incubated at 42°C for 40 min. The reaction was then stopped in an ice bath, and the AsA concentration was determined based on a calibration curve using AsA as the standard (Sigma-Aldrich, São Paulo, Brazil).

For the GSH, a total of 300 mg of leaf tissue was homogenized in 2 mL of a mixture consisting of 0.1 M hydrochloric acid (HCl) and 1 mM EDTA. The homogenate was

centrifuged at 12,000 *g* for 10 min at 4°C. To determine the total glutathione (GSH + GSSG) concentration, 150 µL of the crude extract was added to a reaction mixture consisting of 700 µL of 0.3 mM NADPH and 100 µl of 6 mM 5,5'-dithio-*bis*-2-nitrobenzoic acid (DTNB) (Griffith, 1980). After incubation for 5 min at 30°C, 10 µL of GR (50 U mL^{-1}) was added to the mix, and the absorbance was determined at 412 nm for 2 min. NAPDH, DTNB, and GR solutions were prepared in 125 mM sodium phosphate buffer (pH 7.5) containing 6.3 mM EDTA. For GSSG, the GSH was previously derivatized by adding 200 µL of the crude extract to 12 µL of 2-vinylpyridine; thus, 150 µL was used in the reaction as described above. The GSSG was determined through a calibration curve using GSSG as the standard (Sigma-Aldrich, São Paulo, Brazil), and the GSH concentration was obtained as the difference between the total glutathione and GSSG concentrations.

Oxidative stress-related compounds assays. The oxidative damage in the leaf cells was estimated through the determination of H_2O_2 and MDA concentrations. Samples from the sixth leaf of the main shoot of each plant (a total of 16 leaves per treatment) were collected at 12, 24, 48, 72, 120, and 168 hafs. To determine the concentration of H_2O_2, 100 mg of leaf tissue, previously ground into a fine powder using a mortar and pestle with liquid nitrogen, was homogenized in an ice bath in a volume of 2 mL of a mixture containing 50 mM potassium phosphate buffer (pH 6.5) and 1 mM hydroxylamine (Kuo and Kao, 2003). The homogenate was centrifuged at 10,000 × *g* for 15 min at 4°C, and 100 µL of the supernatant was added to a reaction mixture containing 100 µM ferric ammonium sulfate ($FeNH_4[SO_4]$), 25 mM sulfuric acid, 250 µM xylenol orange, and 100 mM sorbitol in a volume of 2 mL (Gay and Gebicki, 2000). The absorbance was determined at 560 nm after keeping the samples in the dark for 30 min. The controls for the reagents and crude extracts were prepared under the same conditions and subtracted from the sample. The H_2O_2 concentration was estimated based on a standard curve for H_2O_2.

The MDA was expressed as the concentration of total 2-thiobarbituric acid (TBA) reactive substances (Cakmak and Horst, 1991). A total of 100 mg of leaf tissue, previously ground into a fine powder with liquid nitrogen using a mortar and pestle, was homogenized in 2 mL of 0.1 % (w v^{-1}) TCA solution in an ice bath. The homogenate was centrifuged at 12,000 *g* for 15 min at 4°C, and 0.5 ml of the supernatant was added to 1.5 mL of the TBA solution (0.5 % in 20 % TCA) and then incubated for 30 min in a boiling water bath at 95°C. After this period, the reaction was terminated in an ice bath. The samples were centrifuged at 9,000 *g* for 10 min, and the specific absorbance was determined at 532 nm.

The nonspecific absorbance was estimated to be 600 nm and subtracted from the specific absorbance value.

Data analysis. Statistical analyses were performed using SAS software (v. 6.12; SAS Institute, Inc.), and the data from all variables were subjected to an analysis of variance (ANOVA). Within each sampling time, the means from the -Fu and +Fu treatments (for the non-inoculated or inoculated plants) or the means from the non-inoculated and inoculated plants (for the -Fu and +Fu treatments) were compared based on the *t*-test ($P \leq 0.05$). A 2×6 factorial experiment consisting of two fungicide treatments (-Fu and +Fu) and six evaluation times (24, 48, 72, 96, 120, and 144 hai) was considered for the ANOVA of BSS. For AUBSPC, *r*, NL, and FLS, a one-way ANOVA was performed to analyze the effect of the fungicide treatment. For the ANOVA of the antioxidant enzymes and metabolites and the oxidative stress-related compounds, the design was considered to be a $2 \times 2 \times 6$ factorial experiment consisting of the two fungicide treatments, non-inoculated or inoculated plants, and six evaluation times (12, 24, 48, 72, 120, and 168 hafs). Pearson's linear correlation was used to examine the relationships between the variables, and only data from the inoculated plants at 48, 72, 120, and 168 hafs were included in the analysis because these were the only times during which the activities of the antioxidant enzymes, the concentrations of antioxidant metabolites and oxidative stress-related compounds, and the BSS were determined.

RESULTS

Analysis of variance. The factor fungicide spraying (FS) was significant for all of the epidemiological variables, and the factor evaluation time (ET) and the FS × ET interaction were significant for BSS (Table 1). The factors FS, plant inoculation (PI), ET and their double interactions were significant for the activities of most enzymes, and the interaction FS × PI × ET was significant for the activities of POX, APX, GPX, GR, and GST. All of the factors and their interactions were significant for the concentrations of GSH, H_2O_2, and MDA, whereas only the factor FS and the interactions FS × PI and FS × PI × ET were not significant for the AsA concentration.

Symptoms of brown spot. Large, numerous and often coalescing brown spot lesions were noticeable on the leaves of the -Fu plants, whereas the leaves of the +Fu plants showed a few, small non-coalesced lesions (Fig. 1A)

Epidemiological variables. The BSS was significantly lower in the +Fu plants than in the -Fu plants regardless of the evaluation time (Fig. 1B). The BSS increased from 1 to 20% in

the -Fu plants and from 0.1 to 2% in the +Fu plants from 24 to 144 hai. The AUBSPC, *r*, NL, and FLS in the +Fu plants were significantly reduced by 90, 90, 42, and 78%, respectively, compared with the -Fu plants (Fig. 2).

Antioxidant enzymes. Comparison between the non-inoculated and inoculated plants in the -Fu treatment showed significant increase in the activities of SOD (18-72%), POX (165-780%), and GPX (90-271%) from 48 to 168 hafs, of APX (37-134%) and GR (29-115%) from 72 to 168 hafs, and of GST (34%) at 168 hafs for the inoculated plants (Fig. 3). Although a similar trend was obtained for the +Fu plants, such increases were usually later and lower (53-310% for POX from 48 to 168 hafs, 19-28% for SOD, 21-68% for APX, 79-143% for GPX from 72 to 168 hafs, and 20-33% for GR from 120 to 168 hafs). The GST activity was unchanged in the +Fu inoculated plants. Interestingly, the CAT activity in the inoculated plants significantly decreased by 21-28% from 48 to 120 hafs in the -Fu treatment and by 24-34% from 48 to 168 hafs in the +Fu treatment compared to the non-inoculated plants, indicating a CAT inhibition upon the fungal infection (Fig. 3).

The fungicide spraying had a limited effect on the enzyme activity of the non-inoculated plants because it significantly increased only the activities of POX (24%) from 48 to 72 hafs, of GPX (37%) at 168 hafs, and of GR (34%) and GST (33%) at 12 hafs (Fig. 3). The-Fu inoculated plants exhibited significantly higher activities of POX (25-89%) and GPX (12-33%) from 48 to 168 hafs, of SOD (18-33%) from 72 to 168 hafs, and of APX (20-54%), of GR (23-43%) and GST (21-43%) from 120 to 168 hafs than did the +Fu plants.

Antioxidant compounds. In general, the fungicide spraying did not affect the AsA concentration regardless of fungal inoculation and evaluation time. Inoculation with *B. oryzae*, in turn, significantly decreased the AsA concentration by 24-29% from 48 to 72 hai for -Fu plants and by 23% at 48 hai for the +Fu plants (Fig. 4). The GSH concentration increased (40-263% and 75-230% from 48 to 168 hafs for the -Fu and +Fu plants, respectively) upon fungal inoculation and was significantly higher for the -Fu plants (16-30%) than for their +Fu counterparts from 48 to 120 hafs. However, the +Fu plants displayed a significantly higher (27%) GSH concentration than the -Fu plants at 168 hafs. In addition, the fungicide spraying significantly increased the GSH concentration (55-142%) in the non-inoculated plants from 12 to 72 hafs in comparison to the non-sprayed plants.

Oxidative stress-related compounds. The concentrations of H_2O_2 and MDA were determined to assess the oxidative stress triggered by fungicide spraying and fungal

inoculation in the rice leaves. The significantly higher concentrations of H_2O_2 (73-164%) and MDA (49-129%) obtained for the inoculated plants from 48 hafs onwards indicated that an intense oxidative stress occurred in the -Fu plants as a consequence of fungal infection (Fig. 4). The fungicide spraying greatly constrained the *B. oryzae*-induced oxidative stress because later (120 hafs onwards for H_2O_2 and 168 hafs for MDA) and significantly lower increases (57% for H_2O_2 and 39% for MDA) were recorded. Indeed, comparison between the -Fu and +Fu inoculated plants showed that the +Fu plants exhibited significantly lower concentrations of MDA (19-62%) from 48 to 168 hafs and of H_2O_2 (27-56%) from 72 to 168 hafs compared to the -Fu plants, highlighting, therefore, the lowered oxidative stress due to the fungicide spraying in the inoculated plants.

Pearson correlation analysis. Most of the correlations among BSS, the activities of SOD, CAT, POX, APX GPX, GR, and GST and the concentrations of AsA, H_2O_2 and MDA were significant and positive for -Fu plants (Table 2). The GSH concentration was significantly and negatively correlated with the BSS, the activities of CAT and GPX, and the concentrations of H_2O_2 and MDA, but it was significantly and positively correlated with the AsA concentration. For the +Fu plants, the BSS was significantly and positively correlated with the activities of most enzymes and the AsA concentration, whereas the activities of some enzymes were also correlated positively among one other. Significant and negative correlations occurred between the GSH concentration and BSS and among the activities of CAT, GPX and GR and the AsA concentration. The H_2O_2 concentration was significantly and positively correlated with the activities of POX and GPX and with the AsA concentration.

DISCUSSION

Pathogen infection increases ROS production in plants, which, in turn, need to activate a wealth of enzymes and synthesize compounds to prevent or alleviate the cellular damage (Debona et al., 2012; Fortunato et al., 2015). Although strobilurins have been widely recognized for their fungicidal effect, they can also affect the plant's antioxidant system (Wu and Von-Tiedemann, 2001; Zhang et al., 2010), thereby favoring ROS removal. To the best of our knowledge, this study provides the first biochemical evidences that azoxystrobin, by reducing fungal infection but also by modulating the GSH concentration, limited *B. oryzae*-induced oxidative stress in rice leaves.

Superoxide is a primary ROS generated during pathogen infection (Hammond-Kosack and Jones, 1996), but its determination is very difficult because O_2^- is highly

unstable (Ehsani-Moghaddam et al., 2006). Therefore, SOD, the enzyme involved in O_2^- removal, has been very useful as an indicator of changes in O_2^- concentration in plant-pathogen interactions (Ehsani-Moghaddam et al., 2006). In the present study, the increased SOD activity observed in the inoculated plants (irrespective of fungicide treatment) indicates that the O_2^- concentration most likely increased as a result of the *B. oryzae* infection. Similar to these findings, the SOD activity in strawberry and tomato leaves increased in response to *Mycosphaerella fragariae* and *Botrytis cinerea* infection, respectively (Kuzniak and Sklodowska, 2005; Ehsani-Moghaddam et al., 2006), and concurrent increases in SOD activity and O_2^- concentration were reported to occur for soybean-*C. cassiicola* and wheat-*P. oryzae* interactions (Debona et al., 2012; Fortunato et al., 2015). Because the +Fu inoculated plants exhibited lower SOD activity than the -Fu plants, it seems reasonable to assume that the azoxystrobin spraying, by reducing brown spot symptoms rather than by activating SOD, reduced the *B. oryzae*-triggered O_2^- production. The lower concentration of MDA obtained for the +Fu plants compared to their -Fu counterparts provides additional support of this hypothesis because increases in O_2^- concentration as a result of pathogen infection are most often associated with enhanced cellular damage (Debona et al., 2012; Fortunato et al., 2015).

