JONAS ALBERTO RIOS

EFFECTS OF FUNGICIDE AND HOST RESISTANCE ON THE EPIDEMIOLOGY OF WHEAT BLAST AND ON PLANT PHYSIOLOGY AND CHANGES IN THE SOURCE-SINK RELATIONSHIP ON WHEAT DURING THE INFECTION PROCESS OF *Pyricularia oryzae*

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

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BIOGRAFIA

Jonas Alberto Rios, filho de Neuza da Consolação de Souza Rios e Jaci Alves Rios, nasceu em 17 de Novembro de 1985, em Florestal, Estado de Minas Gerais. Ingressou em 2005, no curso de Agronomia da Universidade Federal de Viçosa (UFV) onde lhe foi conferido o título de Engenheiro Agrônomo.

Nesta instituição, foi bolsista de Iniciação Científica em Fitopatologia, atuando no controle de doenças, sob orientação do Prof. Laércio Zambolim.

Em Março de 2010, iniciou o curso de Mestrado em Fitopatologia na Universidade Federal Viçosa, sob orientação do Prof. Fabrício Ávila Rodrigues, defendendo dissertação de mestrado em Fevereiro de 2012.

Neste mesmo ano iniciou o curso de Doutorado em Fitopatologia na Universidade Federal de Viçosa, sob orientação do Prof. Fabrício Ávila Rodrigues, defendendo sua tese de doutorado em fevereiro de 2016.

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SUMÁRIO

RESUMO

RIOS, Jonas Alberto, D.Sc., Universidade Federal de Viçosa, fevereiro de 2016. **Efeito de fungicida e resistência de hospedeiro na epidemiologia da brusone do trigo e fisiologia da planta e alterações na relação fonte-dreno em trigo durante o processo de infecção de *Pyricularia oryzae***. Orientador: Fabrício de Ávila Rodrigues. Coorientador: Fábio Murilo da Matta.

Dois experimentos (Exp. 1 e Exp. 2) foram conduzidos em condições de campo para determinar o desenvolvimento da brusone em espigas, bem como a performance fisiológica e produtiva das cultivares BR-18 (moderadamente resistente) e Guamirim (suscetível) inoculadas com Pyricularia oryzae e tratadas (+F) ou não (–F) com o fungicida epoxiconazole (13,3%) + pyraclostrobina (5%). Relativo a Guamirim –F, BR-18 –F (efeito da resistência) resultou em redução de 44 e 64% na incidência final e severidade final, respectivamente, no Exp. 1, e 3 e 49%, respectivamente, no Exp. 2. Para a Guamirim +F (efeito do fungicida) houve redução de 65 e 77% na incidência final e severidade final, respectivamente, no Exp. 1, e 64 e 95%, respectivamente, no Exp. 2. Similarmente, houve redução maior que 75 % na taxa temporal de progresso no tratamento combinando a aplicação do fungicida e resistência de hospedeiro. Resultados obtidos na análise de regressão indicaram que a severidade encontrada nas espigas e folhas durante o período de 10-14 dias após a antese (daa) proporcionou uma maior perda de produtividade comparado a severidade encontrada aos 18-22 daa, e que a severidade da brusone nas espigas possui maior efeito negativo comparado a severidade encontrada nas folhas. Relativo a Guamirim –F, houve aumento de 0.3 e 16 % na produtividade dos grãos para BR-18 –F, 20 a 61% para Guamirim +F e 26 e 83% para BR-18 +F nos Exp. 1 e Exp. 2, respectivamente. Os tratamentos envolvendo aplicação do fungicida e cultivar resistente manteve a integridade das folhas (baseado em HAD, HLAI, HAA e HRI) e performance fotossintética (baseado em Fv/Fm, Fm, Y(II), e Y(NPQ)) das espigas e folhas em relação ao tratamento cultivar Guamirim –F. Em conclusão, os resultados do presente estudo sugerem que a integração do tratamento com fungicida e resistência de cultivar como prática de manejo para o controle da brusone em espigas. Adicionalmente, dois experimentos foram conduzidos em condições de casa de vegetação para avaliar as alterações associadas com a produção e particionamento de fotoassimilados entre a espiga e a folha bandeira em plantas de trigo infectadas com P. oryzae. Ambos experimentos foram inoculados aos 10 e 20 daa com suspensão de esporos de P. oryzae. Os resultados demonstraram uma redução dos parâmetros de fluorescência da clorofila a (Fm, Fv/Fm YII and Y(NO)) em folhas bandeiras e espigas infectadas associada a menor concentração de clorofila a + b, carotenoides e redução da capacidade de fixação do CO2 pela RuBisCO em folhas bandeiras infectadas. Em folhas bandeiras e grãos obtidos de espigas infectadas, houve redução na concentração de açúcares solúveis com um aumento da razão da concentração de hexoses sobre sacarose em folhas bandeiras. Em folhas bandeiras, houve menor atividade da sacarose fosfato sintase (SPS) e menor expressão do gene sacarose sintase associadas ao aumento da expressão e atividade das invertases. Estas alterações relacionadas a síntese e degradação da sacarose sugerem uma condição dreno dos tecidos foliares infectados. No estágio final de infecção ocorreu uma redução da concentração de amido nos grãos e aumento da sua concentração em folhas bandeiras. Adicionalmente, houve redução na atividade e expressão da ADP-glicose pirofosforilase associada com a menor expressão dos genes β- e α-amilase em folhas bandeiras e espigas. Em conclusão, os efeitos negativos da brusone na qualidade e produtividade dos grãos em trigo podem ser associados com alterações na produção e particionamento de carboidratos durante o processo de formação dos grãos.

ABSTRACT

RIOS, Jonas Alberto, D.Sc., Universidade Federal de Viçosa, February, 2016. **Effects of fungicide and host resistance on the epidemiology of wheat blast and on plant physiology and changes in the source-sink relationship on wheat during the infection process of *Pyricularia oryzae***. Adviser: Fabrício de Ávila Rodrigues. Co-Adviser: Fábio Murilo da Matta.

Two field experiments (Exp. 1 and Exp. 2) were carried out to evaluate the spike blast development as well as the physiological performance and grain yield of wheat cultivars BR-18 (partially resistant) and Guamirim (susceptible) inoculated with *Pyricularia oryzae* and treated or untreated with the fungicide 13.3% epoxiconazole + 5% pyraclostrobin. Relative to Guamirim-untreated, BR-18-untreated (resistance alone) led to 44 and 64% control of final incidence and severity, respectively, in Exp. 1, and 3 and 49% control, respectively, in Exp. 2. Guamirim-treated (fungicide alone) led to 65% control of incidence and 77% control of severity in Exp. 1, and 64% control of incidence and 95% control of severity in Exp. 2. Similarly, there was reduction higher than 75 % in the temporal rate of spike blast progress when host resistance and fungicide were combined. Results from regression analyses indicated that spike and leaf blast severity at 10-14 days after anthesis resulted in greater yield losses (highest negative slope) than severity at 18 to 22 days after anthesis, and that spike blast severity had a greater negative effect on yield than leaf blast severity. Relative to Guamirim-untreated, there was a 0.3 and 16% increase in mean yield for BR-18-untreated (resistance alone), 20 and 61% increase for Guamirim treated (fungicide alone), and 26 and 83% for BR-18 treated (moderate resistance + fungicide) in Exp. 1 and 2, respectively. By virtue of their effects on blast severity, fungicide application and cultivar resistant resulted in higher measures of leaf health (mean HAD, HLAI, HAA and HRI) and photosynthetic performance (based on F_v/F_m, F_m, Y(II), and Y(NPQ)) of both spikes and leaves than the untreated susceptible reference treatment. Results from this study suggesting that the integration of these strategies may be the best approach for managing spike blast and useful for future efforts to develop crop loss models and management guidelines for wheat blast. Additionally, two experiments were carried out in greenhouse conditions to assess the changes associated with photoassimilates production and their partitioning in source-sink relationship on flag leaves and spikes of wheat plants infected with *Pyricularia oryzae*. Flag leaves and spikes were inoculated at 10 days after anthesis (daa) (Exp. 1) and at 20 daa (Exp. 2) with a conidial suspension of *P. oryzae*. There was an impairment on chlorophyll *a* fluorescence parameters (analyzed by F_m, F_v/F_m YII and Y(NO)) on the infected flag leaves and spikes coupled with reduced concentrations of chlorophyll *a* + *b* and carotenoids as well as lower capacity of CO_2 fixation by RuBisCO in the infected flag leaves. In these leaves and grains obtained from the infected spikes, there were lower concentration of soluble sugars and an increase on the hexoses to sucrose ratio on the flag leaves. In the infected flag leaves, there was a lower sucrose phosphate synthase (SPS) activity and lower expression of sucrose synthase (*Susy*) gene coupled with higher expression and activity of acid invertases. These alterations, associated with synthase and degradation of sucrose, suggestss a status sink in the infected flag leaves. At advanced stages of fungal infection, the concentration of starch was reduced on grains whereas on the infected flag leaves its concentration was kept elevated. There were reduction on the activity of ADP-glucose pyrophosphorylase and on the expression of *ADP-glucose pyrophosphorylase* genes and down regulation of *β*- and *α-amylase* expression at late stages of fungal infection on flag leaves and spikes. In conclusion, the greatest effect of blast on both grains quality and yield can be associated with alterations in the production and partitioning of carbohydrates during the grain filling process.

GENERAL INTRODUCTION

Blast, caused by the fungus *Pyricularia oryzae* Sacc. (teleomorph: *Magnaporthe grisea* (Hebert) Barr), is an important disease of wheat (*Triticum aestivum* L.) that causes substantial grain yield and quality losses in Brazil and other South American countries were epidemics occur frequently (Goulart et al. 2007). *P. oryzae* infects all above-ground parts of the wheat plant, (Igarashi et al. 1986), causing symptoms ranging from elliptical to roundish lesions on leaves to premature bleaching and death of individual spikelets or entire spikes (Goulart et al. 2007). After first being reported in Southern Brazil almost 30 years ago, wheat blast was for years treated as a relatively minor disease. However, major epidemics and spread of this disease to new regions over the last decade have led to renewed interest in wheat blast. Several recent studies have been conducted to investigate the diversity and evolution of *P. oryzae* (Maciel et al. 2014), evaluate strategies for management of wheat blast (Maciel et al. 2014; Pagani et al. 2014), and to determine the impact of this disease on crop physiology and yield (Goulart et al. 2007; Debona et al. 2012). However, further research is still needed to better understand how wheat blast develops and affects grain yield and quality.

Although it is clear that wheat blast does impact grain yield and quality, the specific effects of this disease on components of crop physiology and crop loss is largely unknown. Moreover, little is known about the functional relationships among blast, components of wheat health and physiology, and grain yield. Crop loss information is important for disease management decision making (Johnson et al. 1990) and for evaluating the economic benefits of disease management strategies (Jesus Junior et al. 2001). Since crop yield and dry weight are often more strongly correlated with healthy rather than diseased leaf area, several measures of crop health such healthy leaf area duration (HAD) and healthy leaf area absorption (HAA) have been proposed for quantifying the effects of plant diseases on crop loss (Waggoner and Berger 1987). In addition, since damage caused by pathogens is known to affect radiation intercepted (RI) and radiation use efficiency (RUE) (Johnson et al. 1990), RI and RUE have also been used as measures of the effects of diseases on crop loss (Bergamin Filho et al. 1997; Canteri et al. 2005; Moreira et al. 2015).

Plants produce carbohydrates in photosynthetic source organs and export them toward sugar-importing sink organs (Gamm et al. 2011). Wheat grain yield is considered to be a dynamic process determined to a great extent by the capacity of the source leaf tissues to export photoassimilates to the sink grain tissues as well as by the capacity of these sinks to convert these photoassimilates into starch (Tuncel and Okita 2013). During the day, the carbon fixed in the leaves can be converted to sucrose in the cytosol and later on it is transported to the sinks and converted to starch (Zeeman et al. 2007). Some of the fixed CO_2 can be stored as transient starch in chloroplasts and broken down at night to support leaf metabolism and sucrose export (Zeeman et al. 2007). The infection process of pathogens on their hosts' tissue can considerably affect the source/sink relationships via impairments to the photosynthetic performance as well as the carbohydrate composition and partitioning within the infected tissues (Abood and Lösel 2003; Gamm et al. 2011).

The primary objectives of this study were to 1) evaluate the effects of the fungicide 13.3% epoxiconazole + 5% pyraclostrobin and cultivar resistance on incidence and severity of wheat blast on spikes under field conditions; 2) characterize the temporal progress of incidence and severity of wheat blast on spikes as influenced by partial resistance and fungicide treatment; 3) quantify relationships between leaf and spike blast intensity estimated at different times during the growing season and grain yield; 4) quantity relationships between measures of crop health (HAD and HAA) and grain yield; 5) use chlorophyll (Chl) *a* fluorescence imaging to determine the effect of wheat blast on the photosynthetic performance of wheat plants, and 6) determine the individual and integrated effects of a single preventative application of the fungicide 13.3 % epoxiconazole + 5 % pyraclostrobin and partial resistance on grain yield, HAD, HAA, Y(II), Y(NPQ), and F_v/F_m. Finally, we investigated whether the infection of flag leaves and spikes by *P. oryzae* could be possibly associated with reductions on photoassimilate production due to the lower photosynthesis and synthesis of sucrose in the flag leaves reducing, therefore its exportation and conversion into starch in the grains.

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CHAPTER 1

Fungicide and Cultivar Effects on the Development and Temporal Progress of Wheat Blast Under Field Conditions

Abstract

Spike blast of wheat, caused by *Pyricularia oryzae*, is a poorly understood disease that is very difficult to manage. Two field experiments (Exp. 1 and Exp. 2) were conducted in a region of Brazil where blast is not known to be endemic to evaluate its development as influenced by fungicide and host resistance. Plots of wheat cultivars BR-18 (partially resistant) and Guamirim (susceptible) were either treated with the fungicide epoxiconazol + piraclostrobina or left nontreated, and then inoculated with a spore suspension of *P. oryzae* at mid-anthesis. Spike blast incidence and severity, quantified at regular intervals after inoculation, increased over time, and fungicide and cultivar had statistically significant effects ($P < 0.005$) on both measures of disease and their temporal rates of progress. Relative to Guamirim-untreated, BR-18-untreated (resistance alone) led to 44 and 64% control of final incidence and severity, respectively, in Exp. 1, and 3 and 49% control, respectively, in Exp. 2. Guamirim-treated (fungicide alone) led to 65% control of incidence and 77% control of severity in Exp. 1, and 64% control of incidence and 95% control of severity in Exp. 2. For both incidence and severity, fungicide and resistance alone also reduced the temporal rate of progress relative to the susceptible untreated. However, the greatest overall efficacy was observed when resistance and fungicide were combined, with over 70 and 90% control of final incidence and severity, respectively, and over 75% reduction in the temporal rate of spike blast progress. Based on percent control, the integrated effect of resistance and fungicide was additive for incidence, severity, and their temporal rates of progress, suggesting that the integration of these strategies may be the best approach for managing spike blast.

Introduction

The development of wheat blast, caused by the fungus *Pyricularia oryzae* (Cooke) Sacc., is favored by rainy weather, temperatures ranging from 21 to 27°C, cloudy days, and high relative humidity (Goulart et al. 2007). Under such favorable conditions, yield losses as high as 60% have been reported (Goulart et al. 2007). *P. oryzae* infects all above-ground parts of the wheat plants, including leaves, culms, and spikes (Igarashi et al. 1986). On the leaves, typical symptoms are elliptical or roundish lesions with dark-brown margins and grayish centers (Goulart et al. 2007). On spikes, symptoms appear as bleaching and death of infected tissues and dark discoloration of the rachis (Goulart et al. 2007). Although the leaf-blighting stage of wheat blast may affect grain yield and quality, the greatest damage occurs when the spikes are infected (Rios et al. *this issue*), since this often leads to reduced translocation of nutrients to the grains, causing them to become shriveled, deformed, small, and lightweight (Goulart et al. 2007).

Since first being reported in 1985 in the state of Parana, Brazil (Igarashi et al. 1986), wheat blast has become widely distributed across all major wheat-producing areas in Brazil and some neighboring countries (Maciel et al. 2014). Wheat blast is now considered one of the biggest obstacles to the expansion of wheat production in Brazil (Maciel et al. 2014). Current management strategies for minimizing losses caused by wheat blast include the use of resistant cultivars, when available, in association with fungicide application (Maciel et al. 2011; Castroagudín et al. 2015). Although most commercial cultivars are susceptible to blast, BR-18 terena, BRS 229, and MGS3 Brilhante are considered to be moderately resistant (Cruz et al. 2010; Maciel et al. 2008). However, due to the high genetic diversity found in *P. oryzae* populations, partial resistance is generally not durable (Maciel et al. 2014). Of the foliar fungicides recommended for wheat blast management, the Quinone Outside Inhibitors (QoI), marketed either as single active ingredients or as premixes with demethylation inhibitors (DMI), are the most widely used in Brazil (Maciel et al. 2011).

Even after more than 30 years of research, there are still lots of unanswered questions about the epidemiology and management to wheat blast, particularly the spike blast stage of the disease. For instance, the optimum plant growth stage for spikes infection is still a subject of debate; it is unclear whether spike blast is a mono- or a polycyclic disease; the epidemiological importance of leaf blast for spike blast development is largely unknown; and further research is needed to better characterize the incubation and latent periods of spike blast. A thorough understanding of these epidemiological components of blast is important for establishing the optimum time and frequency of fungicide application for disease management. Knowledge gaps in this area may be among the reasons why there have been mixed reports, both anecdotal and published, regarding the efficacy of fungicides against wheat blast on the spikes under field conditions. For instance, Rocha et al. (2014) reported that two applications of tebuconazole, epoxiconazole + pyraclostrobin, or tebuconazole + trifloxystrobin, the first at Zadoks 45 (boot) and the second at Zadoks 65 (mid anthesis), reduced leaf blast incidence (as area under the progress curve) and severity, but were ineffective against spike blast severity. Contrastingly, however, Pagani et al. (2014) reported that two applications of the same three fungicides (the first at early heading followed by a second at the milk growth stage) reduced spike blast severity by 35 to 72%. Furthermore, Rocha et al. (2014) observed that the magnitude of fungicide effects on leaf blast incidence varied among the four wheat genotypes evaluated in the study.

The primary objectives of this study were to: (*i*) evaluate the effects of the fungicide 13.3% epoxiconazole + 5% pyraclostrobin and cultivar resistance on incidence and severity of wheat blast on spikes under field conditions and (*ii*) characterize the temporal progress of incidence and severity of wheat blast on spikes as influenced by partial resistance and fungicide treatment. Plots of a moderately resistant and a susceptible wheat cultivar were either treated with the fungicide or left untreated and then inoculated with a spore suspension of *P. oryzae.* Blast severity was assessed at regular intervals after inoculation, and the resulting data were used to quantify the temporal development of the disease through linear mixed model repeated measures and regression analyses. Results from the analyses were also used to evaluate the individual and integrated effects of 13.3 % epoxiconazole + 5 % pyraclostrobin and moderate resistance on wheat blast development, and to determine if the combined effect was additive.

Materials and Methods

Plot establishment, fungicide treatment and inoculation with *P. oryzae*. Two field experiments were conducted in an experimental area of the Federal University of Viçosa, Viçosa, Brazil, located in the southeastern region of the state of Minas Gerais (20º44’44”S, 42º50’59”W, and 661 m above sea level). The first experiment was carried out from June to September 2013 and the second from August to November 2013. Plots were planted using a Kincaid planter on June 1 and August 8 in experiments 1 and 2, respectively, at a population density of 70 plants per meter of row. Each plot (experimental unit) consisted of five 5-m-long rows, spaced 0.2 m apart, corresponding to a total plot area of 5 m^2. The distance between adjacent plots was 1 m. All plots were managed and maintained according to conventional Brazilian wheat production practices, including fertilizer application before planting based on soil chemical analysis (Embrapa 2013).

The experimental design was a randomized complete block, with a 2 × 2 factorial arrangement of fungicide treatment and cultivar in four replicate blocks. Separate plots of wheat cultivars BR-18 (moderately resistant) and Guamirim (susceptible) were either treated with the fungicide 13.3 % epoxiconazole + 5 % pyraclostrobin (Opera, Basf S.A.-São Paulo, Brazil) at growth stage 65 (mid-anthesis, Zadoks, 1974) at a rate of 0.5 L ha^{-1} or left untreated. Applications were made using a CO_2 pressurized backpack sprayer (3.1 × 10^5 Pa) with Teejet 110.03 nozzles, at a volume of 200 L ha^{-1}.

Approximately 48 hours after fungicide application, plots were spray-inoculated with a suspension containing 10^5 conidia/mL of isolate UFV/DFP-*Po*01 of *P. oryzae*. Leaves and spikes were inoculated at 18:00 hours with approximately 1,000 mL of the inoculum applied to each plot using a CO_2 pressurized backpack sprayer (3.1 × 10^5 Pa) with Teejet 110.03 nozzles. Two hours before inoculation, all plots were mist-irrigated for ten minutes to increase humidity and enhance infection.

Weather data (precipitation, average relative humidity, and maximum, average and minimum temperature) were obtained from an onsite weather station.

Blast assessment and data analysis. Incidence and severity of wheat blast on the spikes were assessed on 40 arbitrarily selected spikes in each plot at 10, 14, 18, and 22 days after inoculation (dai). Incidence was rated as the mean percentage of spikes diseased out of the 40 spikes sampled ([number of diseased spikes/40] × 100), whereas severity was quantified as the mean proportion of diseased spikelet per spike ([diseased spikelets/total spikelets rated] × 100). All incidence and severity data were arcsine-square-root-transformed prior to analysis to stabilize variance, and each experiment and measure of disease was analyzed separately.

Effect of cultivar resistance and fungicide treatment on incidence and severity of wheat blast. To evaluate the integrated effects of cultivar resistance and fungicide treatment on wheat blast, models were fitted to the arcsine-square-root-transformed incidence and severity data with cultivar, fungicide treatment, and disease assessment time as categorical fixed effects and block as a random effect. Since incidence and severity data were collected as temporal repeated measures on the same experimental units and as such were correlated in time (Littell et al. 2006), the random *_residual_* statement and *type* option in GLIMMIX were used to account for, and model, the covariance structure of the within-subject data. Models were fitted using the GLIMMIX procedure of SAS (Littell et al. 2006). The *slice* option in the *lsmeans* statement of GLIMMIX was then used to compare the least squares means among cultivars × fungicide application combinations at each assessment times (growth stages). The model fitted to the data can be written as:

$$y_{ijkl} = \theta + \alpha_i + \beta_j + \tau_l + (\alpha\beta)_{ij} + (\alpha\tau)_{il} + (\beta\tau)_{jl} + (\alpha\beta\tau)_{ijl} + b_k + e_{ijkl} \qquad (1)$$

where: y_{ijkl} is the response (arcsine-square root-transformed incidence or severity), α_i, β_j, and τ_l are the fixed effects of cultivars, fungicide application, and disease assessment time; $(\alpha\beta)_{ij}$, $(\alpha\tau)_{il}$, $(\beta\tau)_{jl}$, and $(\alpha\beta\tau)_{ijl}$ are two- and three-way interactions among cultivars, fungicide application, and assessment time; and θ, b_k, and e_{ijkl} are the constant (intercept), random effect of the *k*-th block, and residual, respectively.

Effect of cultivar resistance and fungicide application on the temporal change in wheat blast intensity on the spikes. For this stage of the analysis, cultivar x fungicide combination, hereafter referred to as treatment (T1 – BR-18-treated, T2 – BR-18-untreated, T3 – Guamirim-treated**,** and T4 – Guamirim-untreated.), was used as the independent variable and fixed effect, block as the random effect, and time of disease assessment as a continuous covariate to estimate regression parameters (slopes and intercepts) for the temporal changes in spike blast incidence and severity. As described above, since repeated measures of incidence and severity are correlated, the random *_residual_* statement and *type* option in GLIMMIX were again used to model the covariance structure of the data.

The significance of and differences between regression slopes (rates of temporal change in blast intensity) were evaluated using *estimate* statements in GLIMMIX. The model fitted to the data can be written as:

$$y_{ijk} = \theta + \alpha_i + \delta X_j + \Delta_i X_j + b_k + e_{ijk} \qquad (2)$$

where: X_j is the j-th observation of the covariable assessment time, δ is the (main) effect of the covariable, Δ_i is the interaction effect of the covariable (effect of treatment on the relationship between y and X), and θ, α_i, b_k, and e_{ijk} are as described above.

Results

Weather conditions and overall blast development. For the period between inoculation and final disease assessment in Exp. 1, temperatures ranged from 5.48 to 26.46ºC, with an average of 16.27ºC; relative humidity from 41.9 to 76.6%, with an average of 60.06%; and total rainfall was 46.95 mm (Fig. 1). For the corresponding period in Exp. 2, temperatures ranged from 13.6 to 31.12ºC, with an average of 21.13ºC; relative humidity from 45.2 to 90.9%, with average of 75.8%; and total rainfall was 112.5 mm (Fig. 1).

The overall level of wheat blast on the spikes varied between the two experiments and among cultivar x fungicide treatment combinations within each experiment (Fig. 2). In both experiments, incidence and severity increased over time, generally reaching the highest levels for all treatments at 22 days after inoculation. In Exp. 1, mean incidence, averaged across replicates, ranged from 8.1 to 19.4% and mean severity from 0.3 to 1.3% at 10 dai compared to 12.5 to 50.0% for incidence and 0.5 to 5.1% for severity at 22 dai. At all assessment times, untreated plots of the susceptible cultivar Guamirim had the highest levels of blast whereas treated plots of the moderately resistant cultivar BR-18 had the lowest (Fig. 2). Similar trends were observed in experiment 2, but mean incidence and severity were considerably higher for all treatment x cultivar combinations, with mean incidence ranged from 15 to 62% at 10 dai, and from 28 to 97% at 22 dai (Fig. 2B). The corresponding ranges for mean severity were 0.4 to 4.5% at 10 dai and 1.5 to 56.8% at 22 dai (Fig. 2D).

Effect of cultivar resistance and fungicide treatment on incidence and severity of wheat blast. Table 1 summarizes the main and interaction effects of cultivar, fungicide treatment, and assessment time on incidence and severity of wheat blast in both experiments. For incidence, all main and interaction effects were statistically significant ($P < 0.05$) in Exp. 1, whereas in Exp. 2, all main and interaction effects, except for interactions between cultivar and fungicide and cultivar and assessment time, were statistically significant (Table 1). For severity, all main effects as well as their interactions were statistically significant in both experiments (Table 1).

As expected, at all assessment times in both experiments, the moderately resistant cultivar (BR-18) generally had significantly lower baseline levels of mean blast incidence and severity than the susceptible cultivar (Guamirim) (based on the untreated check in Table 2), however, the magnitude of the difference between the two cultivars varied among disease assessment times (hence the significant cultivar x assessment time interaction in most cases). For instance, in Exp. 1, incidence was 55% lower on BR-18 compared to Guamirim at 10 dai, but 44% lower at 22 dai (Fig. 2A). Similarly, when compared to Guamirim, mean severity was 75% lower on BR-18 at 10 dai and 64% lower at 22 dai (Fig. 2C). In Exp. 2, when disease levels were much higher, there was a 19% difference in incidence between the two cultivars at 10 dai but only a 3% difference at 22 dai. However, the difference in severity between the two cultivar ranged from 49% (at 22 dai) to 67% (at 10 dai), suggesting that resistance had little effect on infection of the spikes (based on incidence), but substantially reduced the spread of the disease within infected spikes (based on severity).

The magnitude of the differences in mean incidence and severity between the two cultivars in plots treated with 13.3% epoxiconazole + 5% pyraclostrobin tended to be smaller than in the untreated check (Fig. 2). Relative to Guamirim, BR-18 had 7 to 34% lower mean incidence and 22 to 56% lower mean severity in Exp. 1, depending on when disease was assessed. The corresponding differences in Exp. 2 were 19 to 38% for incidence and 15 to 54% for severity. However, with few exceptions (incidence and severity at 22 dai and incidence at 14 dai in Exp. 1), differences in mean blast intensity between the two cultivars in fungicide treated plots were not statistically significant ($P > 0.05$, Table 2). For both cultivars, at all assessment times, treated plots had significantly lower mean incidence and severity than untreated plots ($P < 0.002$, Table 3). The only exception was for incidence and severity on the resistant cultivar (BR-18) at 10 dai in Exp. 1 (when overall disease levels were very low). However, the magnitude of the fungicide effect varied between cultivars and among assessment times. For instance, differences in mean incidence and severity between treated and non-treated plots tended to be greater for the susceptible (Guamirim) than for the moderately resistant (BR-18) cultivar and towards the end of the epidemic (at 18 and 22 dai) than at the beginning (at 10 and 14 dai) (Fig. 2).

Based on linear contrasts, for both incidence and severity, in both experiments moderate resistance alone (BR-18 untreated, T2), fungicide treatment alone (Guamirim treated, T3), and the combination of moderate resistance and fungicide treatment (BR-18 treated, T1) all resulted in significantly lower final (at 22 dai) blast incidence and severity than the untreated susceptible (T4, the worst case scenario). Using T4 as the reference for comparison, percent control of final disease intensity (*C*) was estimated for T1, T2 and T3 as $C = ([\bar{X}_{T4} - \bar{X}_{T1,\ T2\ or\ T3}]\ /\bar{X}_{T4})\bullet 100$, where $\bar{X}_{\bullet}$ represents mean incidence or severity at 22 dai for T1, T2, T3 or T4 (the reference). Relative to T4, resistance alone (T2) led to a 44 and 64% control of final incidence and severity, respectively, in Exp. 1 and a 3 and 49% control, respectively, in Exp. 2 (Table 4). T3 (fungicide alone) led to a 65% control of incidence and a 77% control of severity in Exp. 1 and a 64% control of incidence and a 95% control of severity in Exp. 2. The greatest overall efficacy, based on percent control relative to T4, was observed when the fungicide was combined with moderate resistance (T1), with a 75 and 90% control of incidence and severity, respectively, in Exp. 1 and 71 and 97% control of incidence and severity, respectively, in Exp. 2 (Table 4).

Based on the principle of treatment independence, one can determine whether the combined effect of fungicide and resistance is additive by using the formula $C_{F+R} = 1 - [(1 - C_F) \text{ x } (1 - C_R)]$, where C_{F+R} is the percent control due to fungicide + resistance and C_F and C_R are the percent control due to fungicide alone and resistance alone, respectively (Willyerd et al. 2012). For instance, based on the data shown in Table 4, C_R (T2) and C_F (T3) for blast severity in Exp.1 were 64 and 77%, respectively. Therefore, under the assumption of additivity, $C_{F+R} = 1 - [(1 - 0.77) \times (1 - 0.64] = 0.915$, which is very similar to the value estimated for T1 using the raw data (0.896). Similar results were obtained for severity in Exp. 2 and incidence in both experiments (Table 4). Therefore, one may conclude that the integrated effect of fungicide application and moderate resistant is additive in terms of percent control of spike blast under the conditions of this study.

Temporal change in wheat blast on spikes as influenced by cultivar resistance and fungicide treatment. Results from linear mixed model repeated measures analyses showed that in the absence of fungicide, incidence and severity increased over time in both experiments. Except for comparisons between 18 and 22 dai, in most cases, pairwise comparisons between assessment times in the absence of fungicide application were statistically significant (Table 5), with late assessment times generally having significantly higher mean levels of incidence and severity than early assessment times. However, in the presence of fungicide, most pairwise comparisons of incidence and severity between assessment times were not statistically significant ($P > 0.05$), particularly for the moderately resistant cultivar BR-18 (Table 5). Notable exceptions were for comparisons of incidence between the earliest (10 dai) and later assessment times (14, 18, and 22 dai) when baseline disease levels were low (unfavorable environmental conditions in Exp. 1) and comparisons of severity between these same assessment times when baseline levels of blast were high (favorable environmental conditions in Exp. 2).

To formally quantify the temporal change of wheat blast on spikes and to evaluate the effects of cultivar resistance and fungicide treatment on the rate of disease progress, linear mixed model regression analyses were used to estimate and compare slopes among treatments. A generic model for the temporal change in wheat blast incidence and severity can be written as: $y_{ij} = a_j + b_i$ (dai), where a_j represents a separate intercept for each treatment and b_i a separate slope for each treatment (i.e., a significant treatment × dai interaction [$P < 0.001$]). As indicated by the estimated slopes in Table 6 and by Fig. 3, both incidence and severity increased over time, with highest rates of increase for Guamirim (T4). Pairwise comparisons between slopes were performed using the *estimate* statement in GLIMMIX (Table 7). In both experiments, for both incidence and severity, fungicide treatment alone (T3; Guamirim, treated), resistance alone (T2; BR-18, untreated), and resistance plus fungicide treatment (T1; BR-18, treated) all led to a significant reduction in the rate of wheat blast progress relative to the susceptible untreated (T4). The only exceptions were for the effects of T2 on severity in Exp. 1 and incidence in Exp. 2 (Table

7). Relative to T4, T1 reduced the rate of increase in blast incidence by 81% in Exp. 1 and 75% in Exp. 2, and the rate of increase in severity by 78% and 86% in experiments 1 and 2, respectively. T3 reduced incidence progress rate by 75 and 77% and severity progress rate by 67 and 88% in experiments 1 and 2, respectively. Resistance alone (T2) provided less than a 38% reduction in the rate of progress of both incidence and severity in the two experiments (Table 4). As was the case with final disease severity, the combined effect of 13.3% epoxiconazole + 5% pyraclostrobin and moderate resistance on the temporal chance in spike blast incidence and severity was additive based on percent progress rate reduction relative to the untreated susceptible reference (Table 4).

Discussion

Blast is currently one of the most difficult wheat diseases to manage (Maciel et al. 2014; Goulart et al. 2007), and this may be due in part to the lack of sufficient information about its epidemiology. Previous reports suggest that when used individually, fungicide and resistance are useful approaches for managing wheat blast on the spikes, but their effects have been highly variable (Goulart et al. 1996; Maciel et al. 2011; Urashima et al. 2005), with the latter being influenced by high natural variability in the pathogen population (Maciel et al. 2014). Here we report on the individual and combined effects of host resistance and fungicide application on the development of wheat blast on spikes based on data from two artificially inoculated field experiments conducted under distinct environmental conditions in an area of Brazil where the disease is not known to be endemic. In addition to quantifying the efficacy and additivity of integrating host resistance and fungicide to manage blast, the conditions under which this study was conducted allowed us to investigate the temporal development of blast with and without management interventions from a primary and known source of inoculum.

Using data from the untreated susceptible check, the development of wheat blast on the spikes under unfavorable and favorable field conditions, in the absence of fungicide, was evaluated and characterized. Temperature, relative humidity, and rainfall were within favorable ranges for wheat blast development on more days during Exp. 2 than in Exp. 1. Blast epidemics typically occur in rainy seasons, with temperatures ranging from 21 to 27^{o}C and high relative humidity (Goulart et al. 2007). Consequently, the second experiment had higher levels of blast incidence and severity than the first. Mean incidence at 10 dai was 19% in Exp. 1 compared to 62% in Exp. 2. Correspondingly, mean severity on the susceptible cultivar without fungicide application at 10 dai was 4.5% in Exp. 2 compared to 1.3% in Exp. 1. In both experiments, incidence and severity increased over time, suggesting that wheat blast on spikes was a polycyclic disease and that the temporal change in disease intensity was likely due to the appearance of newly diseased spikes as well as expansion of lesions on previously infected spikes. Under disease favorable conditions (Exp. 2), severity (on the transformed scale) increased almost 6 times faster than under less favorable conditions (Exp. 1); however, the rate of increase in incidence was comparable between the two experiments. These results suggest that once the initial level of infection is high (more than 60% incidence at 10 dai in Exp. 2), spread of the disease within the spike may be more important than new infections for temporal increase in blast intensity under favorable conditions in regions where the disease is not known to be endemic.

Under the conditions of this study, a single preventative application of 13.3% epoxiconazole + 5% pyraclostrobin at mid-anthesis reduced the initial level of spike blast (at 10 dai), the temporal rate of blast increase, and consequently, final blast intensity (at 22 dai), both in terms of incidence and severity. These results suggest that this fungicide reduced wheat blast on spikes by minimizing fungal infection (initial incidence) and suppressing lesion expansion and possibly sporulation, thus reducing temporal disease progress. Indeed, QoI fungicides are known to affect spore germination and penetration of the host tissue (Barlett et al. 2002), both of which are important for successful fungal infection, whereas DMI fungicides affect mycelial growth, which is important for colonization and disease spread within plant tissues (Kuck et al. 2012). However, the overall efficacy of DMI and QoI fungicides against wheat blast on spikes, both as solo active ingredients and premixes, varies considerably among studies. For instance, our results showed that a single application of 13.3% epoxiconazole + 5% pyraclostrobin at mid-anthesis was sufficient to reduce the final severity of wheat blast on spikes and the rate of disease progress by 95 and 88%, respectively, under favorable conditions. Based on results from naturally infected experiments conducted in an area where the disease is endemic, Pagani et al. (2014) demonstrated that two applications of this same fungicide were necessary to reduce blast severity on spikes by 72% in 2010 to 48% in 2011. Rocha et al. (2014) showed that a similar two-treatment fungicide program applied to artificially-inoculated potted plants reduced blast severity on the flag leaf by 96 to 100% but only reduced spike blast severity by 5 to 25%.

Differences among our experiments and those conducted by Pagani et al. (2014) and Rocha et al. (2014) in terms of the timing of the preventative fungicide application relative to infection (inoculation) and the conditions under which blast developed may explain, at least in part, the observed differences in fungicide efficacy. Even without conclusive data regarding the optimum time for *P. oryzae* infection of wheat spikes and the length of the infection window, late-boot to early-grain-fill is often recommended as the window during which fungicides should be applied to manage wheat blast on spike, since the greatest yield losses due to blast usually occurs when the first symptoms are observed shortly after heading and during early grain fill (Goulart et al. 1994; Rios et al. *this issue*). The relatively inferior efficacy of epoxiconazole + pyraclostrobin in the study conducted by Rocha et al. (2014) may be due in part to the fact that fungal infection probably occurred several days after the preventative boot stage (Zadoks 45) application, since plants were inoculated between late-heading and early-anthesis (Zadoks 58-60). It could take up to a week or more for plants to progress from growth stage Zadoks 45 to 60, consequently, the fungicide could have been less effective by the time the spikes were inoculated and infection occurred. In the case of the study by Pagani et al. (2014), which was conducted under natural infection in the Brazilian mid-west where blast is endemic, relatively inferior fungicide efficacy when compared to our study (conducted in a region where blast is not known to be endemic) may be due to multiple fungal infections and infection of secondary tillers, some of which likely occurred after treatment application when the fungicide was no longer effective. Although a second and putatively curative application was made (at the mike stage) in the study by Pagani et al. (2014), it is difficult to discern from the results the curative effect of the fungicide or even its effect on possible later infections or infection of secondary tillers, since single-application treatments at heading alone and at milk alone were not included in the study for comparison with the heading + milk application.

The mean levels of both blast incidence and severity as well as the temporal rate of disease progress were generally lower on the moderately resistant cultivar BR-18 than the susceptible cultivar Guamirim. This is not surprising, since the resistance of BR-18 to wheat blast has been well documented (Goulart and Paiva 1992; Goulart et al. 2007; Arruda et al. 2005), so much so that it is often recommended for use in blast resistance breeding programs (Urashima et al. 2004). However, results from the present study showed that the magnitude of the difference, particularly in incidence, between the cultivars tended to be lower under highly disease-conducive weather conditions, suggesting that host resistance alone was not sufficient to reduce fungal infection (based on incidence) under wet, humid conditions. Indeed, relative to Guamirim, BR-18 led to a 44% reduction in final blast incidence in Exp. 1 when environmental conditions were less favorable for disease development, but only a 3% reduction in Exp. 2 when environmental conditions were much more conducive. In fact, the temporal rate of increase in blast incidence was not significantly different between the two cultivars in Exp. 2. By contrast, BR-18 consistently had lower mean blast severity and lower rates of increase in severity than Guamirim. These results suggest that under favorable environmental conditions, the genetic resistance presented by BR-18 had little effect on fungal infection of the spikes (based on incidence), but substantially reduced the spread of the disease within infected spikes (based on severity) by close to 50% and the temporal rate of spread within the spike by approximately 30%.

While host resistance alone and fungicide application alone both reduced spike blast development relative to the untreated susceptible reference (Guamirim, untreated), combining BR-18 and fungicide application provided the highest overall levels of mean incidence and severity control as well as the greatest percent reduction in the temporal rates of incidence and severity progress relative to the reference. Based on their individual effects on incidence and severity, the findings of the present study suggest that a preventative application of fungicide contributed to spike blast management by reducing both initial fungal infection (based on incidence) and disease spread within the spike (based on severity), whereas BR-18 seemed to act primarily by reducing spread within the spike, particularly under favorable environmental conditions. Consequently, when combined, the two strategies may be complementing each other to provide higher levels of efficacy than when used as individual management strategies. The test of additivity supports this hypothesis. Based on percent control relative to the susceptible untreated, the integration of BR-18 (moderate resistance) and 13.3% epoxiconazole + 5% pyraclostrobin was additive for blast incidence and severity as well as the temporal rate of blast progress. These findings are consistent with those reported for Fusarium head blight on wheat (Willyerd et al. 2012), a spike disease that is very similar to blast. The benefits of integrating multiple management strategies have been previously reported for leaf blast on wheat (Cruz et al. 2011), but few studies have evaluated the integrated management of wheat blast on spike under favorable and unfavorable environmental conditions as was demonstrated in the present study.

Taking advantage of the fact that wheat blast is not known to be endemic in the area of Viçosa, Minas Gerais, Brazil, where small grain crops are not widely cultivated, we conducted inoculated field experiments from which we were able to gain very useful insights into the epidemiology and management of the spike blast stage of the disease. Based on our findings, one may conclude that i) wheat blast on spike is a polycyclic disease since both mean incidence and severity increased over time, ii) once spikes are infected, disease spread within the spike may be the primary means by which disease intensity increases over time; iii) under environmental conditions favorable for the development of wheat blast, partial resistance (at least in BR-18) seems to have little or no effect on primary infection (based on incidence), but reduces disease spread within infected spikes (based on severity); iv) a single anthesis preventative application of the QoI + DMI premix fungicide used in this study contributes to the management of wheat blast on spike by reducing both infection and disease spread within the spike and, consequently, the temporal rate of spike blast progress; and v) the combination of moderate resistance and fungicide has an additive effect on spike blast control, providing greater efficacy than fungicide application or resistance alone.