Although O_2^- dismutation represents a front-line defense against oxidative stress, H_2O_2, another potentially damaging ROS, is generated by the SOD-catalyzed reaction or even spontaneously (Noctor and Foyer, 1998). Consistent with the increased SOD activity, the inoculated plants (mostly those non-sprayed) also displayed higher H_2O_2 concentrations, which were underpinned by the positive correlation between these variables. A positive correlation also occurred between BSS and H_2O_2, suggesting an increased H_2O_2 concentration as the BS progressed. To cope with the increased H_2O_2 generated as a result of the fungal infection, the activities of H_2O_2 scavenger enzymes, such as POX, GPX, and to a lesser extent GST, increased in the inoculated plants, and the highest increases were obtained for the -Fu plants as a reflection of the higher *B. oryzae*-triggered H_2O_2 generation in such plants. Similarly, the activities of POX and GPX in the stem of mango plants from a susceptible cultivar infected with *Ceratocystis fimbriata* increased in contrast to a resistant cultivar (Bispo et al., 2015). The increase of wheat resistance to blast mediated by silicon was not associated with increased activities of most antioxidant enzymes because Si-deprived plants showed higher activities than their Si-supplied counterparts in response to the *P. oryzae* infection (Debona et al., 2014). Taken together, the results from this study suggest that the azoxystrobin constrained the BS

development mainly by its fungicide activity, which, in turn, prevented, to a large extent, the ROS generation, thereby explaining why the +Fu plants did not require increases as prominent as those observed for the -Fu plants.

The activities of APX and GR, both involved in the ascorbate-glutathione pathway, a key player in H_2O_2 metabolism (Noctor and Foyer, 1998), were determined in the present study. Consistent with what was previously reported for other pathosystems (Debona et al., 2012; Resende et al., 2012; Bispo et al., 2015;Fortunato et al. 2015) the activities of both APX and GR increased in response to *B. oryzae* infection, but higher increases were reported for the -Fu plants, which exhibited higher H_2O_2 concentrations. The APX reduces H_2O_2 by using AsA as an electron donor (Noctor and Foyer, 1998), and differences in AsA concentrations were obtained only at 24 and 48 hai, when significant reductions were observed for the inoculated plants relative to their non-inoculated counterparts. This finding suggests that a *B oryzae*-mediated early repression of AsA synthesis or its degradation may contribute to rice susceptibility to BS, as AsA has been associated with potato and soybean resistance to late blight and target spot, respectively (Polkowska-Kowalczyk et al., 2007; Fortunato et al., 2015). The concentrations of GSH, an important antioxidant compound generated by the reduction of oxidized glutathione catalyzed by the NAPDH-dependent enzyme GR (Noctor et al., 2012), increased in response to the *B. oryzae* infection mostly for the -Fu plants until 120 hafs, but lesser increases were recorded as the BS progressed. Interestingly, at 168 hafs, the +Fu plants sustained a higher GSH concentration than did the -Fu plants. These findings, coupled with the higher GSH concentrations observed for the non-inoculated +Fu plants until 72 hafs compared to the -Fu plants, suggest that azoxystrobin modulates glutathione metabolism, which, in turn, may contribute to constraining the BS development in addition to its fungicidal effect. In fact, GSH-deficient mutants of *Arabidopsis* have impaired phytoalexin synthesis and are more susceptible to diseases, though such response may vary according to the growth conditions and pathogen (Noctor et al., 2012). It is believed that the +Fu plants, by sustaining higher GSH concentrations at advanced stages of the fungal infection, were also more able to cope with the *B. oryzae*-induced oxidative stress.

Catalase is another important enzyme involved in H_2O_2 removal, and its activity has been associated with plant resistance to diseases (Magbanua et al., 2007; Debona et al., 2012). In barley, an inverse correlation between CAT activity and resistance to powdery mildew has been suggested because CAT repression leads to an increase in the H_2O_2 concentration that precedes the cell death associated with resistance to biotrophic

pathogens (Vanacker et al., 1998). However, this is not the case for necrotrophic pathogens such as *B. oryzae*, for which increases in ROS concentration, including H_2O_2, are related to heightened susceptibility (Mengiste, 2012). Not surprisingly, therefore, an inhibition in CAT activity and subsequent increases in H_2O_2 concentrations were noticeable for the *B. oryzae*-inoculated plants regardless of the fungicide spray. Intriguingly, a similar inhibition occurred for both the -Fu and +Fu plants, suggesting that *B. oryzae*was able to manipulate CAT activity even though its infection was greatly limited by the azoxystrobin spray. However, the similar inhibition in CAT was not translated into similar H_2O_2 concentrations between the -Fu and +Fu plants. Notably, the -Fu plants displayed H_2O_2 concentrations that were higher than those recorded for the +Fu plants, which may be ascribed to the higher SOD activity observed in the -Fu plants. Similar to these findings, higher H_2O_2 levels occurred in a susceptible wheat cultivar during the necrotrophic phase of *P. oryzae* as a consequence of increased and decreased activities of SOD and CAT, respectively (Debona et al., 2012). In addition, lines of corn resistant to the necrotrophic fungus *Aspergillus flavus* showed increased CAT activity and reduced H_2O_2 concentration (Magbanua et al., 2007).

In a previous study, Dallagnol et al. (2011) suggested that rice plants infected by *B. oryzae* displayed an extensive oxidative stress as indicated by decreases in the concentrations of photosynthetic pigments and increases in MDA concentration. Indeed, in the present study, *B. oryzae* infection was found to increase the H_2O_2 concentration and most likely the O_2^- concentration at the same time that the MDA concentration was kept high, which was supported by the positive correlations that occurred among H_2O_2, MDA, and BSS for the -Fu plants. Because necrotrophic fungi depend on nutrients released from host death cells for their growth and reproduction (Mengiste, 2012), it is not surprising that they produce or induce ROS production to cause oxidative stress. Accordingly, wheat and soybean plants infected by *P. oryzae* and *C. cassiicola*, respectively, displayed increases in H_2O_2, O_2^-, and MDA concentrations (Debona et al., 2012; Fortunato et al., 2015). Oxidative stress has also been associated with toxin production, which is a remarkable feature of hemibitrophic/necrotrophic pathogens, as evidenced by *P. oryzae* and *Exserohilum turcicum* in rice and corn, respectively (Ou, 1985; Chauhan et al., 1997). Germinating conidia of *B. oryzae* produce the non-host-selective toxins ophiobolins A and B (Xiao et al., 1991), which have been suggested to be responsible for the increased MDA concentrations observed in *B. oryzae*-infected rice leaves (Dallagnol et al., 2011). However, in the present study, azoxystrobin was found to prevent, to a certain extent,

increases in the H_2O_2 (and probably O_2^-) and MDA concentrations in the inoculated plants, thereby highlighting the importance of the fungicide in constraining *B. oryzae*-induced oxidative stress.

In conclusion, the present study evidenced that *B. oryzae* infection, regardless of fungicide spray, induced oxidative stress in rice leaves and increased the activities of most antioxidant enzymes, except that of CAT, which was found to be inhibited by the fungal infection. Azoxystrobin limited the *B. oryzae*-induced oxidative stress by constraining the fungal infection rather than by activating antioxidant enzymes. However, a sustained level of GSH at the late stages of fungal infection appeared to contribute to the reduced oxidative stress observed in the azoxystrobin-sprayed plants.

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TABLES AND FIGURES

Table 1. Analysis of variance of the effects of fungicide spraying (FS), plant inoculation (PI), evaluation time (ET), and their interactions on brown spot severity (BSS), area under brown spot progress curve (AUBSPC), progress rate (*r*), number of lesions (NL) per cm^2 of leaf, final lesion size (FLS), activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione reductase (GR), and glutathione-*S*-transferase (GST) and concentrations of ascorbate (AsA), glutathione reduced (GSH), hydrogen peroxide (H_2O_2), and malondialdehyde (MDA).

Variables[a]	FS	PI	ET	FS × PI	FS × ET	PI × ET	FS × PI × ET
BSS	**	-	**	-	**	-	-
AUBSPC	**	-	-	-	-	-	-
r	**	-	-	-	-	-	-
NL	**	-	-	-	-	-	-
FLS	**	-	-	-	-	-	-
SOD	*	**	**	*	*	**	ns
CAT	ns	**	**	ns	*	**	ns
POX	**	**	**	**	**	**	**
APX	*	**	**	**	**	**	**
GPX	*	**	**	**	**	**	**
GR	ns	**	**	**	**	**	**
GST	ns	ns	*	ns	**	ns	ns
AsA	ns	**	**	ns	**	**	ns
GSH	**	**	**	**	**	**	**
H_2O_2	**	**	**	**	*	**	**
MDA	*	**	**	**	*	**	*

[a]Levels of probability: [ns] = nonsignificant, [*] = 0.05 and [**] = 0.01.

Table 2. Pearson correlation coefficients among brown spot severity (BSS), activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione-*S*-transferase (GST) and concentrations of ascorbate (AsA), glutathione reduced (GSH), hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) in the leaves of plants non-sprayed (above diagonal) or sprayed (below diagonal) with the fungicide azoxystrobin and inoculated with *Bipolaris oryzae*.

Variables[a]	BSS	SOD	CAT	POX	APX	GPX	GR	GST	AsA	GSH	H_2O_2	MDA
BSS	–	**0.61**	**0.71**	**0.54**	**0.65**	**0.87**	**0.80**	**0.61**	**0.69**	**-0.85**	**0.90**	**0.73**
SOD	**0.53**	–	0.46	**0.81**	**0.68**	**0.79**	**0.72**	0.49	0.24	-0.27	**0.52**	**0.64**
CAT	**0.71**	0.23	–	0.18	0.20	**0.61**	0.35	0.28	0.31	**-0.74**	**0.80**	**0.59**
POX	**0.50**	0.45	-0.06	–	**0.90**	**0.75**	**0.81**	**0.61**	0.35	-0.14	0.40	0.35
APX	**0.55**	**0.63**	0.31	**0.66**	–	**0.79**	**0.81**	**0.80**	**0.54**	-0.31	0.48	0.34
GPX	**0.89**	**0.60**	**0.51**	**0.70**	**0.75**	–	**0.89**	**0.62**	**0.52**	**-0.62**	**0.81**	**0.66**
GR	**0.67**	0.40	0.29	**0.52**	0.43	**0.74**	–	**0.62**	**0.60**	-0.49	**0.68**	**0.57**
GST	0.02	-0.12	0.35	-0.29	0.15	-0.15	-0.03	–	**0.80**	-0.48	**0.54**	0.45
AsA	**0.71**	0.34	0.31	**0.73**	0.47	**0.80**	**0.57**	-0.24	–	**0.70**	**0.67**	**0.60**
GSH	**-0.84**	-0.28	**-0.55**	-0.45	-0.46	**-0.75**	**-0.58**	-0.09	**-0.64**	–	**-0.83**	**-0.70**
H_2O_2	0.49	0.02	0.33	**0.55**	0.33	**0.51**	0.05	-0.25	**0.58**	-0.49	–	**0.75**
MDA	0.13	0.04	-0.15	0.24	0.09	-0.01	-0.07	0.26	-0.03	-0.07	0.23	–

[a]Values in bold are significant ($P \leq 0.05$) based on the *t*-test.

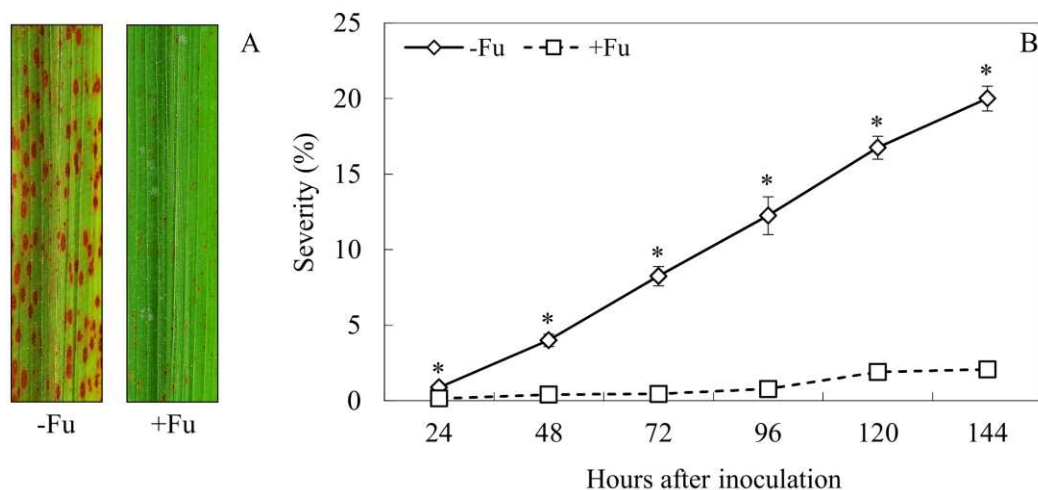


Figure 1. Brown spot (BS) symptoms 144 hours after inoculation (A) and the progress of BS (B) determined on the leaves of plants that were not sprayed (-Fu) or sprayed (+Fu) with the fungicide azoxystrobin and inoculated with *Bipolaris oryzae*. The means from the -Fu and +Fu treatments within each evaluation time that are followed by an asterisk (*) in (B) are significantly different ($P \leq 0.05$) by *t*-test. The bars represent the standard error of the means ($n = 4$).

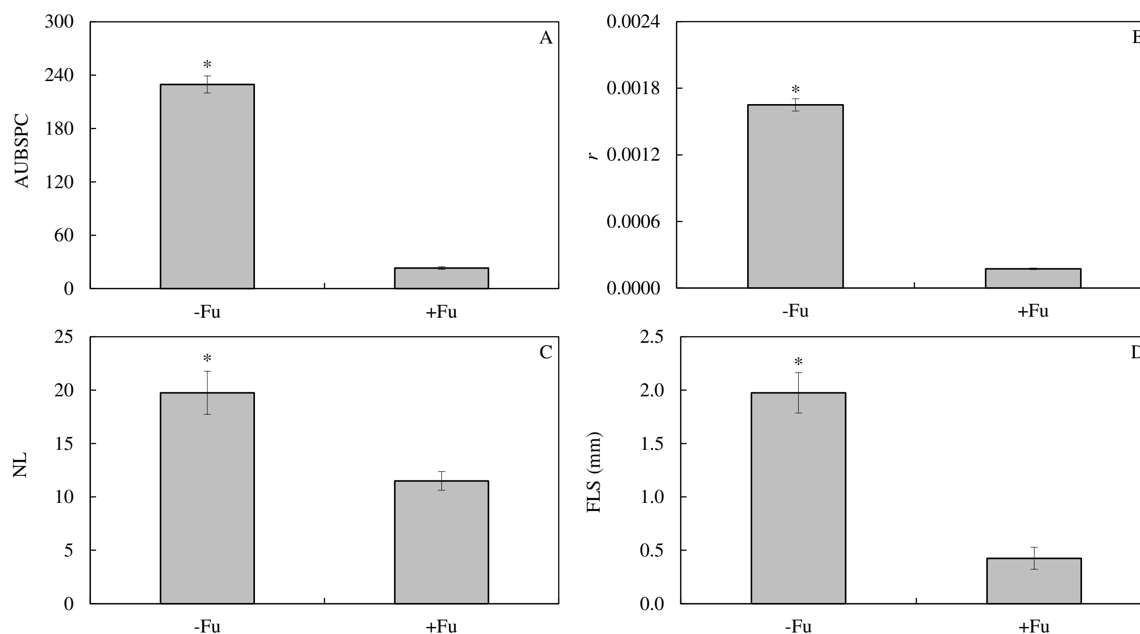
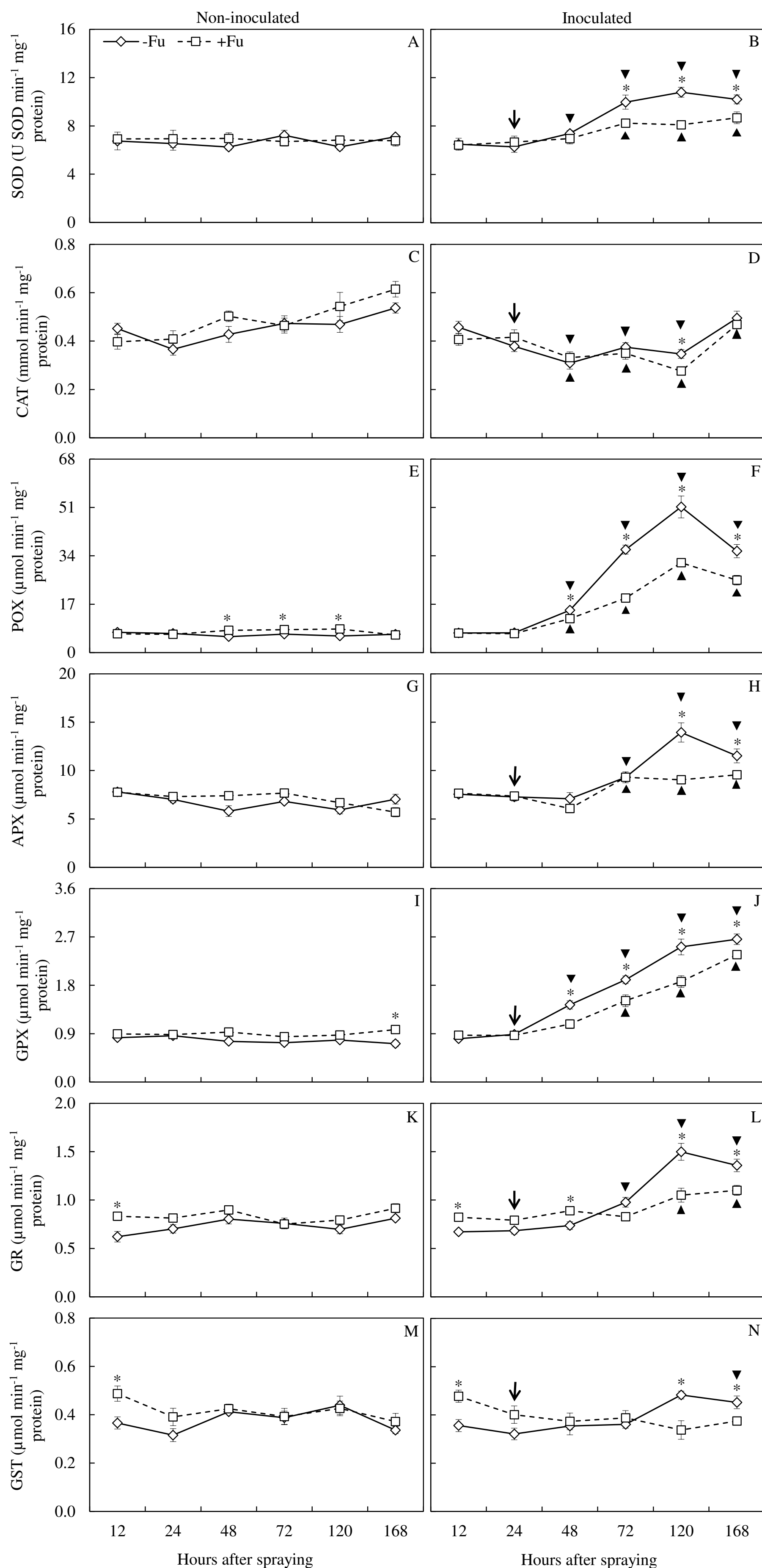


Figure 2. Area under brown spot progress curve (AUBSPC) (A), progress rate (*r*) (B), number of lesions (NL) per cm^2 (c), and final lesion size (FLS) (d) determined on the leaves of plants that were not sprayed (-Fu) or sprayed (+Fu) with the fungicide azoxystrobin and inoculated with *Bipolaris oryzae*. Means from the -Fu and +Fu treatments within each evaluation time that are followed by an asterisk (*) are significantly different ($P \leq 0.05$) by *t*-test. The bars represent the standard error of the means ($n = 4$).



Non-inoculated
Inoculated
-Fu
+Fu
SOD (U SOD min-1 mg-1 protein)
CAT (mmol min-1 mg-1 protein)
POX (µmol min-1 mg-1 protein)
APX (µmol min-1 mg-1 protein)
GPX (µmol min-1 mg-1 protein)
GR (µmol min-1 mg-1 protein)
GST (µmol min-1 mg-1 protein)
A
B
C
D
E
F
G
H
I
J
K
L
M
N
Hours after spraying
Hours after spraying

Figure 3. Activities of superoxide dismutase (SOD) (A, B), catalase (CAT) (C, D), peroxidase (POX) (E, F), ascorbate peroxidase (APX) (G, H), glutathione peroxidase (GPX) (I, J), glutathione reductase (GR), (K, L), and glutathione-*S*-transferase (GST) (M, N) in the leaves of plants non-sprayed (-Fu) or sprayed (+Fu) with the fungicide azoxystrobin and non-inoculated (A, C, E, G, I, K, M) or inoculated (B, D, F, H, J, L, N) with *Bipolaris oryzae*. The means of the -Fu and +Fu treatments within each evaluation time that are followed by an asterisk (*) are significantly different ($P \leq 0.05$) based on the *t*-test. Means of the non-inoculated and inoculated treatments within each evaluation time that are followed by the symbols ▼ or ▲ for the -Fu and +Fu treatments, respectively, are significantly different ($P \leq 0.05$) based on the *t*-test. Arrows indicate the time of *B. oryzae* inoculation. Bars represent the standard error of the means ($n = 4$).

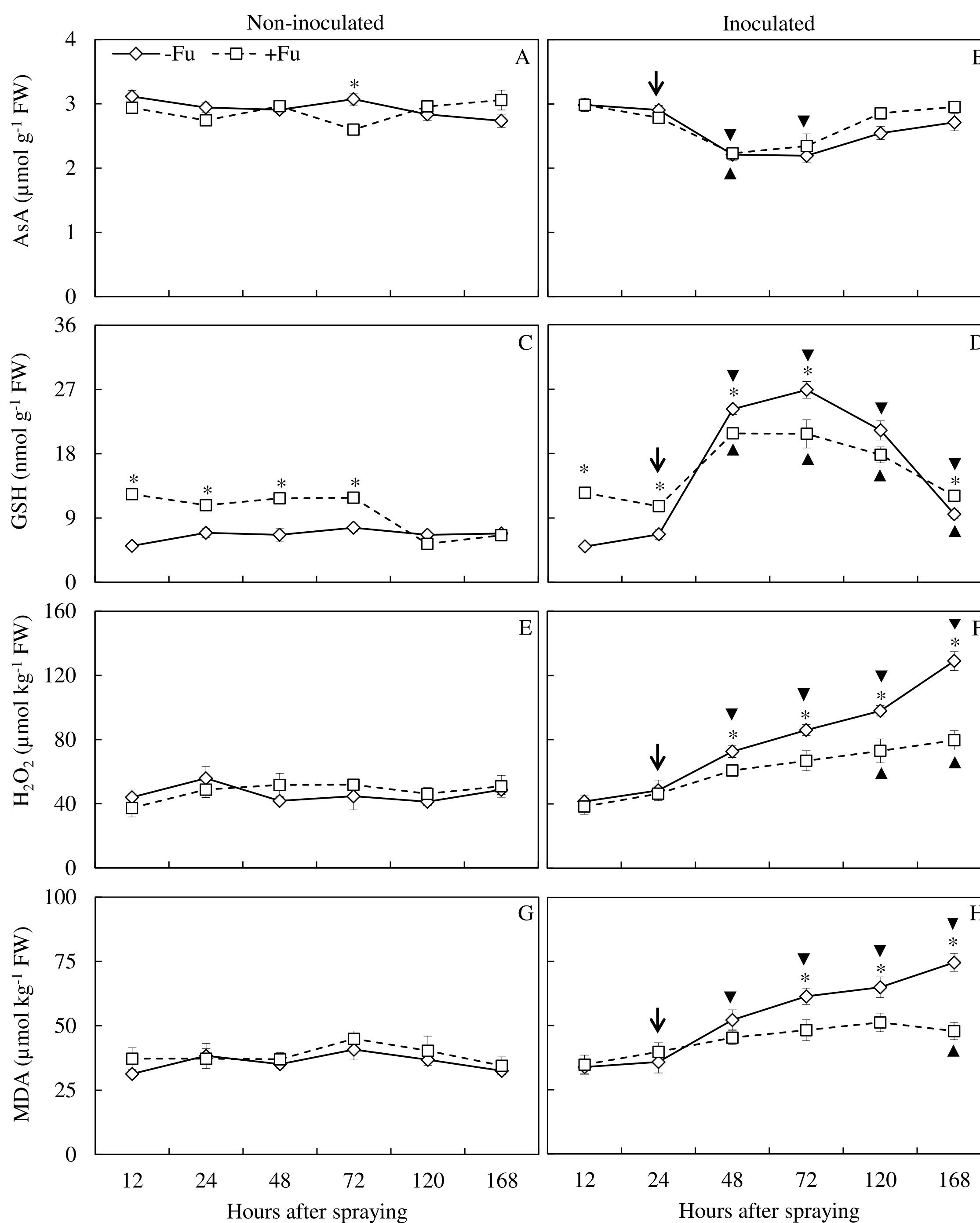


Figure 4. Concentrations of ascorbate (AsA) (A, B), reduced glutathione (GSH) (C, D), hydrogen peroxide (H_2O_2) (E, F) and malondialdehyde (MDA) (G, H) in the leaves of plants non-sprayed (-Fu) or sprayed (+Fu) with the fungicide azoxystrobin and non-inoculated (A, C, E, G) or inoculated (B, D, F, H) with *Bipolaris oryzae*. The means of the -Fu and +Fu treatments within each evaluation time that are followed by an asterisk (*) are significantly different ($P \leq 0.05$) based on the *t*-test. The means of the non-inoculated and inoculated treatments within each evaluation time that are followed by the symbols ▼ or ▲ for the -Fu and +Fu treatments, respectively, are significantly different ($P \leq 0.05$) based on the *t*-test. Arrows indicate the time of *B. oryzae* inoculation. Bars represent the standard error of the means (n = 4). FW = fresh weight.