Since the results summarized above are from artificially inoculated experiments conducted in an area where blast is not endemic, they may not entirely reflect natural in-field blast development (and management) in areas of Bolivia and parts of southern Brazil where the disease is endemic. However, our findings may be of direct relevance to how blast is likely to develop in several major wheat-growing regions of the US Midwest and upper Great Plains. Previous studies have shown the *Lilium* pathotype of *M. oryzae*, the causal agent of gray leaf spot on ryegrass, does not service well under the harsh winter conditions of northern wheat-producing states in the US (Harmon and Latin 2003, 2005). It therefore seems reasonable to assume that if and when wheat blast becomes established in the US, the pathotype that infects wheat will likely not overwinter in the upper Midwest and upper Great Plains and as such will have to be reintroduce (likely from the south) every season. If this is indeed the case, then the temporal progress of spike blast in these regions of the US and its management with fungicides may be comparable to what is reported in this investigation.

However, much research is still needed to better understand the biology and epidemiology of wheat blast and develop and implement effective strategies for its management. For instance, while our results showed that spike blast incidence increased over time from a known primary source of inoculum, questions still remain as to whether this increase was due to new infections or to the development of latent infections. In essence, these are questions about i) latent and incubation periods, ii) optimum growth stage for infection, and iii) associations, if any, among latent/inoculation period, growth stage, and weather conditions. These questions can only be answered with further research, particularly under controlled conditions, and their answers have direct implications for management. For instance, if the infection window is narrow, then a single well-timed fungicide application may be sufficient for effective control, or, depending on the uniformity of the crop, a later application may be necessary to protect secondary tillers, as was shown to be the case for Fusarium head blight, a similar disease (D'Angelo et al. 2014). However, if spikes are susceptible for an extended period and the level of susceptibility is consistently high during that period, then multiple applications will have to be considered as suggested by studies conducted in areas where the disease is endemic (Pagani et al. 2014). Answers to question about infection also have implications for the use of cultural practices such as the management of in-field sources of inoculum like basal leaves, recently reported as a potentially important source of inoculum (Cruz et al. 2015), and planting date, suggested by Goulart et al. (2007) as a strategy for escaping infection.

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Table 1. Summary statistics from linear mixed model analyses of the effects of cultivars (C), fungicide (F), disease assessment time (AT) and their interactions on incidence and severity of wheat blast on wheat spikes in field experiments 1 and 2 conducted in Viçosa, Minas Gerais, Brazil, 2013

Responses[a]	Factors[b]	Experiment 1[c]		Experiment 2[c]	
		F values	*P* values	*F* values	*P* values
Incidence	C	76.50	<0.001	55.91	<0.001
	F	353.57	<0.001	1496.11	<0.001
	C × F	20.12	<0.001	3.92	0.061
	AT	101.58	<0.001	64.42	<0.001
	C × AT	3.94	0.016	2.05	0.169
	F × AT	28.30	<0.001	25.66	<0.001
	C x F x AT	10.75	<0.001	3.66	0.050
Severity	C	208.08	<0.001	30.64	<0.001
	F	309.50	<0.001	275.79	<0.001
	C × F	101.16	<0.001	17.61	0.003
	AT	59.73	<0.001	352.56	<0.001
	C × AT	8.39	<0.001	14.18	<0.001
	F × AT	21.35	<0.001	199.31	<0.001
	C × F × AT	9.36	<0.001	30.64	<0.001

[a] Wheat blast incidence was estimated as the percentage of visually diseased (bleached) spike in a sample of 40 spikes evaluated per plot and severity was estimated as the mean percentage of spike area diseased.

[b] Main and interaction effects of cultivars (C; BR-18, moderately resistant and Guamirim, susceptible), fungicide treatment (F, with and without fungicide) and time of disease assessment (AT; 10, 14, 18, and 22 days after inoculation).

[c] F-statistics and probability values from the fit of linear mixed models to arcsine-square root-transformed blast incidence and severity data. Experiment 1 was conducted between June and September and Experiment 2 between August and November 2013.

Table 2. Probably values for pairwise comparisons of least squares means between cultivars at fixed levels of fungicide treatment and disease assessment time from linear model mixed analyses of the effects of cultivars, fungicide treatment, and assessment time on arcsine-square root-transformed incidence and severity of wheat blast on spike from field experiments 1 and 2 conducted in Viçosa, Minas Gerais, Brazil, in 2013

Fungicide[a]	AT[b]	Contrasts[c]	Experiment 1[d]		Experiment 2[d]	
			Incidence	Severity	Incidence	Severity
Check	10	BR-18 *vs* Guam	<0.001	<0.001	0.050	0.003
	14	BR-18 *vs* Guam	0.326	<0.001	<0.001	<0.001
	18	BR-18 *vs* Guam	<0.001	<0.001	0.300	<0.001
	22	BR-18 *vs* Guam	<0.001	<0.001	0.430	<0.001
Treated	10	BR-18 *vs* Guam	0.672	0.272	0.059	0.175
	14	BR-18 *vs* Guam	0.003	0.091	0.249	0.674
	18	BR-18 *vs* Guam	0.352	0.330	0.262	0.435
	22	BR-18 *vs* Guam	0.013	0.001	0.369	0.418

[a] Plots were either treated with the fungicide 13.3 % epoxiconazole + 5 % pyraclostrobin (treated) or left untreated (check).

[b] Disease assessment time (AT) - Incidence (percentage of visually diseased spike) and severity (mean percentage of spike area diseased) of wheat blast on spike were assessed on 40 arbitrarily selected spikes in each plot at 10, 14, 18, and 22 days after inoculation.

[c] Cultivars BR-18 (moderately resistant) and Guamirim (susceptible).

Table 3. Probably values for pairwise comparisons of least squares means between fungicide treated and the untreated check at fixed levels of cultivars and disease assessment time from linear model mixed analyses of the effects of cultivars, fungicide treatment, and assessment time on arcsine-square root-transformed incidence and severity of wheat blast on spike from field experiments 1 and 2 conducted in Viçosa, Minas Gerais, Brazil, in 2013

Cultivars[a]	AT[b]	Contrasts[c]	Experiment 1		Experiment 2	
			Incidence	Severity	Incidence	Severity
BR-18	10	Treated *vs* Check	0.641	0.672	<0.001	0.015
	14	Treated *vs* Check	<0.001	0.017	<0.001	<0.001
	18	Treated *vs* Check	<0.001	0.001	<0.001	<0.001
	22	Treated *vs* Check	<0.001	<0.001	<0.001	<0.001
Guamirim	10	Treated *vs* Check	<0.001	<0.001	<0.001	0.003
	14	Treated *vs* Check	<0.001	<0.001	<0.001	<0.001
	18	Treated *vs* Check	<0.001	<0.001	<0.001	<0.001
	22	Treated *vs* Check	<0.001	<0.001	<0.001	<0.001

[a] Cultivars BR-18 (moderately resistant) and Guamirim (susceptible).

[b] Disease assessment time (AT). Incidence (percentage of visually diseased spike) and severity (mean percentage of spike area diseased) of wheat blast were assessed on 40 arbitrarily selected spikes in each plot at 10, 14, 18, and 22 days after inoculation.

[c] Plots were either treated with 13.3 % epoxiconazole + 5 % pyraclostrobin (treated) or left untreated (check).

Table 4. Percent control of final mean and reduction of temporal rate of progress of wheat blast incidence and severity on spike for different cultivars × fungicide treatments combinations relative to an untreated susceptible check from field experiments 1 and 2 conducted in Viçosa, Minas Gerais, Brazil, in 2013

		Final blast intensity[c]		Temporal rate of progress[c]	
Responses[a]	Contrasts[b]	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Incidence	T1 vs T4	75 (80)[d]	71 (65)	81 (84)	75 (72)
	T2 vs T4	44	3	38	-23
	T3 vs T4	65	64	75	77
Severity	T1 vs T4	90 (92)	97 (98)	78 (78)	86 (92)
	T2 vs T4	64	49	33	29
	T3 vs T4	77	95	67	88

[a] Wheat blast incidence was estimated as the percentage of visually diseased (bleached) spike in a sample of 40 spikes evaluated per plot and severity was estimated as the mean percentage of spike area diseased.

[b] Cultivars × fungicide treatments combinations, where T1 = BR-18 (moderately resistant), treated with the fungicide; T2 = BR-18 untreated; T3 = Guamirim (susceptible) treated; and T4 = Guamirim, untreated (the reference treatment).

[c] Final wheat blast incidence and severity on spikes rated at 22 days after inoculation. Experiment 1 was between June and September and Experiment 2 between August and November 2013.

[d] Percent control and reduction of temporal progress of wheat blast was estimated as $C = ([\bar{X}_{T4} - \bar{X}_{T1,\ T2\ or\ T3}]\ /\bar{X}_{T4})\bullet 100$, where $\bar{X}_{\bullet}$ represents mean or estimated rate of progress of incidence or severity for treatments T1, T2, T3 or T4 (the reference treatment). Values in parentheses represent expected percent control under the assumption of additivity between fungicide treatment and the level of resistance of the cultivar.

Table 5. Probably values for pairwise comparisons of least squares means between disease assessment time at fixed levels of cultivars and fungicide treatments from linear model mixed analyses of the effects of cultivars, fungicide treatment, and assessment time on arcsine-square root-transformed wheat blast incidence and severity on spike from field experiments 1 and 2 conducted in Viçosa, Minas Gerais, Brazil, in 2013

Cultivars[a]	Fungicide[b]	Contrasts[c]	Experiment 1[d]		Experiment 2[d]	
			Incidence	Severity	Incidence	Severity
BR-18	Treated	10 *vs* 14	0.011	0.716	0.101	<0.001
		10 *vs* 18	0.001	0.129	0.512	<0.001
		10 *vs* 22	0.011	0.057	0.009	0.036
		14 *vs* 18	0.236	0.162	0.809	0.442
		14 *vs* 22	0.739	0.094	0.224	0.566
		18 *vs* 22	0.414	0.586	0.419	0.802
	Check	10 *vs* 14	<0.001	0.007	0.014	<0.001
		10 *vs* 18	<0.001	<0.001	<0.001	<0.001
		10 *vs* 22	<0.001	<0.001	<0.001	<0.001
		14 *vs* 18	0.033	0.012	<0.001	<0.001
		14 *vs* 22	0.796	<0.001	<0.001	<0.001
		18 *vs* 22	0.017	<0.001	0.664	<0.001
Guamirim	Treated	10 *vs* 14	<0.001	0.274	0.539	0.026
		10 *vs* 18	<0.001	0.163	0.445	0.001
		10 *vs* 22	<0.001	<0.001	0.042	0.016
		14 *vs* 18	0.170	0.599	0.613	0.083
		14 *vs* 22	0.769	<0.001	0.161	0.086
		18 *vs* 22	0.293	<0.001	0.707	0.238
	Check	10 *vs* 14	<0.001	<0.001	<0.001	<0.001
		10 *vs* 18	<0.001	<0.001	<0.001	<0.001
		10 *vs* 22	<0.001	<0.001	<0.001	<0.001
		14 *vs* 18	<0.001	0.053	0.097	<0.001
		14 *vs* 22	<0.001	0.088	0.022	<0.001
		18 *vs* 22	0.125	0.991	0.997	<0.001

[a] Cultivars BR-18 (moderately resistant) and Guamirim (susceptible).

[b] Plots were either treated with 13.3 % epoxiconazole + 5 % pyraclostrobin (treated) or left untreated (check).

[c] Wheat blast incidence and severity were assessed in each plot at 10, 14, 18, and 22 days after inoculation.

[d] Wheat blast incidence was estimated as the percentage of visually diseased (bleached) spike in a sample of 40 spikes evaluated per plot and the severity was estimated as the mean percentage of spike area diseased.

Table 6. Estimated regression coefficients (intercepts and slopes) for the effects or different combinations of cultivars and fungicide treatment on temporal change in wheat blast incidence and severity on wheat spikes in field experiments 1 and 2 conducted in Viçosa, Minas Gerais, Brazil, from June to September 2013 and August to November 2013, respectively

Experiments	Coefficients	Factors[a]	Incidence[b]			Severity[b]		
			Estimates	SE	*P*-values	Estimates	SE	*P*-values
Experiment 1	Intercept	T1. BR-18 Treated	0.246	0.059	<0.001	0.036	0.019	0.061
		T2. BR-18 Untreated	0.192	0.059	0.002	-0.008	0.019	0.660
		T3. Guam Treated	0.262	0.059	<0.001	0.026	0.019	0.171
		T4. Guam Untreated	0.161	0.059	0.010	0.052	0.019	0.008
	Slope	T1. BR-18 Treated	0.006	0.004	0.089	0.002	0.001	0.145
		T2. BR-18 Untreated	0.020	0.004	<0.001	0.006	0.001	<0.001
		T3. Guam Treated	0.008	0.004	0.024	0.003	0.001	0.004
		T4. Guam Untreated	0.032	0.004	<0.001	0.009	0.001	<0.001
Experiment 2	Intercept	T1. BR-18 Treated	0.298	0.102	0.007	0.013	0.021	0.556
		T2. BR-18 Untreated	0.221	0.102	0.039	-0.251	0.021	<0.001
		T3. Guam Treated	0.405	0.102	<0.001	0.030	0.021	0.173
		T4. Guam Untreated	0.537	0.102	<.0001	-0.338	0.021	<.0001
	Slope	T1. BR-18 Treated	0.011	0.006	0.077	0.007	0.002	0.004
		T2. BR-18 Untreated	0.054	0.006	<0.001	0.036	0.002	<0.001
		T3. Guam Treated	0.010	0.006	0.106	0.006	0.002	0.009
		T4. Guam Untreated	0.044	0.006	<0.001	0.051	0.002	<0.001

[a] Cultivar × fungicide treatments combinations where T1 = BR-18 (moderately resistant), treated with the fungicide 13.3 % epoxiconazole + 5 % pyraclostrobin; T2 = BR-18 untreated; T3 = Guamirim (susceptible) treated; and T4 = Guamirim untreated (the reference treatment).

[b] Wheat blast incidence was estimated as the percentage of visually diseased (bleached) spike in a sample of 40 spikes evaluated per plot and the severity was estimated as the mean percentage of spike area diseased; Estimate = estimated regression slope or intercept and corresponding standard error (SE) and level of significance (*P*-value) from linear mixed model regression analysis of temporal change in arcsine-square root-transformed blast incidence and severity data.

Table 7. Probably values for pairwise comparisons of estimated regression slopes for the effects for different combinations of cultivars and fungicide treatment on temporal change in wheat blast incidence and severity on spike in field experiments 1 and 2 conducted in Viçosa, Minas Gerais State, Brazil, from June to September 2013 and August to November 2013, respectively.

Responses[a]	Contrasts[b]	Experiment 1[c]			Experiment 2[c]		
		Estimates	SE	*P*-values	Estimates	SE	*P*-values
Incidence	T1 *vs* T4	-0.025	0.005	<0.001	-0.033	0.008	0.001
	T2 *vs* T4	-0.011	0.005	0.031	0.010	0.008	0.264
	T3 *vs* T4	-0.023	0.005	<0.001	-0.034	0.008	0.001
Severity	T1 *vs* T4	-0.007	0.001	<0.001	-0.044	0.003	<0.001
	T2 *vs* T4	-0.002	0.001	0.130	-0.015	0.003	0.001
	T3 *vs* T4	-0.005	0.001	0.001	-0.045	0.003	<0.001

[a] Wheat blast incidence was estimated as the percentage of visually diseased (bleached) spike in a sample of 40 spikes evaluated per plot. Severity was estimated as the mean percentage of spike area diseased.

[b] Cultivar × fungicide treatment combination where T1 = BR-18 (moderately resistant) treated with the 13.3 % epoxiconazole + 5 % pyraclostrobin; T2 = BR-18 untreated; T3 = Guamirim (susceptible) treated; and T4 = Guamirim untreated (the reference treatment).

[c] Estimate = estimated difference in regression slope and corresponding standard error (SE) and level of significance (*P*-value) from linear mixed model regression analysis of temporal change in arcsine-square root transformed of wheat blast incidence and severity.

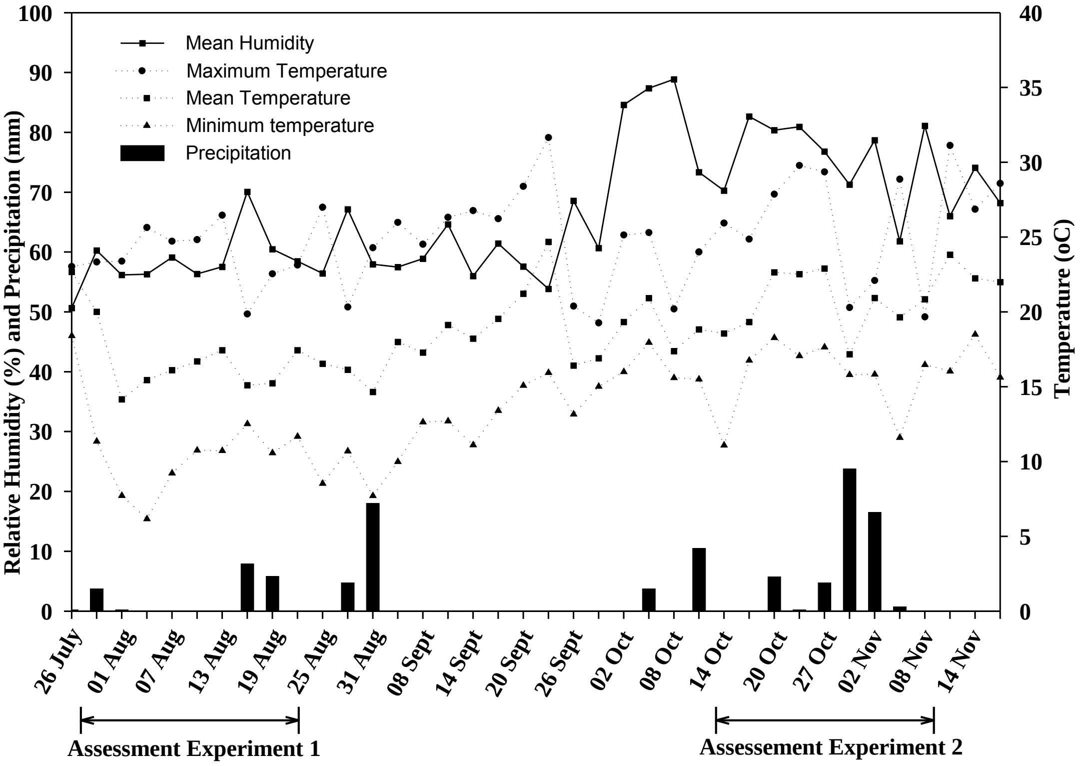

Fig. 1. Mean relative humidity (%), precipitation (mm), and mean, maximum, and minimum temperatures (^{0}C) between July and November 2013. The reference lines below the x-axis show the periods during which wheat blast intensity was assessed.

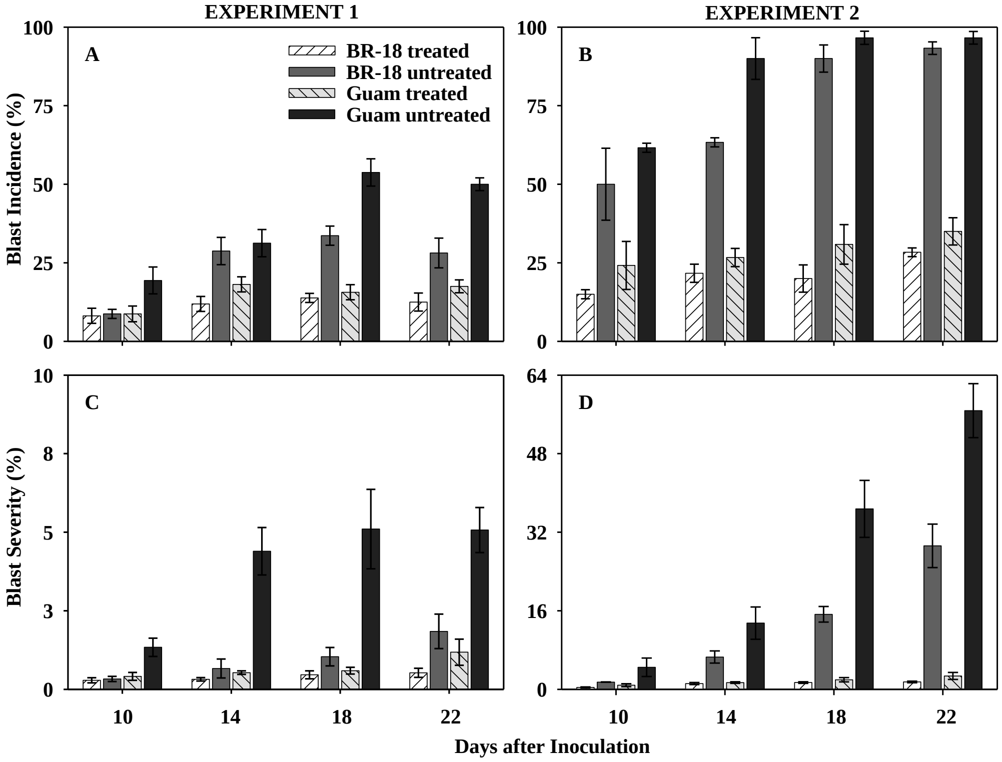

Fig. 2. Mean incidence (A, B) and severity (C, D) of wheat blast on the spikes of wheat plants from cultivars BR-18 (moderately resistant) and Guamirim (susceptible) treated with the fungicide 13.3% epoxiconazol + 5% pyraclostrobin or left untreated. Blast was rated at 10, 14, 18, and 22 days after inoculation in the field experiment 1 (A, C) and 2 (B, D) carried out between June and September 2013 and August and November 2013, respectively, in Viçosa, Minas Gerais State, Brazil. Error bars represent the standard error (*n* = 4) of the mean.