Chapter 3

Rice defense responses to *Bipolaris oryzae* infection are minimally affected by a strobilurin fungicide

ABSTRACT

Strobilurins are one of the most important fungicide classes currently used; beyond that, they can prime plants for enhanced defense against pathogens attack. Here, detailed analyses at the biochemical and microscopic levels were performed to investigate whether azoxystrobin (Az) could enhance defense responses in rice plants (cv. Metica-1) either challenged or not with *Bipolaris oryzae*, the causal agent of brown spot. Az-sprayed plants displayed higher activities of *β*-1,3-glucanase, peroxidase, polyphenol oxidase (PPO) and lipoxygenase (LOX) in the absence of *B. oryzae* inoculation and of phenylalanine ammonia lyase, PPO and LOX at 24 h after inoculation with *B. oryzae* compared to the control plants. Concentrations of total soluble phenols (TSP) were transiently reduced by fungal infection in the Az-sprayed plants, but such plants presented higher TSP levels at 144 hai relative to their non-treated counterparts. Irrespective of the fungicide treatment, *B. oryzae* infection increased activities of all defense enzymes studied, but such increases usually were more prominent for the control than for the Az-sprayed plants. Concentrations of lignin thioglycolic acid derivatives were increased following fungal infection in the control plants. Microscopic analyses revealed that hyphae from *B. oryzae* colonized bulliform, bundle sheath, epidermal, guard, mesophyll and vascular bundle cells besides intercellular spaces, but fewer and smaller fungal cells were noticed in the Az-sprayed than in the control plants. However, the constrained fungal invasion mediated by Az was not accompanied by amplified defense reactions since cells of the control plants reacted by accumulating phenol-like material, whereas such reaction was only marginally reported in cells of Az-treated plants. Therefore, although Az transiently reprogrammed activities of some defense enzymes, which may have contributed for explaining the reduced brown spot severity observed in the Az-treated plants, its fungicidal activity appears to have played a major role in reducing *B. oryzae* infection.

Keywords: *Oryza sativa*, azoxystrobin, brown spot, defense enzymes, phenolics.

INTRODUCTION

Brown spot (BS), caused by *Bipolaris oryzae* (Breda de Haan) Shoemaker (Teleomorph: *Cochliobolus miyabeanus* (Ito and Kuribayashi) Drechs. ex Dastur.) affects millions of rice acres worldwide yearly causing great yield losses (~10% average) and reducing grain quality (Barnwal et al., 2013). Under proper environmental conditions (high temperature and moisture), conidia from *B. oryzae* germinate, produce apressoria from which an infective hyphae penetrates leaf tissue directly or through stomata (Tullis, 1935). Fungal infection is tightly dependent of toxins that are secreted by germlings resulting in quick (~18 h) appearance of typical disease symptoms that include light reddish-brown or gray center lesions surrounded by a dark reddish-brown margin with a bright yellow halo (Ou, 1985; Dallagnol et al., 2011). In the absence of resistant cultivars, brown spot management greatly relies in proper plant nutrition and fungicide spray (Dallagnol et al., 2011; Barnwal et al., 2013).

Plants have evolved a sophisticated defense system which include constitutive or induced, biochemical or structural mechanisms, to ward off pathogen attack. Among such mechanisms some enzymes and compounds have been implicated in rice defense to fungal infection. Rice defense to *B. oryzae* was found to be related to higher activities of peroxidase and chitinase, whereas a role for total soluble phenols (TSP) and lignin was not evident (Dallagnol et al., 2011). In the rice-*Pyricularia oryzae* interaction, accumulation of pathogenesis-related protein 1 (*PR1*) transcripts and diterpenoid phytoalexins was showed to play a pivotal role in host defense to blast (Rodrigues et al., 2004; 2005). In addition, rice plants that displayed higher TSP concentrations and lignin-thioglycolic acid derivatives and greater activities of peroxidase (POX), polyphenoloxidase (PPO), phenylalanine ammonia-lyase (PAL) and lipoxygenase were more resistant to leaf scald (Tatagiba et al., 2014). Furthermore, abscisic and jasmonic acid as well as ethylene have recently emerged as key hormones required for rice defense signaling against brown spot (De Vleesschauwer et al., 2013). Despite the recent progress in dissecting rice defense system, some gaps still remain to be filled, as for example the impact of agrochemicals routinely used in rice defense against pathogens.

Currently, strobilurins are among the most important classes of fungicides used for disease management, controlling a wide range of diseases in economically important crops including rusts, powdery mildews, downy mildews, leaf spots and anthracnoses (Bartlett et al., 2002). Apart from this, such fungicides are able to penetrate plant tissue and translocate, showing fungicide activity in areas that are not directly reached by

sprays (systemic activity). The fungicidal effect of the strobilurins is based on their ability to bind at the Q_o site of cytochrome *b* located at the inner mitochondrial membrane of fungi, thereby inhibiting ATP production and mitochondrial respiration (Ypema and Gold, 1999). In addition, there is an accumulating body of literature showing that strobilurins can reprogram plant metabolism, benefiting growth and enhancing defense against abiotic and biotic stress (Conrath et al., 2015). Regarding strobilurin-mediated plant defense to pathogen infection, it was showed that pyraclostrobin primed tobacco plants for accelerated *PR1* induction after *Tobacco mosaic virus* (TMV) attack (Herms et al., 2002), whereas azoxystrobin enhanced the production of secondary metabolites (phenolics and lignin) and enzymes activities (POX, PPO and PAL) in rice plants infected by *P. oryzae* (Sundravadana et al., 2007).

In spite of these reports, the mechanisms by which strobilurins increase plant disease resistance remain largely unknown. In addition, the effect of azoxystrobin, the main marketed strobilurin, in rice defense responses against *B. oryzae* has not been investigated. To test this hypothesis, assays of activities of a wide range of defense enzymes, concentrations of defense compounds and microscopic analyses were performed in azoxystrobin-sprayed and non-sprayed rice plants either challenged or with *B. oryzae*.

MATERIAL AND METHODS

Plant growth and fungicide spraying. Rice seeds from cv. Metica-1 were surface sterilized in 10% (v v^{-1}) NaOCl for 2 min, rinsed in sterilized water for 3 min, and sown in plastic pots (20-cm-diameter) (Ecovaso, Jaguariúna, São Paulo, Brazil) filled with 2 kg of substrate made from a 1:1:1 mixture of pine bark, peat, and expanded vermiculite (Tropstrato®, Vida Verde, Mogi Mirim, São Paulo, Brazil). A total of ten seeds were sown per pot, and five days after seedlings emergence, each pot was thinned to four seedlings. Substrate in each pot was fertilized with a nutrient solution based on Clark (1975) and contained 1.04 mM $Ca(NO_3)_2.4H_2O$, 1 mM NH_4NO_3, 0.8 mM KNO_3, 0.069 mM KH_2PO_4, 0.931 mM KCl and 0.6 mM $MgSO_4.7H_2O$, 19 µM H_3BO_3, 2 µM $ZnSO_4.7H_2O$, 0.5 µM $CuSO_4.5H_2O$, 7 µM $MnCl_2.4H_2O$, 0.6 µM $Na_2MoO_4.4H_2O$, 90 µM $FeSO_4.7H_2O$, and 90 µM EDTA bisodic. The nutrient solution was prepared using deionized water and applied weekly. Plants were watered as needed with deionized water. The fungicide treatments consisted of plants sprayed or not with azoxystrobin. The fungicide was sprayed 24 hours before *B. oryzae* inoculation on rice plants at V_8 growth stage (Counce et al., 2000). Azoxystrobin (Priori®, Syngenta, 2 mL c.p. l^{-1}) was prepared on the morning of spraying in deionized water + mineral oil (Nimbus®,

Syngenta, 0.5% v v^{-1}). Control plants were sprayed with deionized water + mineral oil (Nimbus®, Syngenta, 0.5% v v^{-1}). The fungicide treatments were sprayed using a CO_2 pressurized backpack sprayer equipped with a flat fan nozzle (XR 110 02®, Teejet) working at a 200,000 Pa pressure to deliver a spray volume of 200 L ha^{-1}.

Inoculation procedure. Rice plants were inoculated with *B. oryzae* at V_8 growth stage (Counce et al., 2000). Pathogen preservation and inoculum preparation were performed according to Dallagnol *et al.* (2011). A conidial suspension of *B. oryzae* (5×10^3 conidia ml^{-1}) was applied as a fine mist to the leaves of each plant until runoff using a VL Airbrush atomizer (Paasche Airbrush Co.). Gelatin (1%, w v^{-1}) was added to the sterile water to aid conidial adhesion to the leaves. Immediately after inoculation, the plants were transferred to a growth chamber with a temperature of 25 ± 2°C and a relative humidity of 90 ± 5% and were subjected to an initial 24-h dark period. After this period, the plants were transferred to a plastic mist growth chamber (MGC) inside a greenhouse for the duration of the experiment. The MGC was made of wood (2 m wide, 1.5 m high, and 5 m long) and covered with 100-µm-thick transparent plastic. The temperature inside the MGC ranged from 25 ± 2°C (day) to 20 ± 2°C (night). The relative humidity was maintained at 92 ± 3% using a misting system in which nozzles (model NEB-100; KGF Company, São Paulo, Brazil) sprayed mist every 30 min above the plant canopy. Relative humidity and temperature were measured with a thermohygrograph (TH-508, Impac, Brazil). The maximum natural photon flux density at plant canopy height was about 900 µmol m^{-2} s^{-1}. Non-inoculated plants were kept in separate chambers, but exposed to the same conditions as the inoculated plants during the experiment.

Experimental design. The experiment was arranged in a 2 × 2 factorial, consisting of two fungicide treatments (with or without azoxystrobin spraying, hereafter referred to as +Fu or -Fu plants, respectively) and two inoculation conditions (inoculated with *B. oryzae* or mock-inoculated with water). Treatments were replicated four times and each replication consisted in a single pot with four plants. The experiment was repeated once.

Quantification of epidemiological variables. The following epidemiological variables were evaluated: brown spot severity (BSS), final lesion size (FLS) and number of lesions by square centimeter (NL). The sixth leaf, from the base to the apex, of the main shoot of each plant (a total of 16 per treatment) was marked and used to evaluate the epidemiological variables mentioned above. BSS on the marked leaf of each plant was scored at 24, 48, 72, 96, 120 and 144 hours after inoculation (hai) using a scale based on the percentage of leaf area showing BS symptoms (Lenz et al., 2010). Area under brown

spot progress curve (AUBSPC) for each leaf in each plant was computed using the trapezoidal integration of brown spot progress curve over time (Shaner and Finney, 1979). The disease progress rate (*r*) was determined based on the linear model Y = a + *r**t, where Y is the disease severity expressed as a proportion, a is the slope of the line, *r* is the disease progress rate, and t is the time (in hours), using the software SAS (v. 6.12; SAS Institute, Inc.). The FLS (mm) of ten random lesions on the marked leaf of each plant was measured at 144 hai employing an electronic digital caliper. The NL of ten random areas on the marked leaf of each plant was determined at 144 hai. For statistical analysis the average of FLS and NL for each leaf was calculated. In addition, leaves were also collected for scanning at 144 hai, when the experiment was finished.

Defense enzymes assays. Samples from the sixth leaf of the main culm of each plant (a total of 16 leaves per treatment) were collected at 12, 24, 48, 72, 120, and 168 hours after fungicide spraying (hafs). The leaf samples were kept in liquid nitrogen during sampling and subsequently stored at 80°C until further analysis. A total of 300 mg of leaf tissue (mix of four leaves collected per replication) was ground into a fine powder in a mortar and pestle with liquid nitrogen to determine the activities of determine the activity of chitinase (CHI), *β*-1,3-glucanases (GLU), peroxidase (POX), polyphenoloxidase (PPO), phenylalanine ammonia lyase (PAL) and lipoxygenases (LOX). The fine powder was homogenized in an ice bath in 2 ml of a solution containing 50 mM potassium phosphate buffer (pH 6.8), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulphonyl fluoride (PMSF), and 2% (w v^{-1}) polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 12,000 *g* for 15 min at 4°C and the supernatant was used as the crude enzyme extract.

CHI activity was determined by adding 20 µl of the crude enzyme extract to a reaction mixture containing 50 mM sodium acetate buffer (pH 5.0) and 0.1 mM *p*-nitrophenyl-β-*D*-*N*-*N'*-diacetylchitobiose (Harman et al., 1993). The reaction mixture was incubated in a water bath at 37°C for 2 h, and the reaction was terminated by adding 500 µl of 0.2 M sodium carbonate. For the control samples, the sodium carbonate was added soon after the addition of the crude enzyme extract to the reaction mixture. The absorbance of the end product released by CHI was measured at 410 nm.

GLU activity was determined according to the method of Lever (1972). The reaction was started following the addition of 20 µl of the crude enzyme extract to a reaction mixture containing 50 mM sodium acetate buffer (pH 5.0) and laminarin (1 mg ml^{-1}). The reaction mixture was incubated in a water bath at 45°C for 1 h. Afterward,

500 µl of this mixture was added to a reaction mixture of dinitrosalicylic acid (DNS). This reaction mixture was then incubated in a water bath for 15 min at 100°C and then cooled in an ice bath until it reached 25°C. The absorbance was measured at 540 nm. A similar procedure was used for the control samples except that the first incubation was excluded.

POX activity was assayed based on the colorimetric determination of pyrogallol oxidation (Kar and Mishra, 1976) after the addition of 20 µl of the crude enzyme extract to 1.98 ml of the reaction mixture containing 25 mM potassium phosphate (pH 6.8), 20 mM pyrogallol, and 20 mM hydrogen peroxide (H_2O_2). The absorbance of coloured purpurogallin was recorded at 420 nm for 1 min at 25°C and used for POX activity determination. PPO activity was determined using the same procedure as for POX, but that H_2O_2 was omitted from the reaction mixture.

PAL activity was assayed following the method proposed by Guo et al. (2007) with some modifications. First, the reaction was started by adding 100 µl of crude enzyme extract to 0.9 ml of a reaction mixture containing 40 mM sodium borate buffer (pH 8.8) and 20 mM *L*-phenylalanine. The reaction mixture was incubated at 30°C for 1 h. For the control samples, the extract was replaced by borate buffer. The reaction was stopped by adding 50 µl of 6 N HCl. The absorbance of the *trans*-cinnamic acid derivatives was recorded at 290 nm.

Activity of LOX was determined by adding 10 µl of the crude enzyme extract to a reaction mixture containing 50 mM sodium phosphate buffer (pH 6.5) and 50 µM sodium linoleate (Axelrod et al., 1981). The reaction mixture was incubated at 25°C, and the absorbance of the product released by LOX for 1 min was measured at 234 nm.

The soluble protein concentrations of the extracts were measured by the method of Bradford (1976) using bovine serum albumin as the standard protein.

Determination of the concentrations of total soluble phenols (TSP) and lignin-thioglycolic acid (LTGA) derivatives. A total of 0.1 g of leaf tissue was ground into a fine powder with liquid nitrogen in a mortar and pestle and homogenized in 1 ml of a solution containing 80% (v v^{-1}) methanol in an ice bath. The homogenate was placed in a shaker at 300 rpm for 2 h at 25°C and then centrifuged at 17,000 × g for 30 min. The supernatant was used to determine TSP concentration, and the pellet was maintained at 20°C to determine the concentration of LTGA derivatives. The concentration of TSP was determined by following the methodology proposed by Zieslin and Ben-Zaken (1993) with modifications proposed by Rodrigues et al. (2005). The reaction was started after the addition of 0.2 M Folin-Ciocalteu phenol reagent to 150 µl of the methanolic

extract and kept at 25°C for 5 min. Next, 0.1 M sodium carbonate was added to the solution, which was maintained at 25°C for 10 min. Afterward, 1 ml of deionized water was added to the mixture and it was incubated at 25°C for 1 h. The absorbance was read at 725 nm, and the TSP concentration was calculated based on a calibration curve using catechol (SigmaAldrich, São Paulo, Brazil) as a standard. For the concentration of LTGA derivatives, the pellet was resuspended in 1.5 ml of deionized water, and the homogenate was centrifuged at 12,000 × g for 15 min. The supernatant was discarded and the pellet was dried at 65°C for 12 h. The alcohol-insoluble dry residue was used to determine the concentration of LTGA derivatives as described by Barber and Ride (1988). The absorbance of the LTGA derivatives supernatant was read at 280 nm, and its concentration was determined by a calibration curve using lignin, alkali, and 2-hydroxypropyl ether (Sigma-Aldrich, São Paulo, Brazil) as a standard.

Light microscopy analysis. Processing of leaf samples for light microscopy analysis followed that proposed by Araújo et al. (2015). Approximately 25 to 30 leaf fragments (≈ 25 mm^2) from each inoculated plant of each replicate and treatment were sampled at 8, 16, 24, 48, 72, 96, and 144 hai. The leaf fragments were carefully transferred to glass vials containing 10 mL of a fixative composed of 3% (v v^{-1}) glutaraldehyde and 2% paraformaldehyde (v v^{-1}) in 0.1 M sodium cacodylate buffer (pH 7.2). The vials were stored at 4°C for two months until they were used for the microscopy observations. Leaf fragments from the -Fu and +Fu plants collected at 72 and 144 hai were washed with 0.1 M sodium cacodylate buffer, subsequently dehydrated through a graded alcohol series (10, 30, 50, 70, 85, 95 and 100%) and then embedded in methacrylate resin (Historesin, Leica Microsystems®, Nussloch/Heidelberg, Germany). During the pre-infiltration and infiltration steps, the leaf fragments were placed in a vacuum chamber for 2 h both in the morning and in the afternoon for three weeks to allow better resin infiltration into the leaf tissue. The samples were stored at 4°C after each vacuum procedure. In total, six blocks of resin, each containing two leaf fragments, were obtained for each treatment at each sampling time. Longitudinal and transverse serial sections (4 µm thick), which were cut from each block using a Leica RM 2245 rotary microtome (Leica Microsystems®, Nussloch/Heidelberg, Germany), were randomly divided and placed on three glass slides and stained with 1% toluidine blue in 2% sodium borate for 5 min. Toluidine blue is a metachromatic dye commonly used for staining plant tissue sections (Vermerris and Nicholson, 2006). Typical host defense mechanisms such as the accumulation of phenolics to prevent further fungal colonization was obtained by staining sections of rice leaves with toluidine blue. When

the specimen samples stained with toluidine blue are viewed under the light microscope, distinct different cell components produce different colors: DNA is bluish-green, RNA is violet, the middle lamella is red, nonlignified cell walls, and soluble phenolics are red-violet, blue violet, blue, or purple, and polymerized phenolics such as lignin become green or bluish-green (Vermerris and Nicholson 2006). The images of the details regarding fungal infection and host defense responses were acquired digitally (Axio Cam HR, Carl Zeiss, Jena, Thuringia, Germany) using a Carl Zeiss Axio Imager A1 microscope (Carl Zeiss, Germany) in the bright-field mode and further processed using AXION VISION v. 4.8.1 software.

Data analysis. Statistical analyses were performed in the SAS software (v. 6.12; SAS Institute, Inc.) and data from all variables were subjected to analysis of variance (ANOVA). Within each sampling time, means from the -Fu and +Fu treatments (for the non-inoculated or inoculated plants) or the means from the inoculated and non-inoculated plants (for the -Fu and +Fu treatments), were compared based on the *t*-test ($P \leq 0.05$). A 2 × 6 factorial experiment consisting of two fungicide treatments (-Fu and +Fu) and six evaluation times (24, 48, 72, 96, 120 and 144 hai) was considered for ANOVA of BSS. For AUBSPC, *r*, NL and FLS one-way ANOVA was performed to analyze the effect of fungicide treatment. For ANOVA of defense enzymes and concentrations of TSP and LTGA derivatives, the design was considered to be a 2 × 2 × 6 factorial experiment consisting of the two fungicide treatments, non-inoculated or inoculated plants and six evaluation times (12, 24, 48, 72, 120 and 168 hafs). Pearson's linear correlation was used to examine relationships between the variables and only data from inoculated plants at 48, 72, 120 and 168 hafs were included in the analysis because these were the only times at which the activities of defense, concentrations of TSP and LTGA derivatives, and BSS were determined.

RESULTS

Analysis of variance. The factor fungicide spraying (FS) was significant for all of the epidemiological variables and the factor evaluation time (ET) and the FS × ET interaction was significant for BSS (Table 1). The factors FS, plant inoculation (PI), ET as well as their double interactions were significant for the activities of most enzymes and the interaction FS × PI × ET was significant for the activities of GLU, POX, PAL, PPO and LOX. All of the factors and their interactions were significant for the concentration of LTGA derivatives, whereas only the factor PI, ET and the interaction PI × ET was significant for the TSP concentration.

Epidemiological variables. Fungicide spray significantly reduced BSS irrespective of the evaluation time; BSS increased from 1.9 to 21.5% in the -Fu plants and from 0.4 to 2.3% in the +Fu plants from 24 to 144 hai (Fig. 1). The +Fu plants displayed values of AUBSPC, *r*, NL and FLS that were 89, 90, 31 and 81% lower than those from the -Fu plants, respectively (Fig. 2).

Defense enzymes. The inoculated plants from the -Fu treatment showed significant increases in the activities of GLU (45-174%), POX (159-735%) and LOX (1179-4203%) from 48 hafs onwards, of PAL (234-821%) and PPO (72-179%) from 72 hafs onwards and of CHI (78-84%) at 120 and 168 hafs compared with the non-inoculated plants (Fig. 3). The *B. oryzae* infection also significantly increased enzymes' activities in the +Fu plants, but such increases were usually less prominent than those recorded for the -Fu plants (45% for CHI, 45-174% for GLU, 47-312% for POX, 77-459% for PAL, 56-128% for PPO and 605-1072% for LOX). Interestingly, PAL activity was significantly increased in response to *B. oryzae* infection at 48 hafs in the +Fu plants, but not in their -Fu counterparts.

Comparisons between -Fu and +Fu treatments across non-inoculated plants displayed significant increases in the activities of LOX (112-337%) from 48 to 168 hafs and of GLU (73-91%), POX (23-41%) and PPO (26-47%) from 72 to 168 hafs as a result of fungicide spraying (Fig. 3). Under *B. oryzae* inoculation, the activities of PAL, PPO and LOX at 48 hafs were significantly higher by 74, 30 and 23% in the +Fu plants than in the -Fu plants. However, the -Fu plants displayed activities that were significantly higher than their +Fu counterparts for POX (25-41%) from 48 hafs onwards, for PAL (55-131%) and LOX (25-61%) from 72 to 168 hafs, for CHI (22-37%) and GLU (32-34%) at 120 and 168 hafs and for PPO (18%) at 168 hafs.

Defense-related compounds. The TSP concentration for the +Fu plants was significantly reduced by 14-17% at 48 and 72 hafs in response to *B. oryzae* infection (Fig. 4). The inoculated plants of the -Fu treatment, in turn, showed significant increases of 22-87% in the concentration of LATG derivatives at 120 and 168 hafs compared to the non-inoculated plants. The concentrations of defense-related compounds were minimally affected by the fungicide spray in the non-inoculated plants. However, the +Fu plants displayed a TSP concentration that was significantly higher by 14% than the -Fu plants at 168 hafs, whereas the LATG derivatives concentration was significantly higher (17-72%) in the -Fu plants than in the +Fu plants at 72 and 168 hafs upon *B. oryzae* inoculation.

Pearson correlation analysis. Most of the correlations among enzymes activities themselves and BSS were significant and positive regardless of the fungicide treatment, whereas there was no significant correlation of concentration of TSP with any variable and of the concentration of LTGA derivatives with both POX activity and the concentration of TSP for the -Fu plants (Table 2). For the +Fu plants, the concentration of LTGA derivatives was positively correlated with POX activity and TSP concentration.

Light microscopy. Hyphae of *B. oryzae* were able to colonize the bundle sheath, bulliform, epidermal, guard, mesophyll and vascular bundles cells besides the intercellular spaces on the leaves of the -Fu plants (Figs. 5 and 6). The massive colonization of *B. oryzae* resulted in extensive disorganization of host cells, particularly of those from the mesophyll. However, some cells reacted to fungal infection by accumulating phenolic-like compounds as indicated by the dark-blue or purple staining, which was particularly evident in vascular bundles, bundle sheath, bulliform and guard cells. As a result of such defense-reactions, some fungal cells appeared to be dead (empty). Although hyphae of *B. oryzae* were able to colonize different types of cells on the leaves of the +Fu plants, fungal colonization was virtually reduced in such plants because fewer and smaller hyphae were noticed in host cells compared with those from the -Fu plants. Cells which reacted to the fungal infection through deposition of phenolic-like compounds also were observed for the +Fu plants, but such response appeared to be less intense than that observed for the -Fu plants. Some fungal cells showing reduced growth and sometimes appearing as empty cells were recorded in host cells of the +Fu plants regardless of any evident accumulation of phenolic-like compounds, which can be ascribed to the fungicide effect that resulted in fungal death

DISCUSSION

In accordance with previous results (Debona *et al.*, 2015), azoxystrobin was highly effective in controlling brown spot in rice as evidenced by reductions in BSS (89%) AUBSPC (89%), *r* (90%), NL (31%) and FLS (81%). It has been reported that disease control promoted by strobilurins may not only be explained through its fungicidal effect but also by their ability to prime plants for enhanced defense against pathogen attack (Herms *et al.*, 2002; Conrath et al., 2015). In this study, we provide compelling evidences at the biochemical and histological levels of the effect of azoxystrobin in rice defense responses against *B. oryzae*.