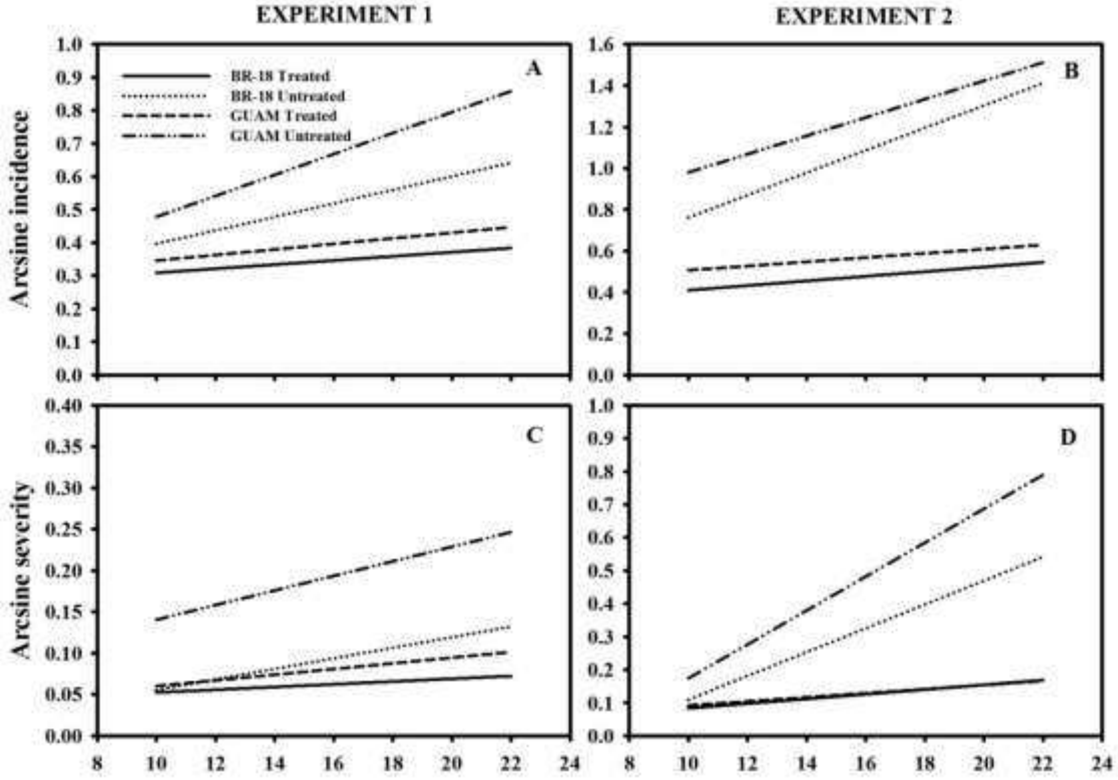

Fig. 3. Temporal change in arcsine-square root-transformed incidence (A, B) and severity (C, D) of wheat blast on the spikes of wheat plants from cultivars BR-18 (moderately resistant) and Guamirim (susceptible) treated with the fungicide 13.3% epoxiconazol + 5% pyraclostrobin or left untreated in field experiments 1 (A, C) and 2 (B, D) conducted between June and September 2013 and August and November 2013, respectively, in Viçosa, Minas Gerais State, Brazil. Lines are based on the regression parameters shown on Table 6 from linear mixed model regression analysis of the effects of cultivar × fungicide treatment combination on transformed wheat blast incidence and severity

CHAPTER 2

Effects of Blast on Components of Wheat Physiology and Grain Yield as Influenced by Fungicide Treatment and Host Resistance

Abstract

Two field experiments (Exp. 1 and Exp. 2) were carried out to assess the physiological performance and grain yield of wheat cultivars BR-18 (partially resistant) and Guamirim (susceptible) inoculated with *Pyricularia oryzae* and treated or untreated with the fungicide 13.3% epoxiconazole + 5% pyraclostrobin. Results from regression analyses indicated that spike and leaf blast severity at 10-14 days after anthesis resulted in greater yield losses (highest negative slope) than severity at 18 to 22 days after anthesis, and that spike blast severity had a greater negative effect on yield than leaf blast severity. Relative to Guamirim-untreated, there was a 0.3 and 16% increase in mean yield for BR-18-untreated (resistance alone), 20 and 61% increase for Guamirim treated (fungicide alone), and 26 and 83% for BR-18 treated (moderate resistance + fungicide) in Exp. 1 and 2, respectively. By virtue of their effects on blast severity, fungicide application and cultivar resistant resulted in higher measures of leaf health (mean HAD, HLAI, HAA and HRI) and photosynthetic performance (based on F_v/F_m, F_m, Y(II), and Y(NPQ)) of both spikes and leaves than the untreated susceptible reference treatment. Results from this study will be useful for future efforts to develop crop loss models and management guidelines for wheat blast.

Introduction

Blast, caused by the fungus *Pyricularia oryzae* Sacc. (teleomorph: *Magnaporthe grisea* (Hebert) Barr), is an important disease of wheat (*Triticum aestivum* L.) that causes substantial grain yield and quality losses in Brazil and other South American countries were epidemics occur frequently (Goulart et al. 2007). After first being reported in Southern Brazil almost 30 years ago, wheat blast was for years treated as a relatively minor disease. However, major epidemics and spread of this disease to new regions over the last decade have led to renewed interest in wheat blast. Several recent studies have been conducted to investigate the diversity and evolution of *P. oryzae* (Maciel et al. 2014), evaluate strategies for management of wheat blast (Maciel et al. 2014; Pagani et al. 2014; Rios et al. *this issue*; Rocha et al. 2014), and to determine the impact of this disease on crop physiology and yield (Goulart et al. 2007; Debona et al. 2014). However, further research is still needed to better understand how wheat blast develops and affects grain yield and quality.

P. oryzae infects all above-ground parts of the wheat plant, (Igarashi et al. 1986), causing symptoms ranging from elliptical to roundish lesions on leaves to premature bleaching and death of individual spikelets or entire spikes (Goulart et al. 2007). Leaf and spike blast impact grain yield and quality by reducing green leaf and spike areas, respectively. In the case of spike blast, infections also lead to reduced translocation of water and nutrients to developing kernels, causing them to become shriveled, deformed, small and lightweight (Goulart et al. 2007). The application of foliar fungicides, particularly Quinone Outside and Demethylation Inhibitors (QoIs and DMIs), and the use of genetic resistance are the strategies most widely recommended for minimizing losses caused by wheat blast (Castroagudín et al. 2015; Cruz et al. 2010, 2011; Maciel et al. 2011, 2014; Pagani et al. 2014; Rios et al. *this issue*; Rocha et al.

2014). In addition, fungicides, particularly the QoIs, are known to have effects on crop physiology of that could affect productivity (Ajigboye et al. 2014; Grossmann et al. 1999; Ruske et al. 2003; Wu and von Tiedemann 2001; Zhang et al. 2010).

Although it is clear that wheat blast does impact grain yield and quality, the specific effects of this disease on components of crop physiology and crop loss is largely unknown. Moreover, little is known about the functional relationships among blast, components of wheat health and physiology, and grain yield. Crop loss information is important for disease management decision making (Johnson et al. 1990) and for evaluating the economic benefits of disease management strategies (Jesus Junior et al. 2001). Since crop yield and dry weight are often more strongly correlated with healthy rather than diseased leaf area, several measures of crop health such healthy leaf area duration (HAD) and healthy leaf area absorption (HAA) have been proposed for quantifying the effects of plant diseases on crop loss (Waggoner and Berger 1987). In addition, since damage caused by pathogens is known to affect radiation intercepted (RI) and radiation use efficiency (RUE) (Johnson et al. 1990), RI and RUE have also been used as measures of the effects of diseases on crop loss (Bergamin Filho et al. 1997; Canteri et al. 2005; Moreira et al. 2015; Polanco et al. 2014).

Photosynthesis, the primary driver of crop yield, is one of the key physiological processes affected by plant pathogens (Bassanezi et al. 2002; Bastiaans 1994; Resende et al. 2012). Chlorophyll fluorescence imaging provides a sensitive, noninvasive and non-destructive method for assessing the effects of biotic and abiotic stresses on photosynthesis (Rolfe and Scholes 2013). This technique has been used successfully in a variety of studies to assess interactions between plants and fungal pathogens, including wheat infected with *Puccinia recondita* or *Blumeria graminis* (Kuckenberg et al. 2009), rice with *Monographella* *albescens* (Tatagiba et al. 2015), and maize with *Stenocarpella macrospora* (Bermúdez-Cardona et al. 2015). Typical host responses observed in plant-fungi interactions include an initial reduction in effective photosystem (PS) II quantum yield (Y(II)), an increase in quantum yield of regulated energy dissipation Y(NPQ), and a decline in maximum PS II quantum yield (F_v/F_m) at advanced stages of pathogen infection (Rolfe and Scholes 2013). Consequently, these indices can be used to evaluate the effect of crop protection strategies such as fungicide treatment and cultivar resistance on the photosynthetic performance of diseased plants (Rolfe and Scholes 2013). For instance, Aucique-Perez et al. (2014) demonstrated that applying silicon to *P. oryzae*-inoculated wheat leaves significantly reduced leaf blast severity and increased F_v/F_m at 96 h after inoculation when compared to non-silicon-treated plants.

The primary objectives of this study were to 1) quantify relationships between leaf and spike blast intensity estimated at different times during the growing season and grain yield; 2) quantity relationships between measures of crop health (HAD and HAA) and grain yield; 3) use chlorophyll (Chl) *a* fluorescence imaging to determine the effect of wheat blast on the photosynthetic performance of wheat plants, and 4) determine the individual and integrated effects of a single preventative application of the fungicide 13.3 % epoxiconazole + 5 % pyraclostrobin and partial resistance on grain yield, HAD, HAA, Y(II), Y(NPQ), and F_v/F_m. Wheat blast severity, HAD, HAA, Y(II), Y(NPQ), F_v/F_m, and grain yield were quantified in fungicide treated and untreated plots of a blast resistant and susceptible wheat cultivar, and then linear mixed models were fitted to the data to determine the effects of treatment and resistance on each of the measured response. Linear mixed model regression analyses were performed to quantify relationships between blast severity, measures of crop health, and grain yield. Separate models were fitted to the data and rates of change in yield per unit increase in spike and leaf blast severity, HAD, and HAA were estimated.

Materials and Methods

Plot establishment, treatment application, wheat blast severity and grain yield. Two field experiments were established in Viçosa in the southeastern region of the state of Minas Gerais, Brazil (20º44’44”S, 42º50’59”W and 661 m above sea level). These experiments and the conditions under which they were conducted are described in detail in Rios et al (*this issue*). In brief, the first experiment was conducted from June to September and the second from August to November 2013, under relatively dry and wet conditions, respectively. In both cases, the experimental design was a randomized complete block with a 2 × 2 factorial arrangement of fungicide treatment (two levels; with and without application of the fungicide 13.3 % epoxiconazole + 5 % pyraclostrobin at mid-anthesis) and cultivar (two levels; BR-18, moderately resistant to wheat blast and Guamirim, susceptible to blast) in four replicate blocks. Plants from all plots were inoculated with a conidial suspension of *P. oryzae* (10^5 conidia/mL) at approximately 48 hours after fungicide application and mist-irrigated for ten minutes to enhance infection and disease development (see Rios et al. (*this issue*) for more details).

Wheat blast intensity and grain yield data were collected from all plots. Incidence and severity of wheat blast on spikes and leaves were assessed on 40 arbitrarily selected tillers in each plot at 10, 14, 18 and 22 days after inoculation (dai). Incidence was rated as the mean percentage of diseased spikes (number of diseased spikes/40 x 100), and severity as the mean proportion of diseased spikelet per spike ([diseased spikelets/total spikelets] x 100). Leaf blast severity (percent leaf area with visual symptoms) was assessed on the flag leaf with the aid of a diagrammatic scale, with severity values ranging from 0.1 to 72% (Rios et al. 2013). The three center rows of each plot, representing an area of approximately 3 m^2, were harvested on 22 September and 27 November in experiments 1 and 2, respectively. Spikes were threshed, grains weighed, and plot yield was estimated in g m^{-2} and then converted to kg ha^{-1} at 12% moisture.

Fluorescence imaging and assessment of crop health and performance. Images and parameters of Chl *a* fluorescence were obtained on leaves and spikes at 10, 14, 18 and 22 dai using the MAXI version of the Imaging-PAM fluorometer and the Imaging Win software (Heinz Walz GmbH, Effeltrich, Germany). The Chl *a* fluorescence emission transients were captured with a CCD (charge-coupled device) camera at a resolution of 640 × 480 pixels in a visible sample area of 24 × 32 mm on each tissue. Prior to imaging, leaves and spikes were dark-adapted for 60 min, after which they were carefully and individually fixed in a support at a distance of 18.5 cm from the CCD camera. These 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 the PS II reaction centers are "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 maximum fluorescence emission (F_m) when all the PS II reaction centers were "closed". From these initial measurements, the maximum PS II photochemical efficiency of the dark-adapted leaves and spikes were estimated through the variable-to-maximum Chl fluorescence ratio, $F_v/F_m = [(F_m - F_0)/F_m)]$. These tissues were then exposed to actinic photon irradiance (530 µmol m^{-2} s^{-1}) for 120 s to obtain the steady-state fluorescence (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’). Following the calculations of Kramer et al. (2004), the energy absorbed by PS II for the following two quenching components for dissipative processes were determined: photochemistry quenching [$Y(II) = (F_m'-F_s)/F_m'$)] and quenching for dissipation by down-regulation [$Y(NPQ) = (F_s/F_m') - (F_s/F_m)$] (Krause and Weis 1991).

Leaves were harvested from a single plant (which corresponds to 4 to 6 tillers) per plot at 10, 14, 18 and 22 dai and then passed through an area meter (LI-3100C, LI-COR Inc., Lincoln, NE, USA) approximately 2-4 hours after being collected to estimate leaf area. Based on the assumption that a wheat plant occupies an area of approximately 0.0028 m^2, leaf area index (LAI) was estimated for each plot as previously described Bergamin Filho et al. (1997) and then used to calculate healthy leaf area index (HLAI), healthy leaf area duration (HAD, days), intercepted radiation (RI), radiation intercepted by the healthy leaf area (HRI, MJ m^{-2}), and healthy leaf area absorption (HAA, MJ m^{-2}) for each treatment × assessment time combination as described by Bergamin Filho et al. (1997). For the purpose of these calculations, plot average leaf blast severity was used when estimating HRI, HLAI, HAD, and HAA, and an extinction coefficient (k) of 0.45 for wheat at anthesis (Whaley et al. 2000) was used when calculating RI and HRI.

Statistical analysis. *Linear mixed model analysis of variance*. To evaluate the effects of cultivar, fungicide treatment, and assessment time on leaf blast severity, HRI, and HLAI, models were fitted with cultivar, fungicide treatment, and disease assessment time as categorical fixed effects and block as a random effect. All models were fitted using the GLIMMIX procedure of SAS, and since all responses were collected as temporal repeated measures on the same experimental units and as such were correlated in time (Littell et al. 2006), the random *_residual_* statement and *type* option in GLIMMIX were used to account for and model the covariance structure of the within-subject data. Similar models were fitted to parameters of Chl *a* fluorescence (maximal photosystem II quantum yield, F_v/F_m; maximal fluorescence, F_m; effective PS II quantum yield, Y(II); and quantum yield of regulated energy dissipation, Y(NPQ)) pooled across experiments.

Linear mixed models were also used to evaluate the effects of cultivar, fungicide treatment and their interaction on variables derived from the integration of wheat blast severity, HLAI, and HRI over time (AUDPC, HAD, and HAA, respectively), as well as grain yield. Since the results from separate analyzes of experiments 1 and 2 were very similar (Table 1), models were also fitted to the pooled data from the two experiments, with experiment and block within experiments as random effects. For all analyses described in this section, the *slice* and *diff* options in the *lsmeans* statement of GLIMMIX were used to test hypotheses and compare least squares means among cultivars, fungicide treatment, and cultivars × fungicide treatment combinations in the case of statistically significant interactions.

Linear mixed model regression analysis. Regression models were fitted to the data to quantify relationships between wheat blast intensity, HAD, HAA, and grain yield. To effectively model these relationships, a wide range of values for independent and dependent variables are required. This was accomplished in this study through the use of cultivars with different levels of resistance to wheat blast, treated and non-treated with a fungicide, and planted under conditions relatively favorable and unfavorable for wheat blast. The data from experiments 1 and 2 were pooled and models were fitted using the GLIMMIX procedure of SAS. Block within experiment was used as random effects in all analyses. To quantify relationships between wheat blast and yield, separate models were fitted for spike and leaf blast severity rated at 10, 14, 18, and 22 dai (labeled as S_10, S_14, S_18 and S_22 for spike blast severity and L_10, L_14, L_18 and L_22 for leaf blast severity). Similarly, separate models were fitted to quantify relationships between HRI (radiation intercepted by the healthy leaf area) at each assessment time (HRI_10, HRI_14, HRI_18, and HRI_22) and grain yield. In addition, for both leaf and spike blast severity, area under the disease progress curve (AUDPC_L and AUDPC_S) was used as continuous covariates in separate models. Model performance was evaluated based on R^2_{LMM} (Nakagawa and Schielzeth 2013), a statistic that provides a measure of the percentage of the variation in the dependent variable (yield in this case) explained by variation in the independent variable. For models with leaf or spike blast severity as the independent variable, regression slopes were compared among models based on *t*-tests (Madden et al. 2007).

Results

Wheat blast severity. Mean wheat blast intensity, based on area under the blast severity progress curve for leaf (AUDPC_L) and spike (AUDPC_S), varied between experiments and between treatments and cultivars within experiments (Fig. 1). Both AUDPC_L and AUDPC_S were higher in Exp. 2 than in Exp. 1, ranging from 35 to 219 and 4 to 70, respectively, in Exp. 1 and from 58 to 264 and 14 to 410, respectively, in Exp. 2. However, in both experiments, the resistant cultivar generally had lower mean AUDPC_L and AUDPC_S then the susceptible cultivar, and plots treated with 13.3% epoxiconazole + 5% pyraclostrobin had lower mean AUDPCs than the untreated checks (Fig. 1).

Table 1 summarizes the main and interaction effects of cultivar and fungicide treatment on AUDPC_L and AUDPC_S for both experiments. Except for the interaction effect of cultivar and fungicide on AUDPC_L in Exp.1, all main and interaction effects were statistically significant ($P < 0.05$), indicating that the cultivar effect on blast was influenced by fungicide treatment and vice versa. However, trends in terms of the mean differences between cultivars and between treatments were similar for both responses in both experiments, but the magnitude of the differences varied, hence the significant interaction (Fig. 1). The moderately resistant cultivar (BR-18) presented significantly lower leaf and spike blast severity than the susceptible cultivar Guamirim (Fig. 1). Indeed, mean AUDPC_L was 19.26% and 19.84% lower on BR-18 than on Guamirim in Exp. 1 and 2, respectively (Fig. 1). Similarly, relative to Guamirim, AUDPC_S was 77.71 and 54.84% lower on BR-18 in experiments 1 and 2, respectively (Fig. 1). The magnitude of the reduction in blast severity was greater in plots treated with 13.3% epoxiconazole + 5% pyraclostrobin when compared to the untreated control (Fig. 1). Relative to Guamirim untreated, AUDPC_L was 75.85 and 74.70% lower for BR-18 treated and 63.32 and 64.98% lower for Guamirim treated in Exp. 1 and 2, respectively. Similarly, AUDPC_S also was reduced by 90.59 and 95.82% in BR-18 treated plots and by 83.05 and 92.89% in Guamirim treated plots, relative to Guamirim untreated (Fig. 1).

HAD, HAA HLAI, and HRI in blast-affected plots. Blast negatively affected variables linked to wheat physiology, and consequently, grain yield (Tables 1 and 3). As was the case with wheat blast, mean HLAI, HRI, HAD, and HAA varied between experiments and between treatments and cultivars within experiments (Fig. 1). For all parameters analyzed, HLAI, HRI, HAD, and HAA, the main effects of cultivar and fungicide were statistically significant ($P < 0.05$) in both experiments, but interactions between the two were not (Table 1). In addition, assessment time (AT) (growth state) and interaction between assessment time and fungicide treatment significantly affected HLAI and HRI, meaning that the fungicide effect depended on when these variables were quantified relative to anthesis.

Compared to Guamirim, when averaged across fungicide treated and untreated plots, BR-18 (the moderately resistant cultivar) had significantly higher mean HAD and HAA (Table 1 and Fig. 1). The former was 22.17 and 58.38% higher and the latter 3.49 and 4.46% higher for BR-18 than for Guamirim in Exp.1 and 2, respectively. Similarly, averaged across cultivars, fungicide treatment had significantly higher mean HAD and HAA than the untreated checks. When compared to untreated Guamirim, in Exp. 1, HAD was 44.95% higher for BR-18 treated and 19.14 % higher for Guamirim treated (Fig. 1). In Exp. 2, these effects were even greater, with mean HAD being 104.55% higher for BR-18 treated and 47.66% higher for Guamirim treated compared to Guamirim untreated. Similar trends were observed for HAA, with BR-18 treated and Guamirim treated having 12.47 and 10.65 % higher mean HAA, respectively, than Guamirim untreated in Exp. 1. The corresponding differences in Exp. 2 were 18.41 and 16.18% (Fig. 1).