As previously demonstrated (Dallagnol *et al*., 2011), activities of CHI and GLU were increased in response to *B. oryzae* infection regardless of the fungicide treatment. CHI and GLU play an important role in disease resistance because they hydrolyze chitin and β-1,3-glucan which comprise major components of fungal cell wall (Keen and Yoshikawa 1983; Mauch et al. 1988). In addition, such enzymes promote the release of oligomers which act as elicitors, therefore amplifying defense responses (Oliveira-Garcia and Valent, 2015). Although GLU activity was transiently induced in non-inoculated plants by azoxystrobin spray, inoculated plants from the -Fu treatment exhibited higher activities of both CHI and GLU than those from the +Fu treatment, indicating that such enzymes played no evident role in the suppression of fungal invasion in the azoxystrobin-sprayed plants, which was also supported by the positive correlation between enzymes activities and BSS. Consistently with the results from the present study, analyses of *CHI* transcripts and activities of CHI and GLU did not show any apparent contribution of them to rice resistance to blast and leaf scald, respectively (Rodrigues *et al*., 2003; Tatagiba *et al*., 2014). The lack of association between CHI and GLU activity and rice resistance to brown spot in azoxystrobin-sprayed plants may be related to the evolution of putative effectors in *B. oryzae* which, as demonstrated for other fungi, may protect their cell walls against or inhibit the activity of lytic enzymes secreted by host cells, thereby rendering ineffective this first defense layer (Oliveira-Garcia and Valent, 2015).

Irrespective of the fungicide treatment, inoculated plants displayed higher activities of PAL, POX and PPO than their non-inoculated counterparts, indicating that such enzymes are integral part of basal resistance to brown spot. Accordingly, rice plants infected with *B. oryzae* or *P. oryzae* showed increases in the activities of such enzymes or in the levels of their transcripts for either compatible or incompatible interactions (Rodrigues *et al*., 2003; Sundravadana *et al*., 2007; Dallagnol *et al*., 2011). The activities of POX and PPO were transiently increased by azoxystrobin spray in the present study. In addition, PPO activity was higher in the +Fu than in the -Fu treatment at 48 hafs for inoculated plants, suggesting that PPO may have contributed to increase rice resistance to brown spot in azoxystrobin-sprayed plants, probably due to its involvement in the production of quinones, compounds which are highly fungitoxic (Mayer, 2006). Taking into account that PAL activity was not affected by the fungicide spray in non-inoculated plants, it is concluded that azoxystrobin primed plants for enhanced defense against *B. oryzae* attack. PAL is a key enzyme in plant immunity because it comprises the first enzyme to integrate the phenylpropanoid pathway and it is

involved in the synthesis of antifungal compounds (soluble phenolics and flavonoids), lignin (important in cell wall reinforcement to confine pathogen invasion and toxin diffusion) and salicylic acid (a key hormone in systemic acquired resistance) (Dixon et al., 2002). Recent quantitative trait loci analyses have revealed a strong relationship between PAL and rice resistance to bacterial blight, blast and sheath blight (Tonnessen *et al*., 2015). Similarly to what was found in the present study, some authors have suggested that strobilurins prime plants for enhanced defense against pathogen infection, fact that was supported by earlier and higher accumulation of *PR1* transcripts in pyraclostrobin-treated plants, which was associated with tobacco resistance to *Pseudomonas syringae* pv. *tobaci* and TMV (Herms *et al*., 2002) as well as by higher activities of PAL, POX and PPO in azoxystrobin-treated plants that was linked to rice resistance to blast (Sundravadana *et al*., 2007).

The decreases in TSP concentrations of inoculated plants from the +Fu treatment at 48 and 72 hafs when compared to those of non-inoculated plants can be ascribed to the higher activity of PPO, suggesting that raised quinone concentration may have been more important than TSP to limit fungal ingress in the +Fu plants. In support to these results, Dallagnol et al. (2013) also found lower TSP concentrations in *B. oryzae*-infected rice plants than in non-infected controls. At advanced stages of fungal infection (144 hai), however, TSP appeared to be important to limit fungal invasion in azoxystrobin-sprayed plants because they sustained a higher TSP concentration than in their -Fu counterparts. Consistent with our findings, rice plants treated with azoxystrobin and inoculated with *P. oryzae* accumulated higher levels of TSP than did the control plants (Sundravadana *et al*., 2007). Many preformed phenolic compounds such as chlorogenic, *p*-coumaric, ferulic, salicylic, cinnamic and caffeic acids are known occur in rice (Kuwatsuka and Oshima, 1962; Varga, 1970) which can become associated to lipids and phospholipids, thereby increasing membrane permeability, electrolyte leakage and cytoplasm aggregation in fungal cells (Southerton and Deverall, 1990; Xiao et al., 1991). TSP, however, can proceed in the phenylpropanoid pathway to drive lignin synthesis, which is a major compound involved in plant disease resistance (Barber and Ride, 1988). As expected, lignin concentration was increased in inoculated plants from the -Fu treatment as result of increases in the activities of PAL, POX and PPO. These findings are in accordance with results obtained by Rodrigues et al. (2003), who found that an induction of *POX* transcripts following *P. grisea* infection corresponded to an increase in the concentration of LTGA derivatives. Since the highest LTGA concentration was recorded in plants that showed the highest BSS (-Fu plants),

lignin was not obviously associated with resistance to brown spot, which was also observed for the rice-*P. grisea* interaction (Rodrigues *et al*., 2003). Additional support to this hypothesis comes from the positive and from the lack of correlation obtained between concentrations of LTGA derivatives and BSS for the -Fu and +Fu plants, respectively. Despite PAL, POX and PPO had been induced in inoculated plants from the +Fu treatment, such increases were not translated in increases in the concentrations of LTGA derivatives probably because TSP accumulation was prioritized over lignin in such plants. Apart from this, the POX induction without correspondent increases in the concentrations of LTGA derivatives suggest that, for azoxystrobin-sprayed plants, POX may have played a more important role in rice resistance to brown spot due to its antioxidant activity than through its ability to enhance cell wall reinforcement as result of phenolics polymerization. These results support conclusions of Dallagnol et al. (2011), who suggested that the relief of oxidative stress by POX played a pivotal role to explain rice resistance to brown spot mediated by silicon.

Consistent with results obtained for other pathosystems (Nascimento et al., 2014; Rios et al., 2014; Fortunato et al., 2015), LOX activity was strongly increased by *B. oryzae* infection irrespective of fungicide treatment, which was supported by the positive correlations between the enzyme activity and BSS. LOX is involved in the synthesis of the hormone jasmonic acid (JA) by catalyzing the first step in the production of oxylipins through oxygenation of polyunsaturated fatty acids to fatty acid hydroperoxides and JA has been implicated in disease resistance in dicots against necrotrophic pathogens (Pieterse et al., 2009). In rice, however, JA seems to be a central node in defense signaling because it was showed to be engaged in resistance against either hemibiotrophs *P. oryzae* and *Xanthomonas oryzae* as well as to the necrotroph *Rhizoctonia solani* (De Vleesschauwer et al., 2013) and LOX activity was linked to blast resistance (Sandhu et al., 2007). The higher activity of LOX exhibited by non-inoculated plants from 48 hafs and thereafter and at 48 hafs by inoculated plants from the +Fu treatment compared to those from the -Fu treatment suggest that azoxystrobin was able to modulate LOX activity which, in turn, could have contributed to constrain *B. oryzae* infection in addition to its fungicide effect at early stages of fungal infection. However, this seems to be a remote possibility because JA has been demonstrated play no apparent role in rice resistance to brown spot (Ahn *et al*., 2005). Indeed, the -Fu plants exhibited higher LOX activity than did the +Fu plants as BS progressed, which was confirmed by the positive correlation between LOX activity and BSS. Although LOX may contribute to plant disease resistance, its sustained high activity can damage

cell membranes, increasing membrane permeability and electrolyte leakage (Brash, 1999) which is thought have facilitated host colonization by *B. oryzae* in the present study. Azoxystrobin spray, therefore, was able to prevent, to a large extent, the fungal-induced increases in LOX activity and the subsequent cellular damage, thereby constraining *B. oryzae* colonization.

Hyphae of *B. oryzae* were able to colonize different rice cells, including bulliform, bundle sheath, epidermal, guard, mesophyll and vascular bundle cells besides intercellular spaces. Comparison between -Fu and +Fu plants, however, showed that fewer host cells were colonized and smaller hyphae occurred in the latter plants. In addition, whereas rice cells (mainly the mesophyll ones) from the control plants exhibited extensive disorganization at 144 hai, most of the cells kept their integrity when plants were treated with azoxystrobin. Despite the massive colonization of *B. oryzae* hyphae noticed in the -Fu plants, some cells, particularly those of the bundle sheath, reacted by accumulating phenol-like compounds as indicated by the dark-blue color, reaction that was only marginally observed in the +Fu plants. In areas where such material accumulated, some fungal cells were dead (empty), corroborating observations made in rice plants infected with *Monographella albescens* and *P. grisea* (Rodrigues et al., 2003; Araújo et al., 2015). In azoxystrobin-treated plants, dead hyphae were also evident, but without a correspondent phenolic-like material surrounding them, indicating that such death was a result from its fungicidal effect. In fact, few bundle sheath cells were colonized in such plants, and the limitation of the penetration of those cells by *B. oryzae* was demonstrated interfere in the lateral spread of the fungus in the leaf (Tullis, 1935).

Taken together, results from the present study showed an extensive reprogramming of defense enzyme activities as a result of *B. oryzae* infection. Although azoxystrobin transiently increased activities of some enzymes and primed plants for enhanced enzyme activities following *B. oryzae* attack, non-treated plants exhibited more extensive defense reactions at advanced stages of fungal infection. Therefore, the constraining of fungal ingress and invasion of rice cells noticed in the azoxystrobin-treated plants seems to be chiefly governed by its fungicidal effect rather than priming effect.

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TABLES AND FIGURES

Table 1. Analysis of variance of the effects of fungicide spraying (FS), plant inoculation (PI), evaluation time (ET) and their interactions on brown spot severity (BSS), area under brown spot progress curve (AUBSPC), progress rate (*r*), number of lesions (NL) per cm^2 of leaf, final lesion size (FLS), activities of chitinases (CHI), *β*-1,3-glucanases, peroxidases (POX), phenylalanine ammonia-lyases (PAL), polyphenoloxidases (PPO) and lipoxygenases (LOX) and concentrations of total soluble phenolics (TSP) and lignin-thioglycolic acid (LTGA) derivatives.

Variables[a]	FS	PI	ET	FS × PI	FS × ET	PI × ET	FS × PI × ET
BSS	**	-	**	-	**	-	-
AUBSPC	**	-	-	-	-	-	-
r	**	-	-	-	-	-	-
NL	**	-	-	-	-	-	-
FLS	**	-	-	-	-	-	-
CHI	ns	**	**	**	**	**	ns
GLU	ns	**	**	**	**	**	**
POX	**	**	**	**	**	**	**
PAL	**	**	**	**	**	**	**
PPO	**	**	**	**	**	**	*
LOX	**	**	**	**	**	**	**
TSP	ns	*	*	ns	ns	**	ns
LTGA	**	**	**	**	**	**	**

[a]Levels of probability: [ns] = nonsignificant, [*] = 0.05 and [**] = 0.01.

Table 2. Pearson correlation coefficients among brown spot severity (BSS), activities of superoxide dismutases (SOD) catalases (CAT), peroxidases (POX), ascorbate peroxidases (APX), glutathione peroxidases (GPX), glutathione reductases (GR) and glutathione-*S*-transferases (GST) and concentrations of ascorbate (AsA), glutathione reduced (GSH), hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) in the leaves of non-sprayed (above diagonal) or sprayed (below diagonal) rice plants with the fungicide azoxystrobin and inoculated with *Bipolaris oryzae*.

Variables[a]	BSS	CHI	GLU	POX	PAL	PPO	LOX	TSP	LTGA
BSS	-	**0.94**	**0.84**	**0.57**	**0.96**	**0.92**	**0.90**	0.11	**0.85**
CHI	**0.85**	-	**0.82**	**0.59**	**0.95**	**0.86**	**0.84**	0.17	**0.78**
GLU	**0.62**	0.40	-	**0.79**	**0.89**	**0.89**	**0.88**	0.12	**0.57**
POX	**0.70**	**0.70**	**0.61**	-	**0.73**	**0.77**	**0.65**	0.18	0.21
PAL	**0.93**	**0.78**	**0.55**	**0.62**	-	**0.96**	**0.90**	0.15	**0.73**
PPO	**0.73**	**0.66**	**0.73**	**0.91**	**0.63**	-	**0.88**	0.15	**0.71**
LOX	**0.94**	**0.83**	**0.62**	**0.71**	**0.93**	**0.72**	-	0.15	**0.77**
TSP	**0.71**	**0.65**	**0.57**	**0.68**	**0.60**	**0.58**	**0.70**	-	0.10
LTGA	0.31	0.48	0.18	**0.55**	0.27	0.36	0.45	**0.50**	-

[a]Values in bold are significant ($P \leq 0.05$) based on the *t*-test.