The effect of cultivar on HLAI and HRI was comparable to its effects on HAD and HAA, with BR-18 again having higher mean values than Guamirim in both experiments. However, the magnitude of the fungicide effect on HLAI and HRI varied with assessment time (growth stage). For both responses, mean differences between fungicide treated plots and untreated checks were greater at 18 and 21 days after anthesis than at 10 and 14 days after anthesis. The only exception was for HLAI in Exp. 2 where the greatest differences were observed at 14 and 22 days after anthesis. In both experiments, HRI were significantly lower at 18 and 22 days after anthesis than at 10 and 14 days after, with the magnitude of these differences being higher in untreated than in treated plots. Somewhat similar trends were observed for HLAI in Exp. 1 but not in Exp. 2, where mean HLAI were significantly higher late compared to early in the season and differences between assessments times were greater in treated plots than in the checks.

Parameters of Chl *a* fluorescence on blast-affected leaves and spikes. The first visual alterations in the Chl *a* fluorescence image of leaves and spikes were evident at 10 dai (anthesis) in all treatments (Fig. 2). In all cases, the negative effect of blast increased over time as lesions expanded on leaves and spikes became more bleached and died. However, the extent to which photosynthetic activity was affected, as indicated by the necrotic areas in the images, depended on the treatment and the cultivar, being greater on untreated leaves and spikes, particularly those of the susceptible cultivar Guamirim. These areas reflected damage to the photosynthetic apparatus, with an associated loss of the optical properties of the colonized tissues leading, therefore, to changes in the values of the F_m, F_v/F_m, YII and YNPQ parameters (Fig. 3).

Table 2 summarizes the main and interaction effects of cultivar, fungicide treatment, and assessment time on parameters of Chl *a* fluorescence for leaves and spikes. Results from the linear mixed model analyses showed that main and two-way interaction effects of cultivar, fungicide treatment and assessment time on F_m, F_v/F_m, YII and YNPQ were generally statistically significant ($P < 0.05$), whereas three-way interactions were not or were only marginally significant. The only exceptions were for the main effect of cultivar on F_m on leaves and the two-way interaction effects of cultivar and fungicide on Y(II), F_m, and

Y(NPQ) on leaves; cultivar and assessment time on F_m on leaves and Y(II) on spikes; and fungicide and assessment time on Y(II) on leaves (Table 2).

For both cultivars, mean F_v/F_m was significantly higher on fungicide-treated leaves than untreated leaves at all assessment times. For spikes, fungicide-treated plots had higher mean F_v/F_m than untreated plots at 18 and 22 dai but not at 10 and 14 dai on BR-18 and at all assessment times on Guamirim (Fig. 3 A and B). The resistant cultivar had significantly higher mean F_v/F_m on leaves from untreated plots at all assessment times but not from treated plots. Similar differences were observed for F_v/F_m on spikes, with BR-18 having significantly higher mean F_v/F_m than Guamirim at all but the last assessment time for untreated but not treated spikes (Fig. 3B). For effective PSII quantum yield (YII), the resistant cultivar also tended to have higher means than the susceptible cultivar, but the effect varied among assessment times for leaves and between fungicide treatments for spikes. For instance, BR-18 had significantly higher mean YII on leaves than Guamirim at 10 and 18 dai, but not at 14 and 22 dai. In the case of YII on spikes, BR-18 had significantly higher mean values than Guamirim for untreated spikes but not for treated spikes (Fig. 3C and D). Treated tissues consistently had higher mean YII than untreated, but the magnitude of the difference on spikes was significantly greater at 18 and 22 dai than at 10 and 14 dai.

Averaged across cultivars, mean F_m (maximum fluorescence) was significantly higher on treated than untreated leaves at all but the first assessment time (Fig. 3E). Similarly, mean F_m was higher on treated than untreated spikes at all assessment times, with the differences being statistically significant in all cases for Guamirim and at 14 and 22 dai for BR-18 (Fig. 3F). In terms of cultivar effect on mean F_m, this response was highly variable, with BR-18 having significantly higher means than Guamirim on treated spikes at 22 dai and untreated spikes at 18 dai. For all other treatment × assessment time combinations, differences were numerical but not statistically significant (Fig. 3E and F). As expected, opposite trends to those seen for F_m, F_v/F_m, and YII were observed for YNPQ, with treated leaves having significantly lower means than untreated leaves at all but the first assessment time, regardless of cultivar, and the resistant cultivar having significantly lower mean YNPQ that the susceptible cultivar at all but the second assessment time (Fig. 3G). On spikes, the effects of cultivar, fungicide, and assessment time on mean YNPQ were less consistent. However, for cases in which the effects were statistically significant, the responses were comparable to those observed on leaves. For instance, BR-18 had significantly lower mean YNPQ than Guamirim on untreated spikes (averaged across assessment times) and at 22 dai (averaged across treatments). In terms of the fungicide treatment effect, treated spikes had significantly lower mean YNPQ than untreated spikes for Guamirim but not BR-18, and at 18 dai but not at the other growth stages (Fig. 3H).

Grain yield and blast-yield, HRI-yield, and HAA-yield relationships. Baseline yields varied between experiments and among cultivar × fungicide treatment combinations within each experiment (Fig. 1), ranging from 2,300 to 3,630 kg ha^{-1}, with a mean of 2,931 kg ha^{-1} in Exp. 1 and from 1,517 to 3,767 kg ha^{-1}, with an mean of 2,665 kg ha^{-1} in Exp. 2. In both experiments, the highest mean yield was observed in plots of the resistant cultivar (BR-18) treated with the fungicide (3,310 and 3,483 kg ha^{-1} in Exp. 1 and 2, respectively) and the lowest in untreated plots of Guamirim, the susceptible cultivar (2,623 and 1,900, kg ha^{-1} in Exp. 1 and 2, respectively). Fungicide treatment had a significant effect on yield in both experiments (Table 1). In Exp. 1, mean yield was 25.75% higher for BR-18 treated and 20.49% higher for Guamirim treated when compared to the corresponding untreated plots. In

Exp. 2, the effect of cultivar was also statistically significant and mean yield was 57.73% higher for BR-18 treated and 61.40% higher for Guamirim treated when compared to their respective untreated means. Averaged across fungicide treatments, BR-18 had 0.38 and 16.22% higher mean yield than Guamirim in Exp. 1 and 2, respectively.

By virtue of the effects of cultivar and fungicide treatment on wheat blast intensity, components of wheat physiology, and grain yield (Table 1 and Fig. 1), and the influence of weather conditions across the two experiments on these responses, a range of values was obtained for the pooled data, making it possible to quantify relationships between blast and yield, HRI and yield, and HAA and yield. All measures of wheat blast had significant negative functional relationships with grain yield (Fig. 4A and B), whereas radiation intercepted by the health leaf area (HRI, Fig. 4C) and healthy leaf area absorption (HAA, Fig. 4C) had significant positive functional relationships with yield ($P < 0.001$). For relationships between leaf blast and yield, as indicated by the R^2_{LMM} values in Table 3, between 60 and 65% of the variation in yield was explained by the variation in disease severity, and the rate of yield reduction per percent increase in severity ranged from 62.2 to 173.5 kg ha^{-1}/% severity (negative slopes, Table 3). Similar trends were observed for relationships between spike blast and yield, with 56 and 69% of the variation in yield explained by spike blast severity and rates of yield reduction per unit increase in severity ranged from 23.4 to 321.5 kg ha^{-1}/% severity. For both leaf and spike blast, significantly higher rates of yield reduction occurred for severity at 10 days after anthesis than at 14, 18, or 22 days after anthesis (Table 3 and Fig. 4). Spike blast severity at 10 days after anthesis had a greater impact on yield (high negative slopes in Table 3) than leaf blast severity at the same growth stage. Interestingly, for leaf as well as spike blast, a greater percentage of the variation in yield was explained by disease severity integrated over the four assessment times (10 to 22 days after anthesis) than individual assessments, as indicated by the higher R^2_{LMM} values (66 to 77%) for AUDPC-yield models in Table 3. Total AUDPC explained about 10% more of the variation in yield than leaf (AUDPC_L) or spike (AUDPC_S) severity AUDPC alone.

As expected, opposite trends to those observed for relationships between measures of wheat blast and yield were observed for HRI-yield relationships. Between 60 and 65 percent of the variation in yield was explained by the variation in HRI, with the highest rate of yield increase associated with HRI at 10 days after anthesis and the lowest at 22 days after anthesis. The regression slope was significantly greater at the 5% level of significance for the HRI_10 model than the HRI_14, HRI_18, or HRI_22 models and significantly lower at the 10% level of significance for the HRI_22 model than the other three models (Table 3 and Fig. 4). Like HRI, healthy leaf area absorption (HAA) also had a significant positive linear relationship with grain yield with an R^2_{LMM} of 65% and a 709 kg ha^{-1} increase in yield per unit increase in HAA. Healthy leaf area duration (HAD) did not have a significant linear relationship with grain yield ($P > 0.05$). Less than 0.5% of the variation in yield was explained by the variation in HAD (data not shown).

Discussion

In this study, we demonstrated the effects of wheat blast on components of crop physiology and grain yield; documented the individual and combined effects of host resistance and fungicide treatment on parameters related to photosynthesis on blast-affected leaves and spikes; and qualified relationships between blast, grain yield, and components of wheat physiology from two artificially inoculated field experiments conducted under distinct environmental conditions. Photosynthetic performance was greater in PSII on infected flag leaves and spikes treated with 13.3% epoxiconazole + 5% pyraclostrobin, particularly on the resistant cultivar BR-18. Fungicide treatment and cultivar resistance reduced blast intensity, leading to healthier leaves and spikes, and consequently greater light interception and absorption. By reducing the negative effects of blast on components of wheat physiology and increasing photosynthetic performance, fungicide and host resistance also resulted in higher mean grain yield when compared to the untreated susceptible check. Results from regression analyses showed that the greatest rates of yield reduction per unit increase in blast intensity (based on regression slopes) were associated with spike and leaf blast severity between 10 and 14 days after anthesis (inoculation), suggesting that management during early grain fill would be critical for minimizing yield loss.

Greater yield reduction due to blast was observed in Exp. 2 than in Exp. 1 due in part to the fact that leaf and spike blast severity was higher in Exp. 2. Based on data from the untreated susceptible checks (the treatment combination with the lowest mean yield) and the moderately resistant cultivar treated with 13.3% epoxiconazole + 5% pyraclostrobin (the treatment combination with the highest mean yield) yield loss was 20.78 % in Exp. 1 compared to 45.45 % in Exp. 2. For both leaf and spike blast, there was a significant negative relationship between disease severity and grain yield, with the greatest rates of yield reduction per unit increase in disease was associated with blast severity at 10 days after anthesis. This suggests that wheat blast is likely to have a greater effect on grain yield when it occurs during the early stages of grain development than during mid-late grain-fill. This is consistent with results from a study conducted by Goulart et al. (2007) which showed that yield loss in wheat provoked by blast under natural field conditions was 63.4% when infection occurred early compared to 46.0% for late infection.

These results suggest that both leaf and spike blast should be managed during early grain-fill to minimize yield loss. Indeed, when compared to the untreated susceptible check, a preventative application of 13.3% epoxiconazole + 5% pyraclostrobin at mid-anthesis increased yield in blast-affected plots by 26 and 21% on the moderately resistant and susceptible cultivars, respectively, in Exp. 1, and by 58 and 62% on the two cultivars in Exp. 2. Our results corroborated those of Pagani et al. (2014) which showed that in experiments with naturally occurring blast epidemics, mean grain yields were approximately two times higher (93 to 120% yield increase over the check) in plots treated twice with epoxiconazole + pyraclostrobin (the first at early anthesis and the second at milk) for wheat blast control than in the untreated check. However, contrary to what was observed in our study, Pagani et al. (2014) reported greater mean yield increases due to fungicide in the experiment with relatively lower baseline levels of blast (mean spike blast severity in the untreated check was 89.7%) than in the experiment with higher blast intensity (mean spike blast severity in the untreated check was 98.9%). Managing wheat blast with partial resistance alone (BR-18 untreated) also increased grain yield relative to the untreated susceptible, but the magnitude of the yield increase (0.3% in Exp. 1 and 16% in Exp.. 2) was considerably lower than that achieved with the fungicide alone (20 and 61% in Exp. 1 and Exp. 2, respectively) or moderate resistance + fungicide (26 and 83%, in Exp. 1 and Exp. 2, respectively). This emphasizes the value of integrating fungicide application and cultivar resistance to manage wheat blast, as this approach has been shown to have an additive effect on blast in terms of percent control (Rios et al. this issue).

The effect of wheat blast of grain yield is clearly related to its effect on the health, integrity, and photosynthetic activity of the plant. The cultivar x fungicide combination with the lowest mean spike and leaf blast intensities generally had the highest mean HLAI, HAA, HRI, F_v/F_m and Y(II) values and the lowest mean Y(NPQ) values. Moreover, the significant positive relationships between HAA and yield and HRI and yield, and the fact that regression slopes for the latter relationship were greater for HRI at 10 and 14 than at 18 and 22 days after anthesis, emphasize the importance of the health and activity of the plants, particularly during grain-filling, for grain yield. Photosynthesis of spikes and leaves during grain-filling represents an important source of photoassimilates for grain development and yield (Sanchez-Bragado et al. 2014; Tuncel and Okita 2013). As suggested by Debona et al. (2014) and Aucique-Perez et al. (2014), blast negatively affects photosynthesis by lowering gas exchange capacity, reducing chlorophyll concentration, and possibly via biochemical effects related to reduced Rubisco activity. Therefore, the negative effect of wheat blast on grain yield may be associated with reduction of the plant's capacity to produce and translocate photoassimilates.

Here we demonstrated through analyses of parameters of Chl *a* fluorescence an impairment of the photosynthetic capacity of diseased leaves and spikes due to lower efficiency of PSII. Blast symptoms provoked a range of damages at the photochemical level on leaves and spikes, and these dysfunctions were much more severe on leaves and spikes of plants from the susceptible cultivar that were not treated with 13.3% epoxiconazole + 5% pyraclostrobin. For instance, collectively, treated leaves and spikes showed F_v/F_m values of approximately 0.80, which suggested the absence of chronic photoinhibition to photosynthesis (Rolfe et al. 2010). In wheat, F_v/F_m was shown to be strongly related to higher PSII efficiency under field conditions, and consequently, to higher plant biomass and grain yield (Ajigboye et al. 2014). Additionally, fungicide-treated tissues had higher levels of Y(II), which is indicative of higher apparent electron transport activity (Krause et al. 1991). Higher F_v/F_m and Y(II) values reflect greater photoprotection capacity, which reduces photooxidative damage to infected tissues (Rolfe et al. 2010). Conversely, the lower observed Y(NPQ) values of treated leaves and spikes suggested reduced damage to the reaction centers of PSII coupled with higher capacity to transfer excitation energy from the antenna to the reaction centers (Baker et al. 2008). Ajigboye et al. (2014) showed that in addition to reducing disease intensity, the effects of QoI and DMI fungicides combined with increased PSII efficiency were associated with increased biomass and, consequently, higher grain yield under field conditions.

The HAD, HAA, HLAI and HRI results from this study further support the importance of maintaining the wheat leaves healthy, particularly during early grain-fill, and provide additional models that can be used to estimate yield/loss in blast-affected fields. Understandably, based on data from the untreated susceptible, blast affected the amount of light intercepted and absorbed by the wheat leaves, both of which are important for photosynthesis, and consequently, yield (Long et al. 2006). Untreated plots had the highest levels of blast and correspondingly the lowest measures of leaf health (mean HAD, HLAI, HAA and HRI) and photosynthetic performance (based on F_v/F_m, Y(II), and Y(NPQ)). Conversely, treated plots of the moderately resistant cultivar BR-18 had the healthiest leaves, and as a consequence, greater light interception and absorption. As discussed above, both

HAA and HRI had significant positive relationships with grain yield, explaining between 60 and 65% of the yield variation. Interestingly, contrary to what has commonly been reported for other pathosystems (Jesus Junior et al. 2001), based on R^2_{LMM}, these and other measures of leaf health were no better at explaining the variation in wheat yield than measures of wheat blast intensity. In fact, the relationship between HAD and yield was not statistically significant under the conditions of the present study. Bergamin Filho et al. (1997) suggested that for decision-making it is better to develop models using HRI or HLAI at various times during a cropping season to estimate yield (as was done for HRI here) rather than integrating these variables to obtain HAD and HAA.

Results from the regression analyses of relationships between blast and yield provide a measure of the relative impact of leaf and spike blast severity and the time of disease onset on yield loss. Slopes for relationships between severity at 10 days after anthesis and yield were significantly greater (higher negative values) than slopes for relationships between severity at 14, 18 and 21 days after anthesis and yield. In addition, slopes for relationships between severity at 10 and 14 days after anthesis and yield were greater for spike blast than leaf blast. These results suggest that for both spike and leaf blast, the highest rates of yield reduction (highest negative slope) are associated with disease develops within the first 10-14 days after anthesis and that spike blast severity during this early grain filliling period has a greater effect on yield than leaf blast severity. Results from inverse regression analyses of relationships between blast severity and relative yield (Madden and Paul 2009; Salgado et al. 2015) support this hypothesis. For leaf blast, 50% yield reduction was estimated to occur at 10 and 19% leaf blast severity at 10 and 14 days after anthesis, respectively, whereas for spike blast, this same level of yield loss was estimated to occur at lower levels of severity; approximately 5% severity at 10 days after anthesis and 14% at 14 days later.

The relatively greater effect of spike blast than leaf blast on yield may be due in part to reduced translocation of photoassimilates to developing grain due to death of diseased rachis tissues (Goulart et al. 2007). Based on these results, one may be inclined to think that spike blast provides a better estimate of the impact of this disease on wheat yield. However, the strength of the relationship between disease severity integrated over the growing season (AUDPC) was similar for spike and wheat blast, both explaining 66% of the variation in yield. In fact, of all the blast x yield models evaluated in this study, the model with blast represented as the sum of leaf and spike blast AUDPC explained the highest percentage of the yield variation, 77%. This suggests that when the two stages of the disease occur, they should both be considered when evaluating the effects on yield. However, from a practical standpoint, when quantifying wheat blast for developing yield loss models and establishing management decision thresholds, leaf and spike blast severity at anthesis and early grain-fill may be more useful.

Quantification of the effects of disease on plant physiology is one of the first steps to understanding crop losses (Bastiaans et al. 1994). Based on inoculated experiments conducted in a region of Brazil where wheat blast is not endemic, we successfully quantify some of these effects and developed models for relationships between blast and yield and between measures of wheat health. We showed that under blast-favorable conditions, early infections of leaves and spikes may negatively impact components of wheat photosynthesis. However, under the conditions of this study, the combination of a preventative anthesis application of 13.3% epoxiconazole + 5% pyraclostrobin and partial resistance successfully reduced these negative effects and maintained the photosynthetic performance of leaves and spikes, leading to higher grain yield. These results will be extremely useful as efforts of understand the epidemiology of, and develop management programs for, this complex wheat disease continues. However, further research will be needed to evaluate the models developed here under conditions of natural infection, particularly in regions where the disease is endemic.

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Table 1. Probability values (levels of significance) from linear mixed model analyses of the effects of cultivars (C), fungicide (F), disease assessment time (AT) and their interactions on area under the disease progress curve for wheat blast severity on leaves (AUDPC_L) and spikes (AUDPC_S), healthy leaf area index (HLAI), radiation intercepted by the healthy leaf area (HRI), healthy leaf area duration (HAD), healthy leaf area absorption (HAA), and grain yield (YLD) in field Experiment 1 conducted between June and September and 2 between August and November 2013 in Viçosa, Minas Gerais, Brazil, 2013

Experiment	Factors[a]	AUDPC_L	AUDPC_S	HLAI	HRI	HAD	HAA	YLD
Exp. 1	C	0.003	<0.001	<0.001	<0.001	<0.001	0.002	0.397
	F	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	C × F	0.415	<0.001	0.689	0.195	0.679	0.215	0.456
	AT	…	…	<0.001	<0.001	…	…	…
	C ×AT	…	…	0.150	0.026	…	…	…
	F × AT	…	…	0.005	<0.001	…	…	…
	C × F × AT	…	…	0.690	0.186	…	…	…
Exp. 2	C	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	0.006
	F	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	C × F	0.041	0.002	0.950	0.114	0.893	0.086	0.606
	AT	…	…	<0.001	<0.001	…	…	…
	C ×AT	…	…	0.066	0.104	…	…	…
	F × AT	…	…	0.005	<0.001	…	…	…
	C × F × AT	…	…	0.049	0.954	…	…	…

[a] Main and interaction effects of cultivars (C; BR-18, moderately resistant and Guamirim, susceptible), fungicide treatment (F, with and without application of the fungicide 13.3 % epoxiconazole + 5 % pyraclostrobin) and time of disease assessment (AT; 10, 14, 18, and 22 days after inoculation).

Table 2. Probability values (levels of significance) from linear mixed model analyses of the effects of cultivars (C), fungicide (F), disease assessment time (AT) and their interactions on chlorophyll *a* fluorescence parameters on blast-affected wheat leaves and spikes from field experiments conducted in Viçosa, Minas Gerais, Brazil, 2013[a]

Factors[b]	Leaf[c]				Spike[c]			
	F_v/F_m	Y(II)	F_m	Y(NPQ)	F_v/F_m	Y(II)	F_m	Y(NPQ)
C	<0.001	0.010	0.495	0.002	<0.001	<0.001	0.007	0.008
F	<0.001	0.011	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
C × F	<0.001	0.080	0.622	0.232	0.003	0.009	0.119	0.004
AT	<0.001	<0.001	<0.001	0.004	<0.001	<0.001	<0.001	<0.001
C × AT	<0.107	<0.001	0.310	<0.001	0.003	0.216	0.003	<0.001
F × AT	<0.001	0.886	<0.001	0.014	0.017	0.008	0.001	<0.001
C × F × AT	0.407	0.633	0.494	0.128	0.047	0.079	0.008	0.332

[a] *Fv/Fm* = Maximal photosystem II quantum yield, Y(II) = effective PS II quantum yield, *Fm* = maximum fluorescence, and Y(NPQ) = quantum yield of regulated energy dissipation

[b] Main and interaction effects of cultivars (C; BR-18, moderately resistant and Guamirim, susceptible), fungicide treatment (F, with and without application of the fungicide 13.3 % epoxiconazole + 5 % pyraclostrobin) and time of disease assessment (AT; 10, 14, 18, and 22 days after inoculation).