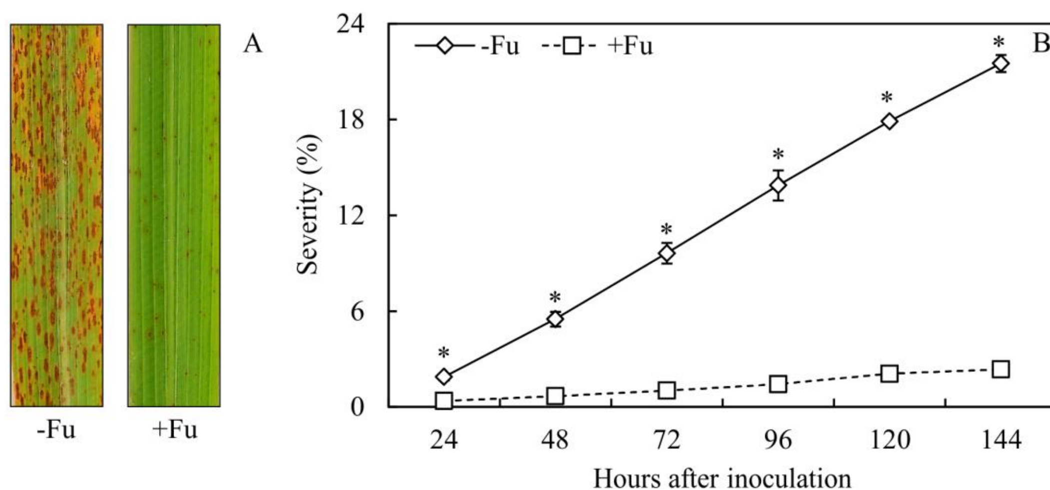

Figure 1. Brown spot (BS) symptoms 144 hours after inoculation (A) and the progress of BS (B) determined on the leaves of plants that were not sprayed (-Fu) or sprayed (+Fu) with the fungicide azoxystrobin and inoculated with *Bipolaris oryzae*. The means from the -Fu and +Fu treatments within each evaluation time that are followed by an asterisk (*) in (B) are significantly different ($P \leq 0.05$) by *t*-test. The bars represent the standard error of the means ($n = 4$).

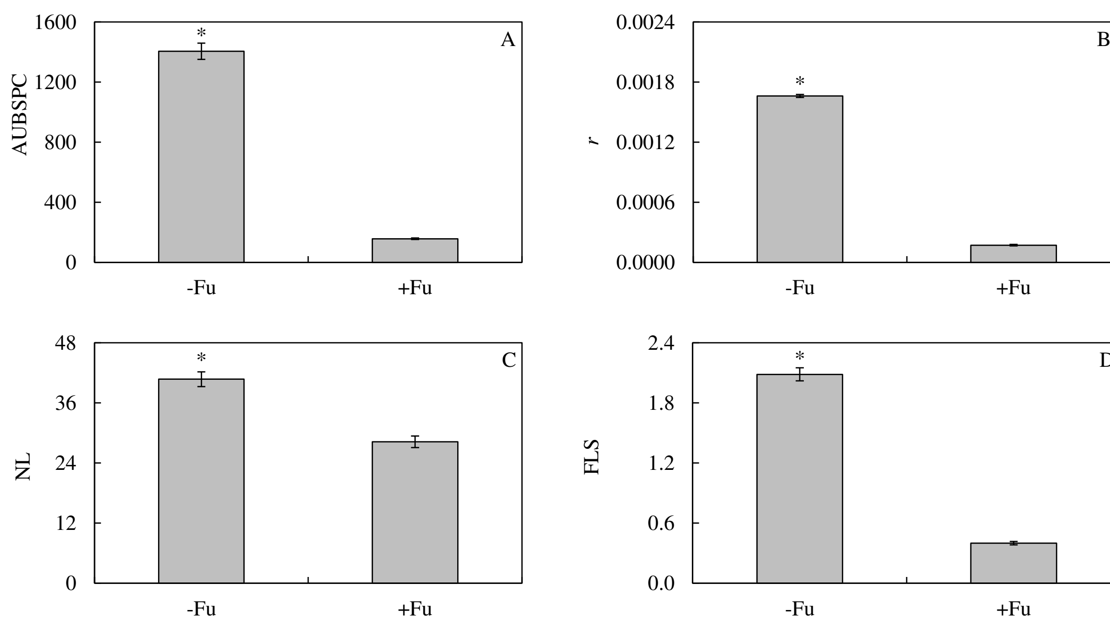

Figure 2. Area under brown spot progress curve (AUBSPC) (A), progress rate (*r*) (B), number of lesions (NL) per cm^2 (c), and final lesion size (FLS) (d) determined on the leaves of plants that were not sprayed (-Fu) or sprayed (+Fu) with the fungicide azoxystrobin and inoculated with *Bipolaris oryzae*. Means from the -Fu and +Fu treatments within each evaluation time that are followed by an asterisk (*) are significantly different ($P \leq 0.05$) by *t*-test. The bars represent the standard error of the means ($n = 4$).

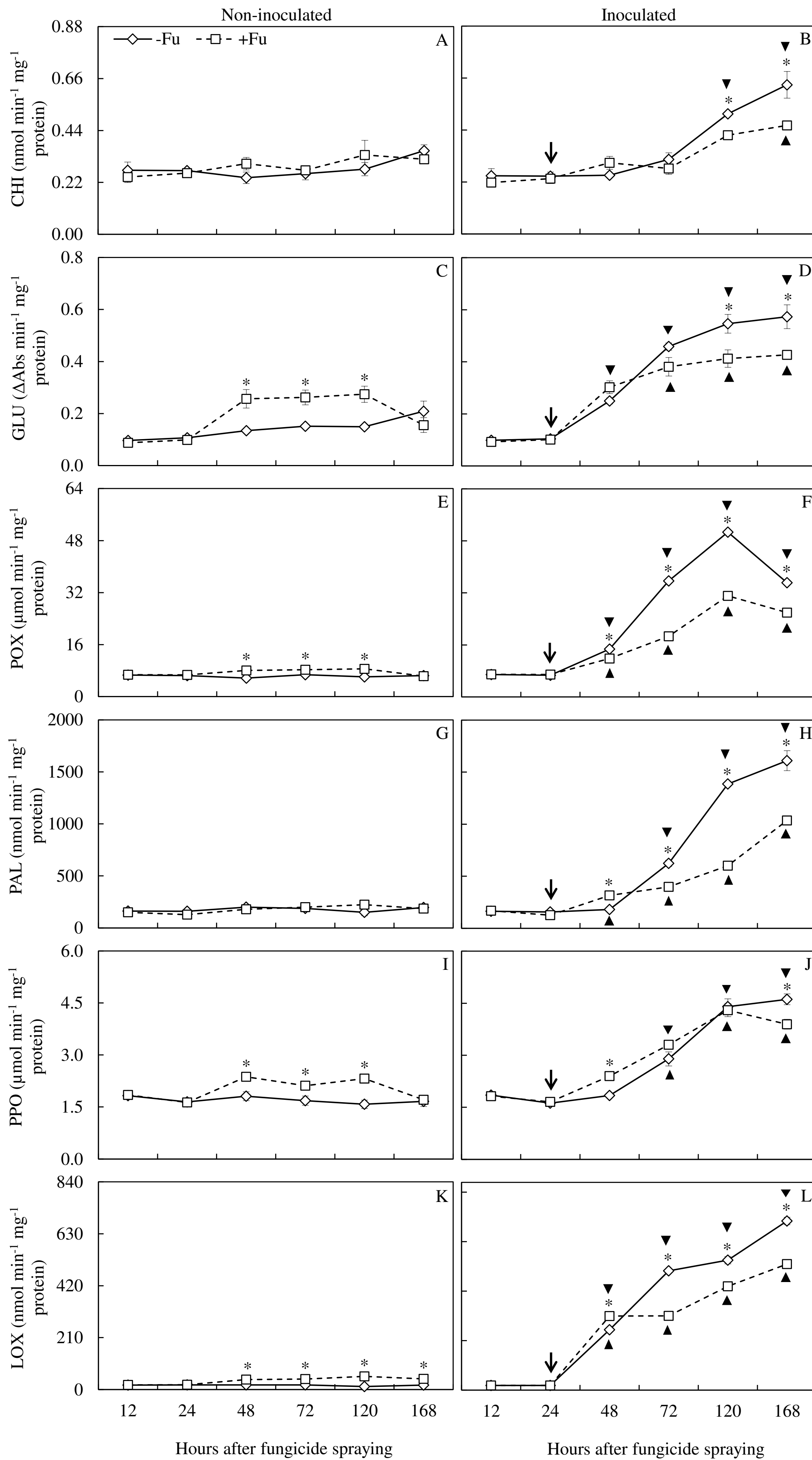
Non-inoculated
Inoculated
-Fu
+Fu
A
B
C
D
E
F
G
H
I
J
K
L
CHI (nmol min-1 mg-1 protein)
GLU (ΔAbs min-1 mg-1 protein)
POX (μmol min-1 mg-1 protein)
PAL (nmol min-1 mg-1 protein)
PPO (μmol min-1 mg-1 protein)
LOX (nmol min-1 mg-1 protein)
12
24
48
72
120
168
Hours after fungicide spraying
Hours after fungicide spraying

Figure 3. Activities of chitinases (CHI) (A and B), *β*-1,3 glucanases (C and D), peroxidases (POX) (E and F), phenylalanine ammonia-lyases (PAL) (G and H), polyphenoloxidases (PPO) (I and J) and lipoxygenases (GR), (K and L) in leaves of non-sprayed (-Fu) or sprayed (+Fu) rice plants with the fungicide azoxystrobin and non-inoculated (A, C, E, G, I and K) or inoculated (B, D, F, H, J and L) with *Bipolaris oryzae*. Means of -Fu and +Fu treatments within each evaluation time that are followed by an asterisk (*) are significantly different ($P \leq 0.05$) based on the *t*-test. Means of non-inoculated and inoculated treatments within each evaluation time that are followed by the symbols ▼ or ▲ for the -Fu and +Fu treatments, respectively, are significantly different ($P \leq 0.05$) based on the *t*-test. Arrows indicate the time of *B. oryzae* inoculation. Bars represent the standard error of the means ($n = 4$).

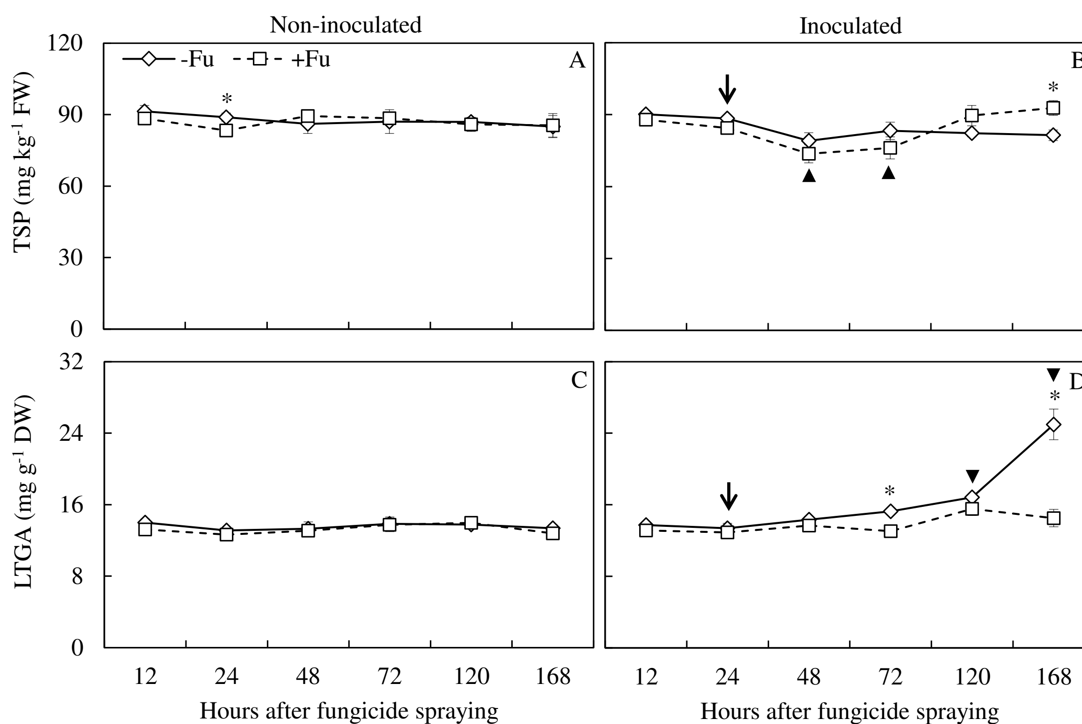

Figure 4. Concentrations of total soluble phenolics (TSP) (A and B) and lignin-thioglycolic acid (LTGA) derivatives (C and D) in leaves of non-sprayed (-Fu) or sprayed (+Fu) rice plants with the fungicide azoxystrobin and non-inoculated (A and C) or inoculated (B and D) with *Bipolaris oryzae*. Means of -Fu and +Fu treatments within each evaluation time that are followed by an asterisk (*) are significantly different ($P \leq 0.05$) based on the *t*-test. Means of non-inoculated and inoculated treatments within each evaluation time that are followed by the symbols ▼ or ▲ for the -Fu and +Fu treatments, respectively, are significantly different ($P \leq 0.05$) based on the *t*-test. Arrows indicate the time of *B. oryzae* inoculation. Bars represent the standard error of the means ($n = 4$). FW and DW = fresh and dry weight, respectively.