[c] Analyses are for pooled data from field experiments conducted between June and September and August and November 2013.

Table 3. Regression coefficients (intercepts and slopes) and corresponding statistics for relationships between different measures of wheat blast severity, radiations intercepted by the healthy leaf area (HRI), healthy leaf area duration (HAD), and healthy leaf area absorption (HAA) and wheat grain yield.

Predictor	Intercept	se (intercept)	Slope		se (slope)	R^2_{LMM}
L_10	3,491.99	128.76	-173.50^{**}	a	25.33	60
L_14	3,545.23	128.27	-91.97^{**}	b	9.82	61
L_18	3,707.95	133.42	-73.16^{**}	b c	5.91	61
L_22	3,771.98	131.22	-62.21^{**}	c	5.25	65
S_10	3,111.76	75.48	-321.47^{**}	A	54.17	56
S_14	3,113.55	69.29	-109.98^{**}	B	15.52	69
S_18	3,024.83	74.31	-38.88^{**}	C	6.46	63
S_22	3,020.13	67.71	-24.40^{**}	C	3.79	66
HRI_10	-13,970.00	2,415.19	$17{,}109.00^{**}$	…	2462.99	60
HRI_14	-5,646.63	910.89	$8{,}749.19^{**}$	…	937.99	60
HRI_18	-3,609.23	529.85	$7{,}247.20^{**}$	…	586.00	62
HRI_22	-2,444.95	454.64	$5{,}837.64^{**}$	…	493.23	65
AUDPC_L	3,707.45	125.69	-6.76^{**}	…	0.58	66
AUDPC_S	3,048.36	70.35	-4.19^{**}	…	0.65	66
AUDPC	3,343.01	89.34	-2.93^{**}	…	0.33	77
HAA	-5,114.85	674.24	709.31^{**}	…	59.74	65

[a] L_10, 14, 18 and 22 = leave blast severity at 10, 14, 18 and 22 days after inoculation (dai) respectively; S_10, 14, 18 and 22 = spike blast severity at 10, 14, 18 and 22 dai, respectively; HRI_10, 14, 18 and 22 = radiations intercepted by the healthy leaf area at 10, 14, 18 and 22 dai; AUDPC_L and AUDPC_S = area under leaf and spike blast severity progress curve.

[b] se = standard error of the estimated intercepts and slopes.

[c] Slope = estimated rate of change in grain yield per unit increase in the predictor. ** indicate slope significantly different form zero at the 1% level of probability. Slopes from leaf blast severity models (L_10, L_14, L_18, and L_22) followed by the same lower case letters and those from spike blast severity models (S_10, S_14, S_18, and S_22) followed by the same upper case letters are not significantly different at the 5% level of probability based on *t*-tests.

[d] R^2_{LMM} = proportion of the variation in grain yield explained by variation in the predictor estimated through linear mixed model regression analysis as described by Nakagawa and Schielzeth (2013).

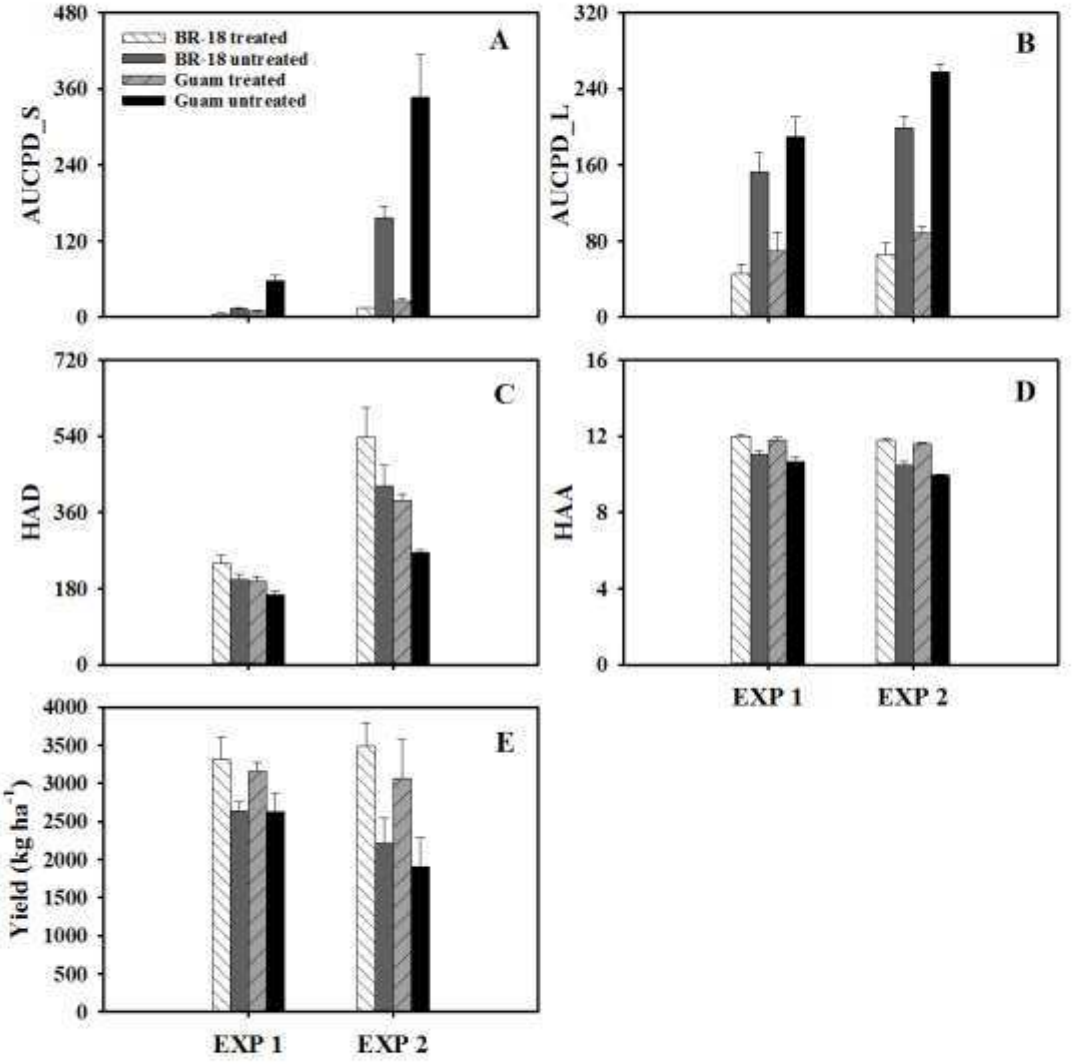
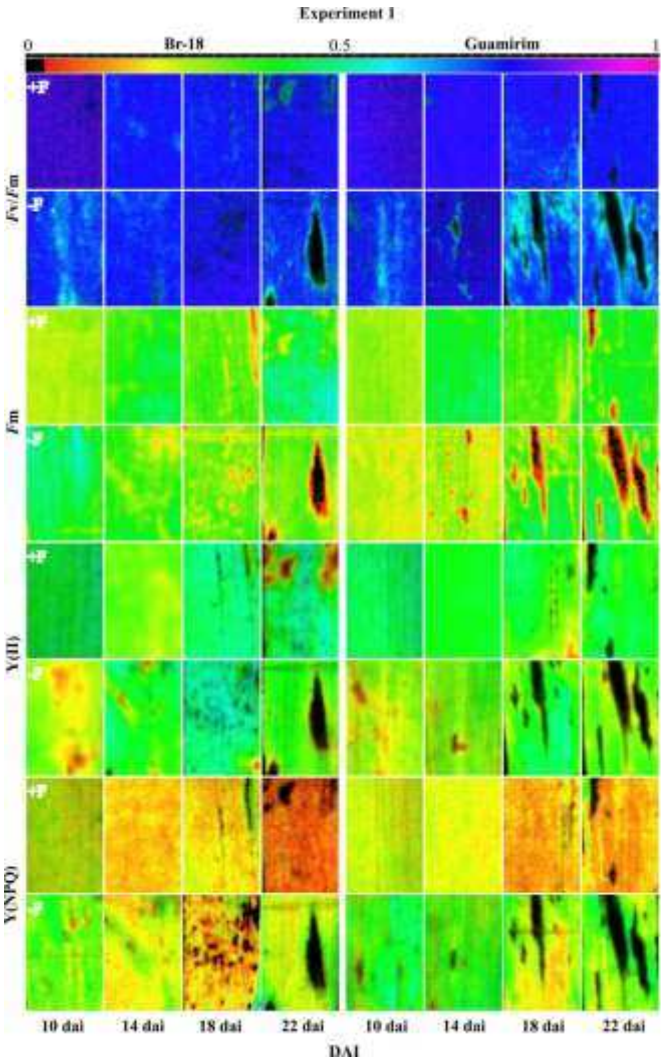


Fig. 1. Area under the disease progress curve for spike (AUDPC_S) (**A**) and leaf (AUDPC_L) (**B**), healthy leaf area duration (HAD) (C), healthy leaf area absorption (HAA) (**D**) and mean grain yield (kg/ha) (**E**) for cultivars BR-18 (moderately resistant) and Guamirim (susceptible) treated with the fungicide 13.3% epoxiconazol + 5% pyraclostrobin or left untreated. Experiments 1 and 2 were conducted between June and September and August and November 2013, respectively, in Viçosa, Minas Gerais, Brazil. Error bars represent standard error (*n* = 4) of the mean.


Experiment 1
0
Br-18
0.5
Guamirim
1
+F
-F
Fv/Fm
+F
-F
Fm
+F
-F
Y(II)
+F
-F
Y(NPQ)
10 dai
14 dai
18 dai
22 dai
10 dai
14 dai
18 dai
22 dai
DAI

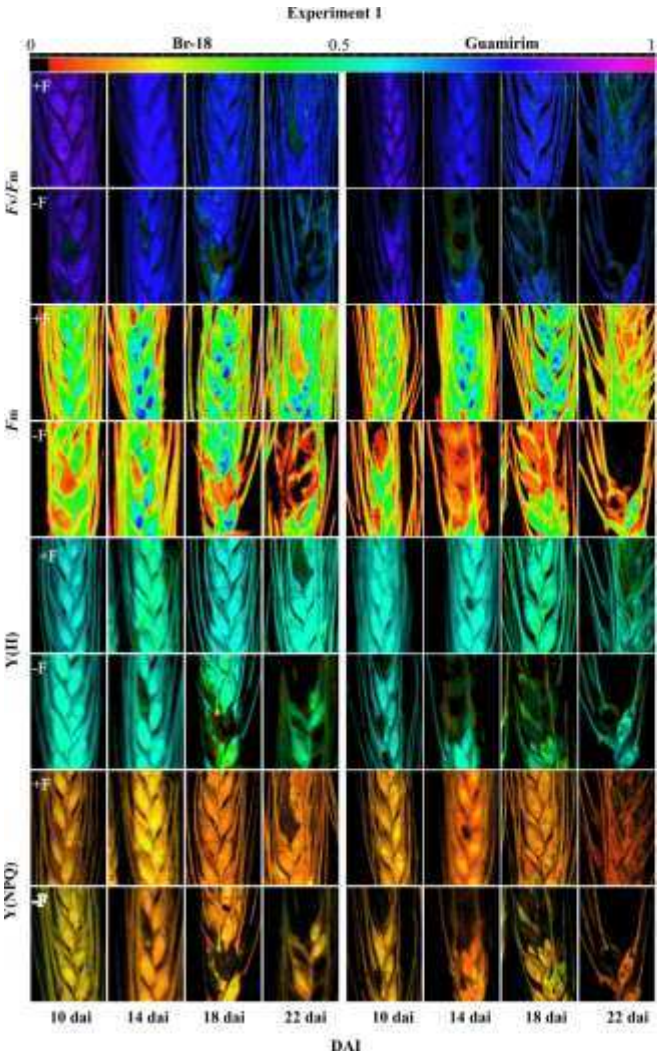

Experiment 1
0
Br-18
0.5
Guamirim
1
+F
-F
Fv/Fm
+F
-F
Fm
+F
-F
Y(II)
+F
-F
Y(NPQ)
10 dai
14 dai
18 dai
22 dai
10 dai
14 dai
18 dai
22 dai
DAI

Fig 2. Parameters of chlorophyll *a* fluorescence, maximal photosystem II quantum yield (F_v/F_m), maximal fluorescence (F_m), effective PS II quantum yield (Y(II)), quantum yield of regulated energy dissipation (Y(NPQ)) on wheat blast infected leaves and spikes of cultivars BR-18 (moderately resistant) and Guamirim (susceptible) treated (+F) with the fungicide 13.3% epoxiconazol + 5% pyraclostrobin or left untreated (-F). Parameters were rated at 10, 14, 18, and 22 days after inoculation (dai).

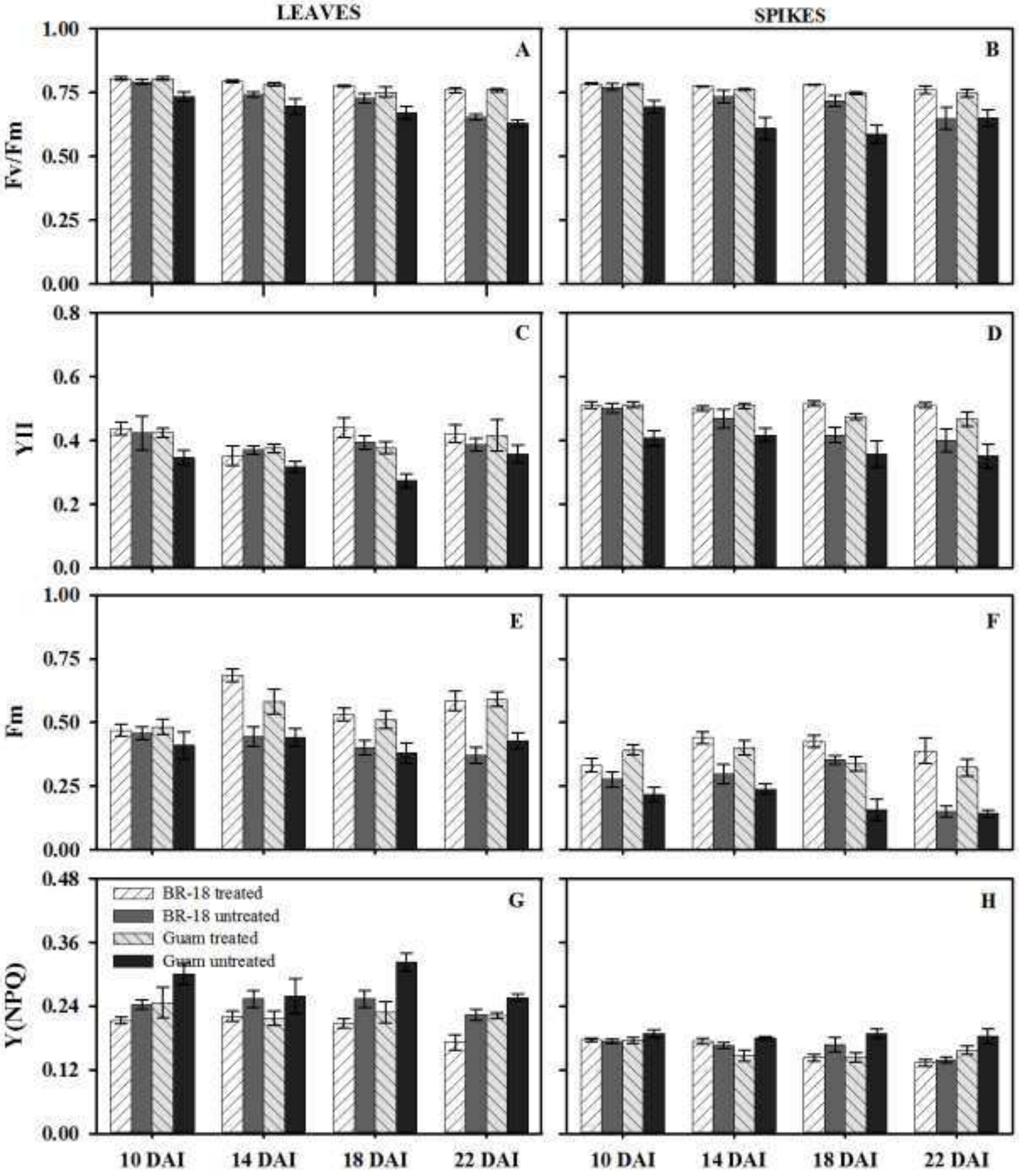


Fig. 3. Maximal photosystem II quantum yield (F_v/F_m) (**A** and **B**), maximal fluorescence (F_m) (**C** and **D**), effective PS II quantum yield (YII) (**E** and **F**), quantum yield of regulated energy dissipation (Y(NPQ)) (**G** and **H**) from wheat blast infected leaves (**A**, **C**, **E** and **G**) and spikes (**B**, **D**,**F** and **H**) of cultivars BR-18 (moderately resistant) and Guamirim (susceptible) treated with the fungicide 13.3% epoxiconazol + 5% pyraclostrobin or left untreated. Parameters were rated at 10, 14, 18, and 22 days after inoculation. Error bars represent the standard error of the mean (*n* = 20).

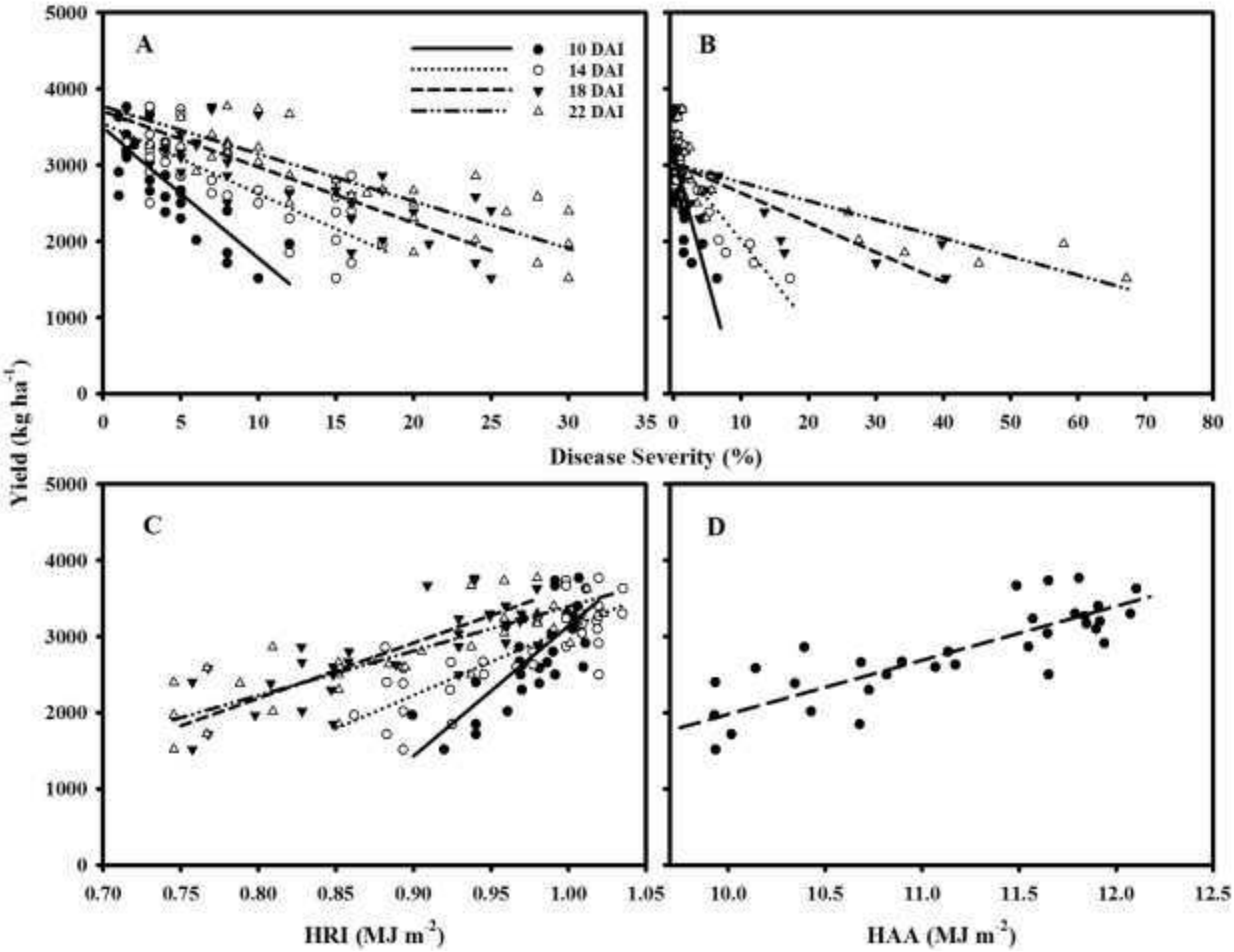


Fig 4. Relationships between **A**, leaves blast severity, **B**, spikes blast severity, **C**, radiation intercepted by the healthy leaf area (HRI), **D**, healthy leaf area absorption (HAA) and grain yield (kg/ha). Dots represent observation from cultivars BR-18 (moderately resistant) and Guamirim (susceptible) treated with the fungicide 13.3% epoxiconazol + 5% pyraclostrobin or left untreated in two field experiments conducted between June and September and August and November 2013 in Viçosa, Minas Gerais, Brazil. Lines represent predicted grain yield from the fit of the regression models in Table 3. dai = days after inoculation, respectively.

CHAPTER 3

Changes in the Source-Sink Relationship on Wheat During the Infection Process of *Pyricularia oryzae*

Abstract

Two experiments were carried out to assess the changes associated with photoassimilates production and partitioning in source-sink relationship on flag leaves and spikes of wheat plants infected with *Pyricularia oryzae*, the causal agent of blast. Flag leaves and spikes were inoculated at 10 days after anthesis (daa) (Exp. 1) or at 20 daa (Exp. 2) with a conidial suspension of *P. oryzae*. There was an impairment on chlorophyll *a* fluorescence parameters (analyzed as F_m, F_v/F_m YII and Y(NO)) on both the infected flag leaves and spikes coupled with reduced concentrations of chlorophyll *a* + *b* and carotenoids as well as lower capacity for CO_2 fixation by RuBisCO in the infected flag leaves. In these leaves and grains (obtained from the infected spikes) there were lower concentration of soluble sugars and an increase on the hexoses-to-sucrose ratio on the flag leaves. In the infected flag leaves, there was a lower sucrose phosphate synthase (SPS) activity and lower expression of sucrose synthesis (*Susy*) gene while there were higher expression and activity of acid invertases. . At advanced stages of fungal infection, the concentration of starch was reduced on grains whereas on the infected flag leaves its concentration was kept high. There were reduction on the activity of ADP-glucose pyrophosphorylase and on the expression of *ADP-glucose pyrophosphorylase* genes and down-regulation of *β*- and *α-amylase* expression at late stages of fungal infection on flag leaves and spikes. In conclusion, the greatest effect of blast on both grains quality and yield can be associated with alterations in the production and partitioning of carbohydrates during the grain filling process.

Keywords: Pyricularia oryzae, blast, sucrose, starch, wheat.

Introduction

Blast, caused by the fungus *Pyricularia oryzae* (Cooke) Sacc., is one of the most important diseases occurring on wheat and can cause yield losses of approximately 60% (Goulart & Paiva, 2007). This fungus can infect all aerial organs of the plants (Igarashi et al., 1986). On leaves, the symptoms are elliptical or rounded lesions with dark brown margins and gray centers (Goulart & Paiva, 2007). However, it is on the spikes that the blast symptoms are much more pronounced, which including the bleaching and death of the infected tissues in the rachis (Goulart & Paiva, 2007). The management strategies for wheat blast includes the use of resistant cultivars in association with the application of fungicides especially the Quinone Outside Inhibitors formulated as a single active ingredient or in mixture with demethylation inhibitors (Maciel et al., 2011).

Plants produce carbohydrates in photosynthetic source organs and export them toward sugar-importing sink organs (Gamm et al., 2011). Wheat grain yield is considered to be a dynamic process determined to a great extent by the capacity of the source leaves to export photoassimilates to the sink grains tissues as well as by the capacity of these sinks to convert these photoassimilates into starch (Tuncel & Okita, 2013). During the day, the carbon fixed in the leaves can be converted to sucrose in the cytosol and later on it is transported to the sinks and converted to starch (Zeeman et al., 2007). Some of the fixed CO_2 can be stored as transient starch in chloroplasts and broken down at night to support leaf metabolism and sucrose export (Zeeman et al., 2007). The infection process of pathogens on their hosts' tissue can considerably affect the source/sink relationships via impairments to the photosynthetic performance as well as the carbohydrate composition and partitioning within the infected tissues (Abood & Lösel, 2003, Gamm et al., 2011). In the grapevine leaves infected with *Plasmopora viticola*, for example, a higher starch accumulation was noticed due to a higher AGPase activity (the enzyme that catalyzes the rate-limiting step of starch biosynthesis) and modifications in the starch degradation pathway (Gamm et al., 2011).

Invertases are ubiquitous enzymes that are produced not only by plants, but also by bacteria and fungi to ensure their nutritional supply (Voegele et al., 2006). These are found in several isoforms that differ by their subcellular localization, optimum pH and isoelectric points; these enzymes promote the cleavage of sucrose to hexoses that are then transported into the sink cells (Roitsch et al., 2003). The cell wall invertases are important in phloem unloading and is a determinant of the sink strength in plants (Berger et al., 2007). The increase in invertase activities at the pathogen's infection sites appears to be a common feature reported to occur in many host-pathogen interactions (Fotopoulos et al., 2003; Roitsc et al., 2003; Chou et al., 2000, Berger et al., 2007). An increase on invertase in the infected tissues is associated with a reduction on photoassimilate export and unloading of sucrose into cells adjacent to fungal hyphae indicating, therefore, a status sink in the infected tissue (Berger et al., 2007).. On wheat leaves infected by *Blumeria graminis* f. sp. *tritici* there was higher uptake of sugars associated with an increase on the activity of cell-wall invertases (Sutton et al., 2007).

Reduction on wheat yield caused by the infection of *P. oryzae* on the spikes has been suggested to be associated with a reduction in the capacity of translocation of photoassimilates in the infected rachis making, therefore, the grains to become shriveled, deformed, small and lightweight (Goulart & Paiva, 2007). Considering the importance of wheat blast to dramatically decrease wheat yields, the present study aimed to investigate whether the infection of flag leaves and spikes by *P. oryzae* play a role in reducing thephotoassimilate production via lower photosynthesis and synthesis of sucrose in the flag leaves reducing, therefore its exportation and its impacts on the conversion into starch in the grains. In order to test this hypothesis, we determined the expression of genes and the activity of enzymes involved in the carbohydrate metabolism, the chlorophyll *a* fluorescence parameters as well as the quantification of the concentrations of carbohydrates, pigments and metabolites in order to feasible decipher the mechanism responsible for reduction on grain yield on wheat plants infected with *P. oryzae*.

Material and Methods

Plant growth

Wheat seeds (*Triticum aestivum*) from BRS-Guamirim, a commercially grown Brazilian cultivar which is susceptible to blast (Cruz et al., 2010), were surface sterilized in 10% (vol/vol) NaOCl for 2 min and sown in plastic pots (20-cm diameter) (Ecovaso, Jaguariúna, São Paulo, Brazil) filled with 1 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 1.63 g of calcium phosphate monobasic was added to each plastic pot. A total of twelve seeds were sown per pot and at five days after seedlings emergence, each pot was thinned to six seedlings. The substrate in each pot was fertilized with a nutrient solution containing the following in g L^{-1}: 6.4 KCl, 3.48 K_2SO_4, 5.01 $MgSO_4.7H_2O$, 2.03 $(NH_2)_2CO$, 0.009 $NH_4MO_7O_{24}.4H_2O$, 0.054 H_3BO_3, 0.222 $ZnSO_4.7H_2O$, 0.058 $CuSO_4.5H_2O$ and 0.137 $MnCl_2.4H_2O$ (Xavier Filha et al., 2011). A volume of 15 mL of nutrient solution containing 0.27 g L^{-1} $FeSO_4.7H_2O$ and 0.37 g L^{-1} EDTA bisodic was also applied after seedlings emergence. The nutrient solution was prepared using deionized water. A total of 30 ml of the nutrient solution was also applied to each pot weekly.

Plant inoculation with *P. oryzae*

Leaves and spikes of plants were inoculated at 10 days after anthesis (daa) (growth stage 75) (Exp. 1) and at 20 daa (growth stage 83) (Exp. 2) (Zadoks et al., 1974) with a conidial suspension of *P. oryzae* (10^5 conidia mL^{-1}). Twenty-five milliliters of suspension was sprayed as a fine mist in the leaves and spikes of each plant until runoff using a VL Airbrush atomizer (Paasche Airbrush Co., Chicago, IL). Gelatin (1%, w v^{-1}) was added to the suspension to aid conidial adhesion to the leaf blades and spikes. 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 experiments. 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 24 ± 1°C (day) to 18 ± 2°C (night). The relative humidity was maintained at 90 ± 5% 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 thermo-hygrograph (TH-508, Impac, São Paulo, Brazil). The maximum natural photon flux density at plant canopy height was approximately 950 µmol m^{-2} s^{-1}.

Blast assessment

Blast severity on the flag leaves and spikes was assessed at 48, 72 and 96 hours after inoculation (hai). On the flag leaves, blast severity was quantified by using a diagrammatic scale (Rios et al., 2013) while on the spikes it was quantified as the mean proportion of diseased spikelet per spike (diseased spikelets/total spikelets rated × 100).

Chlorophyll *a* (Chl *a*) fluorescence imaging and parameters

Images and parameters of Chl *a* fluorescence were obtained from the flag leaves and spikes of wheat plants at 48, 72 and 96 hai using the MAXI version of the Imaging-PAM fluorometer and the Imaging Win software (Heinz Walz GmbH, Effeltrich, Germany). The Chl *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 tissue. The performance photosynthetic was determined in spikes and flag leaves ass described earliest by Tatagiba et al., (2015). Following the calculations of Kramer et al. (2004), the energy absorbed by PSII for the following two yield components for dissipative processes were determined: the yield of photochemistry [Y(II) = (F_m’ - F_s)/F_m’)] and the yield for other non-photochemical (non-regulated) losses [Y(NO) = F_s/F_m] (Kramer et al., 2004).

Determination of the concentration of photosynthetic pigments

The concentrations of chlorophyll *a* (Chl *a*), Chl *b* and carotenoids were determined using the dimethyl sulfoxide (DMSO) as an extractor (Santos et al., 2008). Five disks of flag leaves (1 cm in diameter) were collected at 48, 72 and 96 hai. These collected disks were immersed in glass tubes containing 5 mL of saturated DMSO solution and calcium carbonate ($CaCO_3$) (5 g L^{-1}) (Wellburn, 1994) and kept in the dark at room temperature for 24 h. The absorbances of the extracts were read at 480, 649.1 and 665.1 nm using a saturated solution of DMSO and $CaCO_3$ as a blank.

Extraction and determination of the concentrations of carbohydrates, amino acids, malate, fumarate and proteins

Samples of both flag leaves and grains obtained from the inoculated spikes were collected at 48, 72 and 96 hai from plants at 10 and 20 daa. Samples (250 mg of fresh weight (FW)) were lyophilized (- 48°C) and grounded in a cell disruptor using metal balls (3.2 mm in diameter) following agitation at40 *g* for 5 min (Mini-Bead beater-96, Bio Spec Products, Bartlesville, OK, USA). Samples (15 mg) were homogenized with 600 µL 98% (v/v) ethanol and then incubated at 80ºC for 20 min following centrifugation at 14000 *g* for 5 min. This process was repeated after homogenizing the pellet with 80 and 50% ethanol, respectively. The concentrations of sugars (glucose, fructose and sucrose), amino acids (Gibon et al., 2004), malate and fumarate (Nunes-Nesi et al., 2007) were determined in the supernatant solutions while the concentrations of starch (Fernie et al., 2001) and proteins (Bradford, 1976) were determined in the insoluble fractions. The ratio of the sugar concentrations (RCS) was estimated as RCS = ($X_{48,\ 72\ and\ 96\ \mathrm{hai}}/\bar{X}_{control}$) where $\bar{X}$ represents mean value of concentrations of carbohydrates from non-inoculated plants (control) at 48, 72 and 96 hai.

RNA extraction and real time quantitative PCR (RT-qPCR)

Flag leaves and spikelets obtained from the inoculated spikes (a total of 5 flag leaves and 5 spikelets per replication of each treatment) were collected at 48, 72 and 96 hai for the gen expression analysis. The samples were maintained in liquid nitrogen during samplings and stored at -80°C until further analysis. Samples of flag leaves and spikelets were ground to powder under liquid nitrogen for RNA extraction using the RNAqueous® kit (Applied Biosystems, Foster City, USA) according to the manufacturer's instructions. After extraction, the total RNA concentration was determined using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, USA) and RNA quality was assessed by the integrity of the bands of ribosomal RNA on agarose gel 1.5%. The RNA purity was determined by the ratio of absorbance at 260 and 280 nm. Next, total RNA was treated with RNase Free DNase (Promega, São Paulo, Brazil) according to the manufacturer's recommendations. The total RNA of the samples was again quantified and evaluated for integrity as previously described. First-strand cDNA was synthesized using 1 µg of total RNA, M-MLV reverse transcriptase (Invitrogen, São Paulo, Brazil) and the primer oligo (dT) 12-18 (Sigma-Aldrich, São Paulo, Brazil) according to the manufacturer’s instructions. The investigated genes, the designed primer sequences and the RT-qPCR primer sequences are listed in Table 1. The primers sequences for the amplification of the *cell wall invertases (Inv (CW)),* s*ucrose synthase (Susy), glucose-1-phosphate adenylyltransferase* (large subunit 1) (*AGPase*), *α-amylase* and *β-amylase* were designed using the Primer Express software (Applied Biosystems, Foster City, USA) and confirmed in accordance with the sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov/). The relative quantification of gene transcripts was carried out using the method $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001). The accumulation of transcripts for each gene was normalized using the expression of the constitutive genes *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and *ubiquitin* (*UBIQ*) as references. The RT-qPCR reactions contained 90 ng of cDNA template, 10 µL of 2 × SYBR-green master PCR mix (Applied Biosystems, São Paulo, Brazil) and primers at a concentration of 200 nM for each forward and reverse primer. The amplification conditions were two stages of 50°C for 2 min and 95°C for 10 min followed by 40 cycles at 95 and 60°C, respectively, for 30 s each. The dissociation curve was observed after amplification to ensure that only a single amplicon was produced in each reaction. Amplification of the specific regions of targeted genes and the real-time detection of amplicon production was undertaken in a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., São Paulo, Brazil).

Determination of the activities of sucrose synthase (Susy, EC 2.2.7.27)), ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO, EC 4.1.1.39), ADP-glucose pyrophosphorylase (AGPase, EC 2.2.7.27), acid invertases (EC 3.2.1.26) and sucrose-phosphate synthase (SPS, EC 2.4.1.14)

Samples of flag leaves and spikelets obtained from the inoculated spikes were collected at 48, 72 and 96 hai in two timepoints, at 10 and 20 daa. Samples were kept in liquid nitrogen during samplings and then stored at -80°C until further analysis. The enzymes were extracted from frozen samples as described by Nunes-Nesi et al. (2007). The activities of Susy towards break of sucrose (Nunes-Nesi et al., 2005), Rubisco (Sulpice et al., 2007), AGPase (Gibon et al., 2004), acid invertases (Praxedes et al., 2006) and SPS (under saturating – V_{max} and limiting – V_{sel} substrate conditions) (Jenner et al., 2001) were determined. These enzymes activities were expressed on FW and the RuBisCO activation state (%) was calculated as the ratio of initial activity to final activity.

Experimental design and statistical analysis

Two experiments (both leaves and spikes inoculated at 10 daa (Exp. 1) and 20 daa (Exp. 2) were arranged in completely randomized design with four replications. Each experimental unit consisted of one plastic pot containing five plants. Data obtained from each variable evaluated were subjected to analysis of variance. Means of blast severity on flag leaves and spikes were compared using the Tukey´s test ($P \leq 0.05$). For enzyme activities, Chl *a* fluorescence parameters as well as the concentrations of pigments and metabolites, means for the treatments non-inoculated and inoculated plants at each inoculation period (10 or 20 daa) were compared by the *t*-test ($P \leq 0.05$). Analysis of the temporal changes (up or down rate during the time course evaluated) on the expression of the genes studied and on the concentration of carbohydrates was performed using the interval of confidence. All statistical analysis were performed using the software SAS (v. 6.12; SAS Institute, Inc.).

Results

Disease development

At 10 daa, blast severity significantly increased by 8, 26 and 58% on the flag leaves and by 11, 44 and 72% on the spikes, respectively, at 48, 72 and 96 hai. At 20 daa, blast severity significantly increased by 5, 22 and 48% on the flag leaves and by 7, 38 and 60% on the spikes, respectively, at 48, 72 and 96 hai (Fig. 1).

Parameters of Chl *a* fluorescence

Chl *a* fluorescence analysis demonstrated that the photosynthetic performance was negatively affected upon plant inoculation (Fig. 2). The first visual alterations on the images of Chl *a* fluorescence in the infected leaves and spikes were already evident at 48 hai. In all cases, the damage caused by fungal infection increased over time as lesions expanded on the leaves and the spikes became more bleached and died. Coupled with an increased on blast severity, progressive loss of photosynthetic activity occurred mainly after 72 hai on leaves and at 96 hai in the spikes as indicated by the black areas in the images. These areas reflected damage to the photosynthetic apparatus as indicated by the intense necrosis in association with a complete loss of the optical properties of the infected tissues (Fig. 2). Changes on the values of F_m, F_v/F_m, Y(II) and Y(NO) in the flag leaves and spikes occurred at early stages of fungal infection at both 10 and 20 daa (Fig. 3). Indeed, the values of the parameters F_m and F_v/F_m significantly decreased for the infected flag leaves and spikes during the time course evaluated. For flag leaves and spikes, there were reduction on Y(II) at 72 and 96 hai at 10 and 20 daa. Conversely, the Y(NO) significantly increased for the infected flag leaves and spikes at 48, 72 and 96 hai at 10 daa as well as at 72 and 96 hai at 20 daa (Fig. 3).

RuBisCO activity

Compared with the non-infected flag leaves, initial RuBisCO activity was significantly reduced by 12, 65 and 72% in the infected flag leaves at 48, 72 and 96 hai at 10 daa and by 15 and 48% at 72 and 96 hai at 20 daa (Fig. 5). Similarly, the final RuBisCO activity was reduced by 21, 71 and 81% at 10 daa and by 38 and 53% at 20 daa at 72 and 96 hai, respectively. Additionally, the RuBisCO activation state was of 63, 71 and 88% for non-infected flag leaves in comparison to 57, 58 and 60% for infected flag leaves at 10 daa. At 20 daa, its activation state was of 57, 63 and 57% for non-infected flag leavesand of 51, 46 and 52% for infected flag leaves.

Photosynthetic pigments

The concentrations of Chl *a*, Chl *b* and carotenoids were significantly lower at 48, 72 and 96 hai in the infected flag leaves compared to the non-infected ones (Fig. 4). At 10 daa, there were reductions of 32, 31 and 28% for Chl *a*, of 32, 30 and 26% for Chl *b* and of 31, 33 and 49% for carotenoids at 48, 72 and 96 hai, respectively. At 20 daa, reductions of 25, 36 and 55% for Chl *a*, of 15, 43 and 54% for Chl *b* and of 27, 25 and 29% for carotenoids occurred at 48, 72 and 96 hai, respectively (Fig. 4).

Concentrations of carbohydrates, amino acids, malate, fumarate and proteins

At 10 and 20 daa, the concentrations of sucrose, fructose and glucose were significantly reduced for the infected flag leaves compared to the non-infected ones (Fig. 6). At 10 daa, reductions on the concentrations of sucrose, fructose and glucose were greater than 60% during the time course evaluated. At 20 daa, reductions on the concentrations of sucrose, fructose and glucose were greater than 26%, except for sucrose at 48 hai. At 10 daa, the concentration of starch for the infected flag leaves significantly increased by 25% at 96 hai while at 20 daa, these increases were of 12 and 16% at 72 and 96 hai, respectively. For the grains obtained from the inoculated spikes, the reduction on the concentration of all soluble sugars quantified, except for fructose at 48 hai, was greater than 27% at 10 and 20 daa compared to the non-inoculated ones. Starch concentration was reduced by 25, 23 and 45% at 10 daa and by 29, 30 and 45% at 20 daa for the grains obtained from the inoculated spikes at 48, 72 and 96 hai, respectively. At 10 daa, the temporal analysis of the RCS for the infected flag leaves showed significant reduction on sucrose concentration during the time course evaluated and for sucrose, fructose and glucose at 20 daa. The starch concentration for the infected flag leaves at 10 and 20 daa significantly increased at 96 hai. For the grains obtained from the inoculated spikes, there were significant reductions on the concentration of soluble sugar and starch at advanced stages of fungal infection (Fig. 6).

For the infected flag leaves, the malate concentration was significantly reduced by 16, 54 and 68% at 10 daa and by 13, 18 and 30% at 20 daa at 48, 72 and 96 hai, respectively, compared to the non-infected ones. For the infected flag leaves, the concentration of fumarate was reduced by 41 and 54% at 10 daa and by 34 and 45% at 20 daa at 72 and 96 hai, respectively. The concentration of proteins was significantly reduced by 12% at 10 daa and by 6, 18, and 32% at 20 daa, respectively, at 48, 72 and 96 hai for the infected flag leaves compared to the non-infected ones. The amino acids concentration was significantly reduced by 65, 71 and 62% at 10 daa, respectively, at 48, 72 and 96 hai and by 26 and 64% at 20 daa, respectively, at 48 and 96 hai for the infected flag leaves compared to the non-infected ones (Table 2). For the grains obtained from the inoculated spikes, these reductions were of 12 and 18% at 10 daa and of 20 and 14% at 20 daa at 72 and 96 hai, respectively. For the grains obtained from the inoculated spikes, there were significant increases of 31% at 96 hai at 10 daa and of 31 and 17%, respectively, at 48 and 72 hai at 20 daa. For the grains obtained from the inoculated spikes, there were significant reductions on the concentration of proteins of 28, 10 and 23% at 10 daa and of 9, 7 and 13% at 20 daa, respectively, at 48, 72 and 96 hai.