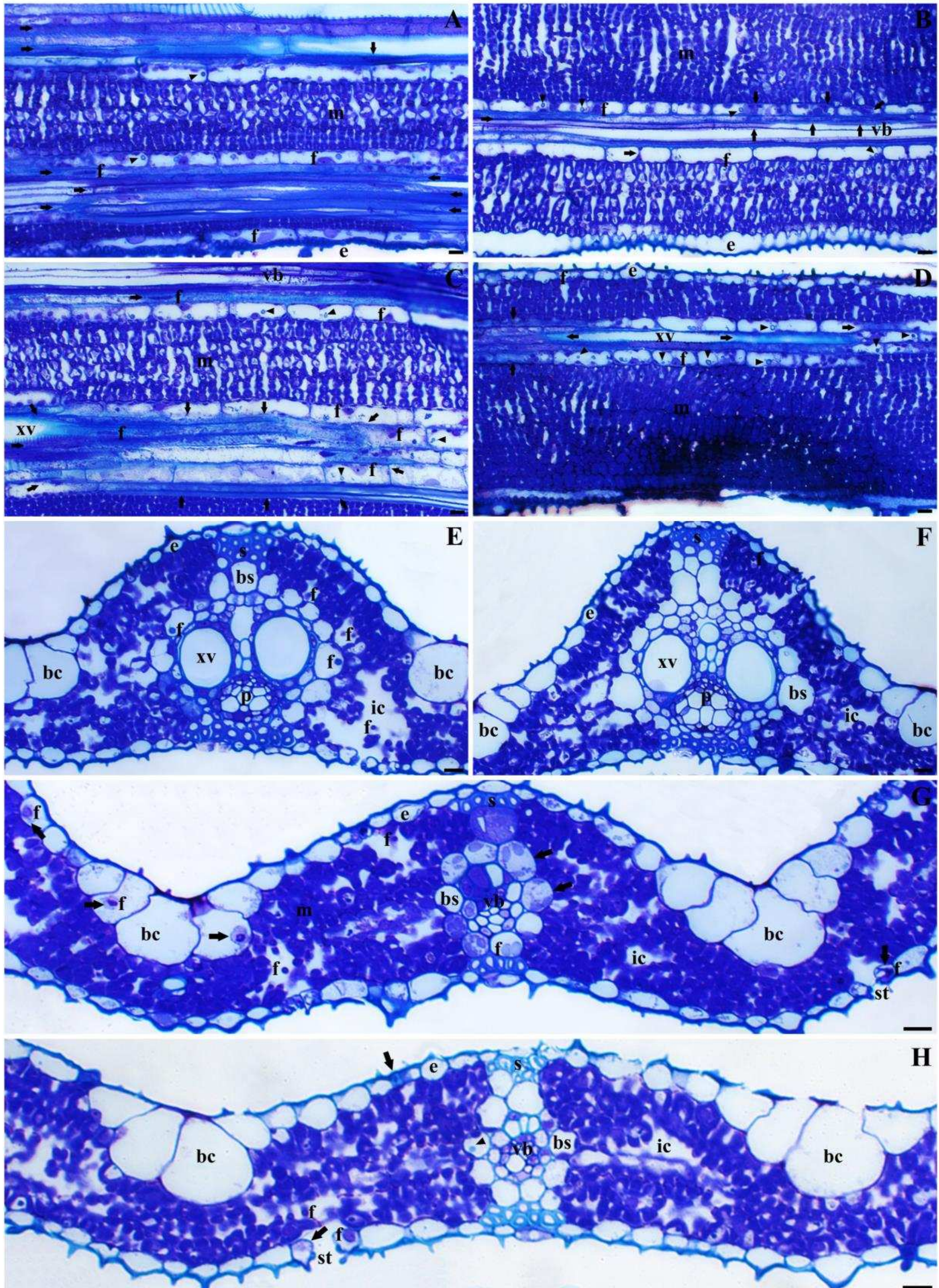
A
m
f
e
B
vb
C
xv
D
E
s
bs
xv
p
bc
ic
F
G
st
H

Figure 5. Light micrographs of longitudinal (A, B, C and D) and tranverse (E, F, G and H) sections of the leaf tissue of non-sprayed (-Fu) (A, C, E and G) or sprayed (+Fu) rice plants (B, D, E and H) with the fungicide azoxystrobin at 72 hours after inoculation with *Bipolaris oryzae*. Fungal hyphae colonized bulliform, bundle sheath, epidermal, guard, mesophyll and vascular bundle cells besides the intercellular spaces of both -Fu and +Fu plants, but fungal colonization was greatly constrained in the +Fu plants. Mesophyll cells of -Fu plants showed extensive disorganization as a result of *B. oryzae* colonization. Vascular bundle, bundle sheath, bulliform and guard cells reacted to the fungal infection by accumulating phenolic-like compounds as indicated by the staining dark-blue or purple (arrows) whereas some cells accumulated amorphous granular material around the fungal hyphae (arrows), reaction that was more prominent for the +Fu plants. Some fungal hyphae appeared dead (arrowheads). Bundle sheath (bs), bulliform cell (bc), epidermis (e), fungal hyphae (f), intercellular space (ic), mesophyll cells (m), phloem (p), slerenchyma (s), stomata (st), tracheal elements (te), vascular bundle (vb) and xylem vessels (xv). Scale bars: 10 µm.

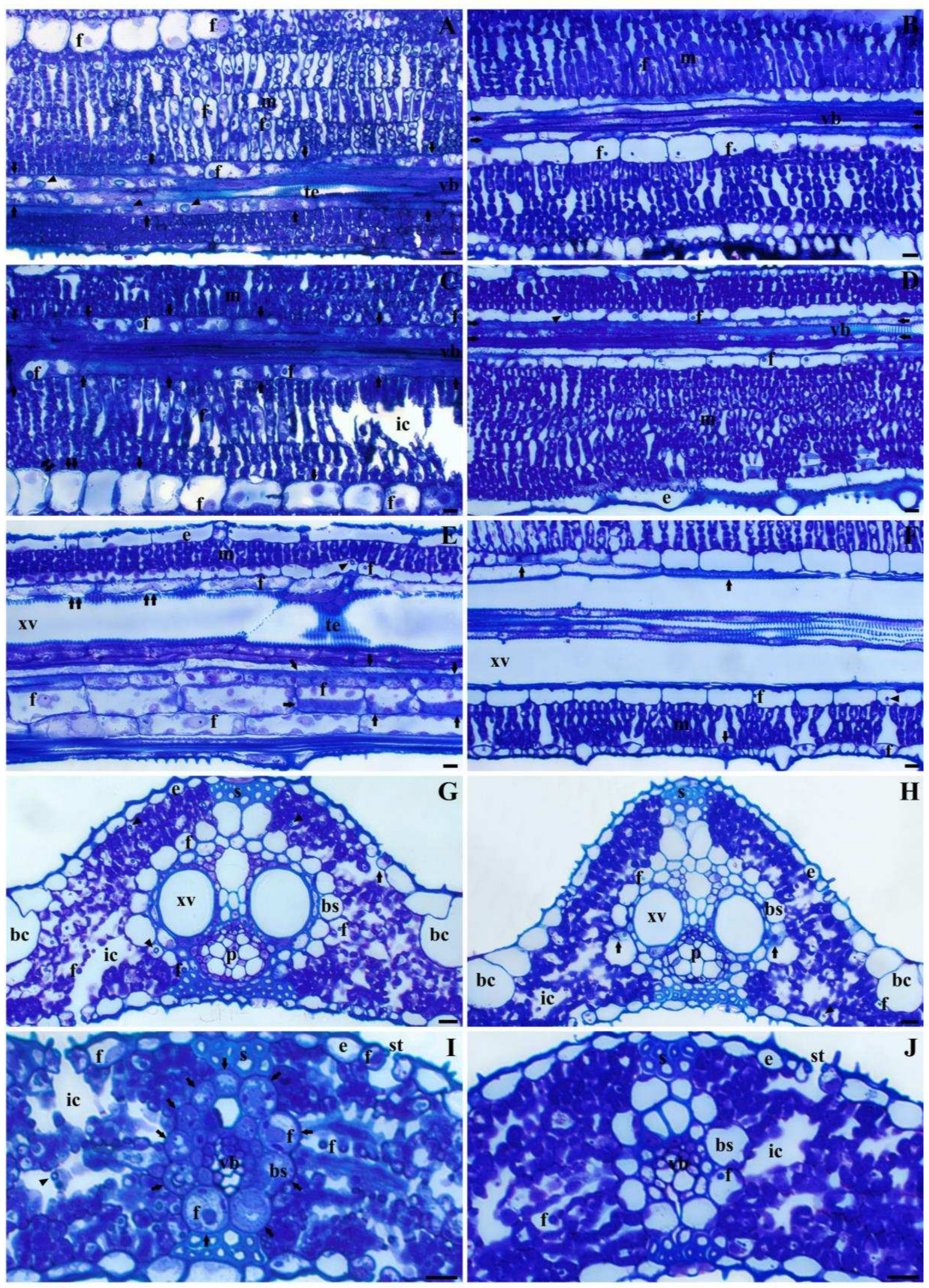
A
f
m
vb
te
B
vb
C
ic
D
e
E
xv
F
G
s
bs
bc
ic
p
H
I
st
J

Figure 6. Light micrographs of longitudinal (A, B, C and D) and tranverse (E, F, G and H) sections of the leaf tissue of non-sprayed (-Fu) (A, C, E and G) or sprayed (+Fu) rice plants (B, D, E and H) with the fungicide azoxystrobin at 144 hours after inoculation with *Bipolaris oryzae*. Fungal hyphae colonized bulliform, bundle sheath, epidermal, guard, mesophyll and vascular bundle cells besides the intercellular spaces of both -Fu and +Fu plants, but fungal colonization was greatly constrained in the +Fu plants. Mesophyll cells of -Fu plants showed extensive disorganization as a result of *B. oryzae* colonization. Vascular bundle, bundle sheath, bulliform and guard cells reacted to the fungal infection by accumulating phenolic-like compounds as indicated by the staining dark-blue or purple (arrows) whereas some cells accumulated amorphous granular material around the fungal hyphae (arrows), reaction was more prominent for the +Fu plants. Some fungal hyphae appeared dead (arrowheads). A cell membrane dislodgement was also evident in the -Fu plants (E) (double arrows). Note the more extensive disorganization of mesophyll cells (C) and accumulation of phenolic-like material in bundle cells (I) when compared to 72 hours after inoculation. Bundle sheath (bs), bulliform cell (bc), epidermis (e), fungal hyphae (f), intercellular space (ic), mesophyll cells (m), phloem (p), slerenchyma (s), stomata (st), tracheal elements (te), vascular bundle (vb) and xylem vessels (xv). Scale bars: 10 µm.

GENERAL CONCLUSIONS

1. Azoxystrobin decreases rice photosynthesis by diffusive contrainsts at the stomatal level.
2. *Bipolaris oryzae*-induced reductions in rice photosynthesis are chiefly governed by biochemical constraints.
3. *Bipolaris oryzae* infection causes chronic photoinhibition to photosynthesis, pigment degradation, activation of a wide range of defense responses as well as an extensive oxidative stress in rice.
4. Azoxystrobin preserves, to a large extent, the ability of *B. oryzae*-infected rice plants to capture, use and dissipate the light energy and relieves the fungal-induced oxidative stress.
5. The fungicidal effect of azoxystrobin rather than the priming one plays a major role in constraing brown spot development.