Gene expression

At 10 daa, the infected flag leaves displayed increases in the expression of *Susy* and *AGPase* (1.4-fold) at 48 hai and of *β-amylase* and *α-amylase* (1.9 to 2.5-folds) at 48 and 72 hai relative to the non-infected ones. The expression of *Susy* and *AGPase* (0.28 to 0.95-folds) were repressed at 72 and 96 hai and the *β-amylase* and *α-amylase* (0.46 to 0.97-folds) at 96 hai. At 20 daa, there were increases on the expression of *Susy*, *AGPase*, *α-amylase* and *β-amylase* (2.55 to 2.28-folds) at 48 and 72 hai, but their expressions were repressed (0.19 to 0.64-folds) at 96 hai. At 10 daa, there were increases in the expression of *Susy* (1.3 to 1.7-folds) at 48 and 72 hai, *α-amylase* (1.3-fold) at 72 hai and *β-amylase* (1.3 to 1.7-folds) at 48, 72 and 96 hai for the inoculated spikes relative to the non-inoculated ones. There were reductions on the expression of *Susy* (0.2-fold) at 96 hai, *α-amylase* (0.4 to 0.9-folds) at 48 and 96 hai and *AGPase* (0.15 to 0.50-folds) at 48, 72 and 96 hai. For the inoculated spikes at 20 daa, there were increases in expression of *Susy*, *AGPase* and *α-*

amylase (1.2 to 3.0-folds) at 48 hai and *β-amylase* (2.0 to 2.2-folds) at 48 and 72 hai. There were reductions on the expression of *Susy*, *AGPase* and *α-amylase* (0.31 to 0.81-folds) at 72 and 96 hai and *β-amylase* (0.93-fold) at 96 hai. At 10 and 20 daa, there were increases on the expression of *invertases* (1.1 to 3.4-folds) at 72 and 96 hai for the infected flag leaves and spikes (Fig. 7).

Enzymes activities

The infected flag leaves infected hadd significant reductions on the AGPase activity of 42 and 33%, respectively, at 72 and 96 hai at 10 daa and of 28, 8 and 22% at 48, 72 and 96 hai, respectively, at 20 daa relative to the non-infected ones. There were significant reductions on the AGPase activity for the inoculated spikes of 30, 50 and 40% at 10 daa and of 18, 20 and 30% at 20 daa, respectively, at 48, 72 and 96 hai. The SPS activity was significantly reduced by 21, 48 and 44% on infected flag leaves and by 18, 30 and 27% on inoculated spikes at 48, 72 and 96 hai, respectively, at 10 daa in comparison to the non-inoculated controls. At 20 daa, reduction greater than 35% on SPS activity in the infected flag leaves and spikes occurred at 72 and 96 hai. The Susy activity on infected flag leaves was significantly higher by 36, 32 and 29% at 10 daa and by 50, 26 and 45% at 20 daa at 48, 72 and 96 hai, respectively. For the inoculated spikes, there were significant reductions on Susy activity of 29 and 20% at 48 and 72 hai at 10 daa and of 63% at 96 hai at 20 daa. The cell wall acid invertase activities in the infected flag leaves were significantly higher by 19, 21 and 53% at 10 daa and by 16, 20 and 56% at 20 daa at 48, 72 and 96 hai, respectively. At 96 hai for the inoculated spikes, the cell wall acid invertases activity was reduced by 15% at 10 daa and by 20% at 20 daa (Fig. 8).

Discussion

The current study provides, to the best of the authors´ knowledge, the first evidence that the photosynthetic performance of infected flag leaves and spikes by *P. oryzae* was down-regulated through impairment in photochemistry (denoted by a lot of alteration in Chl *a* fluorescence parameters coupled with reductions (or degradation of photosynthetic pigments) and biochemistry (denoted by decreases in RuBisCO activity and activation state) . Additionally, novel evidence that the infection of flag leaves and spikes of wheat plants by *P. oryzae* affected carbohydrates production and partitioning during two important grain-filling stages is also presented. Changes in the metabolism of carbohydrates have been reported to occur in many host-parasite interactions (Abood et al., 2003; Maust et al., 2003; Gamm et al., 2011).

At 10 and 20 daa, the photochemical performance of infected flag leaves and spikes progressively decreased as the disease developed. In the present study, the decreases in the values of F_m, F_v/F_m and Y(II) associated with increase of the Y(NO) values reflected the lost of the capacity of photoprotection, which resulted in increase of the photooxidative damage on theses infected host tissues. The maximal PSII photochemical efficiency, as determined by the F_v/F_m, decreased to values below 0.80 on infected flag leaves and spikes compared to the non-inoculated ones suggesting, therefore, the occurrence of photoinhibition to photosynthesis (Rolfe et al., 2010). Additionally, the lower values for Y(II) suggested impairment on the apparent electron transport activity on infected flag leaves and spikes. This effect resulted from fungal infection in the flag leaves was coupled with a progressive loss of pigments, especially Chl *a*, which is a preferentially photobleached relative to Chl *b* (Murchie et al., 1997). The photooxidative damage in the plant tissue is associated with higher Y(NO) values and reflects the inability of the plant to regulate its mechanisms of protection (Rolfe et al., 2010). The Y(NO) suggests that the fraction of energy could be passively dissipated in the form of heat and fluorescence mainly due to closed PSII reaction centers (Klughammer et al., 2008). The dysfunctions at the photochemical level in infected plant tissue suggest lower provision of ATP and reduced power for CO_2 assimilation (Rolfe et al., 2010). Nonetheless, in the present study, is expected to be the CO_2 fixation additionally presumably impaired given that RuBisCO activity remarkably decreased in the infected flag leaves. Previous studies have also demonstrated via gas gas exchange analyses , that the photosynthetic reduction on the wheat leaves infected by *P. oryzae* was due chiefly to biochemical constrains linked to presumable lower RuBisCO activity (Debona et al., 2014, Aucique-Pérez et al., 2014). Given that photosynthesis was drastically affected in the infected flag leaves and spikes a lower the availability of photoassimilates to be exported towards the sink tissues is, therefore, to be expected.

The concentration of sucrose, the main form of sugar transported in plants, was reduced in the infected flag leaves and on the grains obtained from infected spikes suggesting alterations in the source-sink metabolism. Sucrose synthesis was likely to be decreased due to lower substrate availability (less trose-phosphate and lower CO_2 fixation) and lower SPS activity, the enzyme that catalyzes the rate-limiting step in sucrose biosynthesis. This is an important regulatory enzyme in the sucrose synthesis for catalyzes the conversion of UDP-glucose and fructose-6-P to produce UDP and sucrose-6-P. (Winter & Huber, 2000). Apart from lower sucrose synthesis,increase in invertases activities and gene expression on infected flag leaves and spikelets suggest this contributing further to depress the sucrose concentrations increased breakdown of sucrose. In the present study, the concentrations of fructose and glucose was also dramatically decreased as the disease developed in the flag leaves and grains. According to Berger et al. (2004), changes in sugar levels in response to pathogen infection depends on the pathogen´s life style compared to the regulation of genes expression. Despite reduction on the concentration of soluble sugars in the infected flag leaves, the decrease in the concentration of sucrose was stronger than the fructose and glucose. Consequently, the hexoses-to-sucrose ratio increased as the disease developed correlating, therefore, with an induction on cell wall invertases. The results of the present study are supported by the findings of Berger et al. (2004) who observed significant reduction on the concentration of sugars and an increase in the hexoses-to-sucrose ratio in tomato leaves infected with *Pseudomonas syringae* and *Botrytis cinerea*. As has been reported for other host-parasite interactions (Gamm et al., 2011; Maust et al., 2003), for the wheat-*P. oryzae* interaction in particular, the association of reduced photosynthesis with an increase on invertase activities coupled with increases for the hexoses-to-sucrose ratio suggest, therefore, a status sink in the infected tissues. Additionally, the concentrations of malate and fumarate were lower in the infected flag leaves. The reduction on their synthesis may be associated to a lower level of carbohydrates in the cytosol. Malate and fumarate are involved in various metabolic pathways in plants in order to provide a source for carbon and energy for the biosynthesis of defense compounds (Casati et al., 1999). Therefore, the manipulation of the concentrations of malate and fumarate may be a strategy adopted by *P. oryzae* in order to establish a successful infection.

Long-distance transport of assimilates from source tissues into sink is driven by differences in hydrostatic pressure generated by loading and unloading of sucrose (Eschrich, 1980). Source strength refers to be ability of the source tissues to export photoassimilates being the SPS activity an indicator important regulatory enzyme in the process (Champigny et al., 1995; Wang et al., 2015). In the present study, infected flag leaves showed impairment of the photosynthetic performance coupled with lower sucrose synthesis by SPS activity suggesting, therefore, reduction of their capacity as a source of photoassimilates. On the other hand, the sink strength refers to the ability of the grains to absorb and convert these photoassimilates into starch (Wang et al., 2015). Once sucrose is unloaded to sink organs, it can be used either by cytosolic invertases or SuSy. The SuSy activity utilizes sucrose to produce fructose and UDP-glucose, which is the first major step of sucrose utilization in the grains (Winter & Huber, 2000). According to Li et al. (2013), a higher SuSy activity may potentially increase the sucrose flow towards the sink tissues which ultimately may lead to increased starch synthesis. Thus, the lower SuSy activity coupled with reduced sucrose concentration suggests an impairment of the sink ability in the infected spikes. The *Inv(CW)* expression was induced in infected grains, which was not associated with an increase, but with a reduction, on invertase activity at advanced stages of fungal infection. Previous studies demonstrated that sugars and stress-related stimuli independently activate differently signal transduction pathways on plants (Roitsch et al., 2004). Thus, invertase activity could be influenced by a post-translational regulation being important to control sucrose unloading (Gibon et al., 2004; Gamm et al., 2011). Therefore, the decrease on the concentration of sucrose on the grains obtained from the infected spikes could be also resulted of the lower invertases activity that negatively impaired its transport capacity.

In the present study, starch was unexpectedly accumulated in the infected leaves at 96 hai, given that less substrate for starch synthesis was produced (reduced photosynthesis) coupled with lower *AGPase* expression concomitant with lower a AGPase activity. Nonetheless, starch accumulation in the infected leaf tissue seems to be a common feature in many host-parasite interactions. There we proposed that such an accumulation was associated with a down- regulation of the starch degradation pathway, given that expression of α- and β- amylases was repressed in the flag leaves these enzymes accounted by initial attack on the starch granule, resulting in release of malto-oligosaccharides and maltose, respectively (Scheiidig et al., 2002 In contrast to the flag leaves, in the grains obtained from the infected spikes, the concentration of starch dramatically decreased during the time course evaluated mainly because of a lower sucrose transport from the photosynthetic organs coupled with lower activity and expression of AGPase.

Additionally, the expression of α- and *β-amylase* was induced at early stages of fungal infection indicating, therefore, an increase of the breakdown of starch in the infected grains. According to Lawlor & Paul (2014), the rate of grain filling is highly regulated when shortage of photoassimilates occurs, this resulting in limited duration of filling and, consequently, small and shrunken grains. Thus, lower starch synthesis and accumulation may compromise not only the total grain dry weight (and consequently the wheat yield) (Tuncel & Okita, 2013), but also the finality of the grain.

In the present study, the lower concentration of amino acids in the infected flag leaves was associated with a reduced photosynthetic performance. Photosynthesis provides reducing power necessary for the reduction of nitrate into ammonia and carbon skeletons that will be used for the amino acid synthesis (Wang et al., 2010). This reduction could also be associated with the negative effect of *P. oryzae* on the infected tissues that may act as an additional sink of amino acids. Additionally, the concentration of proteins in the infected flag leaves and on the grains obtained from the infected spikes was low. According to Dupont et al. (2003), the accumulation of proteins on the grains is an important factor associated with their development and, therefore, with the quality of the flour. Thus, the reduction on the quality of the infected grains will reflect in a decrease in the concentration of proteins.

It can be concluded that the deleterious effects of blast on grain quality and yield can be associated with photosynthetic dysfunctions and impairment on sucrose synthesis in the flag leaves due to reduced SPS activity and *Susy* expression. Conversely, there were increases on cleavage of sucrose by invertases reducing, therefore, its exportation from infected flag leaves to grains. Consequently, lower synthesis of starch was achieved due to lower activity and expression of AGPase associated to its higher breakdown by α- and β-amylase at early stages of fungal infection. Therefore, it can be concluded that the effect of

P. oryzae in the source-sink relationship was mainly due to alterations in the production assimilate and partitioning during the grain filling process.

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List of Tables and Figures

Table 1. Genes and primers sequences used to study the metabolism of carbohydrates in the flag leaves and spikes of wheat plants non-inoculated or inoculated with *Pyricularia oryzae* through the quantitative reverse transcription qRT-PCR.

Target genes[a]	Forward primers sequences (5´ to 3´)	Reverse primers sequences (5´ to 3´)
Inv (CW)	TTCACGTTTTCAACAACGGC	CTGGCTTCTTCATCTCCCAC
Susy	GGCCATATCCGTTGGATCTC	TTGGTGTCACAGATGTAGCG
AGPase	GTACGGGCTAGTGAAGTTCG	ATCGTCACCCTTTGGCTTTT
α-amylases	TACGACCATGTGTTCGACTG	GATGGATCCCGTTCCTTGAC
β-amylases	ATGCCGGACAGTACAATGAC	CTCCCCTGCTCAGTGAGATA
GAPDH	CCTTCCGTGTTCCCACTGTTG	ATGCCCTTGAGGTTTCCCTC
UBIQ	CCTTCACTTGGTTCTCCGTCT	AACGACCAGGACGACAGACACA
MAG2	ACAGATCGCGTCAAGATTGTTTT	CACAGGGTTGGCCGAGTT

[a] *Inv (CW)*: *Cell wall invertases, Susy: sucrose synthase*; *AGPase*: *ADP-glucose pyrophosphorylase*; *α-amylases and β-amylases*; *GAPDH*: *glyceraldehyde-3-phosphate dehydrogenase*; *EF1α*: *elongation factor 1α*; *UBIQ: ubiquitin*; *MAG2*: *glycogen synthase from Pyricularia oryzae*.

Table 2. Concentrations of malate (nmol mg^{-1} DW) , fumarate (nmol mg^{-1} DW), proteins (mg^{-1} DW) and amino acids (mmol mg^{-1} DW) in the flag leaves and on grains obtained from spikes of wheat plants non-inoculated (NI) or inoculated (I) with *Pyricularia oryzae*. DW = dry weight.

Treatments	daa[a]	hai[b]	Malate		Fumarate		Protein		Amino acids	
			NI	I	NI	I	NI	I	NI	I
Flag leaves	10	48	107.5*	89.6	8.2$^{\text{ns}}$	8.6	45.7*	39.6	7.9*	2.7
		72	169.6*	77.0	11.6*	6.8	43.4*	38.1	8.7*	2.5
		96	241.8*	76.3	11.8*	5.4	41.3*	35.4	7.5*	2.8
	20	48	86.4*	74.9	6.2$^{\text{ns}}$	5.7	50.0 *	46.6	4.2*	3.1
		72	101.1*	83.6	6.9*	4.5	55.6*	45.3	3.6	3.4
		96	131.9*	92.1	4.4*	2.4	47.4*	32.1	5.9*	2.1
Grains	10	48	79.5$^{\text{ns}}$	75.6	9.0$^{\text{ns}}$	8.9	68.3*	48.8	6.1$^{\text{ns}}$	5.9
		72	85.5*	75.8	8.1$^{\text{ns}}$	8.5	58.4*	52.6	6.1$^{\text{ns}}$	5.9
		96	92.2*	74.7	6.0*	8.8	65.0*	49.5	5.7$^{\text{ns}}$	5.6
	20	48	61.2$^{\text{ns}}$	61.0	4.4*	6.4	41.6*	37.6	3.3$^{\text{ns}}$	3.3
		72	76.4*	61.0	4.9*	5.9	40.2*	37.7	2.9$^{\text{ns}}$	3.6
		96	70.3*	60.1	5.5$^{\text{ns}}$	5.8	45.2*	39.2	2.6$^{\text{ns}}$	3.3

[a] Plants were inoculated at 10 (growth stage 75) and 20 (growth stage 83) days after anthesis (daa).

[b] Samples from flag leaves and spikes were collected from inoculated plants (48, 72 and 96 hours after inoculation (hai)) at 10 and 20 daa.

Means from NI and I treatments followed by an asterisk (*), within each evaluation time, are significantly different ($P \leq 0.05$) by the *t*-test. DW: dry weight.

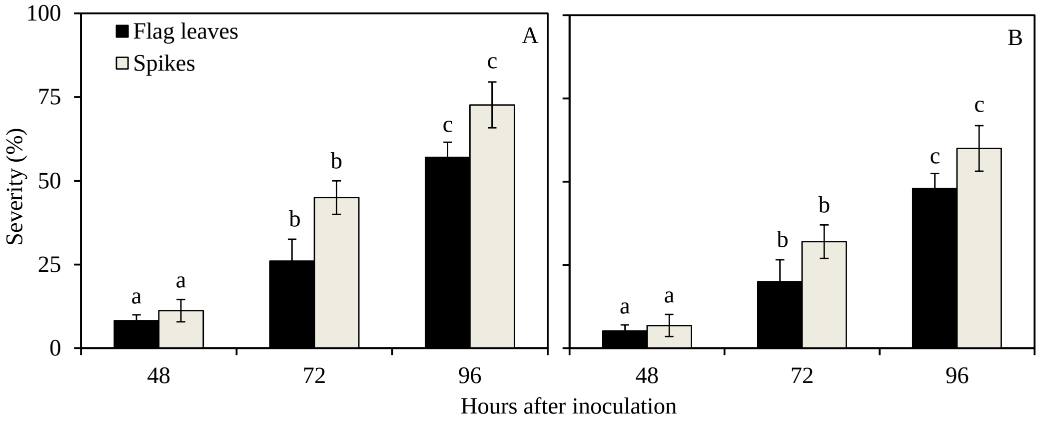

Fig. 1. Blast severity on the flag leaves and spikes of wheat plants inoculated with *Pyricularia oryzae*. Means of blast severity on either flag leaves or spikes of wheat plants at 10 (A) or 20 (B) days after anthesis followed by the same letter, within each evaluation time, are significantly different ($P \leq 0.05$) by Tukey´s test. Bars represent the standard error of the mean. $n = 10$.

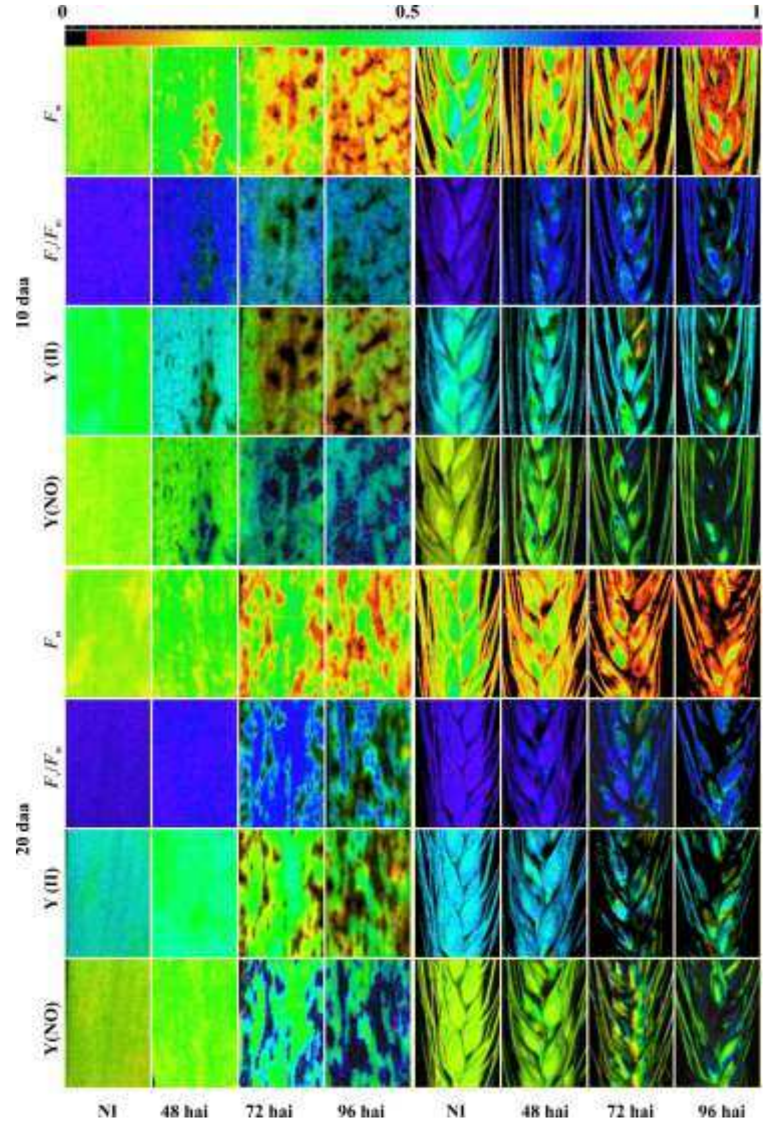

Fig. 2. Maximal photosystem II quantum yield (F_v/F_m), maximal fluorescence (F_m), effective PSII quantum yield (Y(II)) and quantum yield of non-regulated energy dissipation (Y(NO)) determined in the flag leaves and spikes of wheat plants at 10 and 20 days after anthesis that were non-inoculated (NI) or inoculated (I) (48, 72 and 96 hours after inoculation) with *Pyricularia oryzae*. Means from NI and I treatments followed by an asterisk (*), within each evaluation time, are significantly different ($P \leq 0.05$) by the *t*-test.

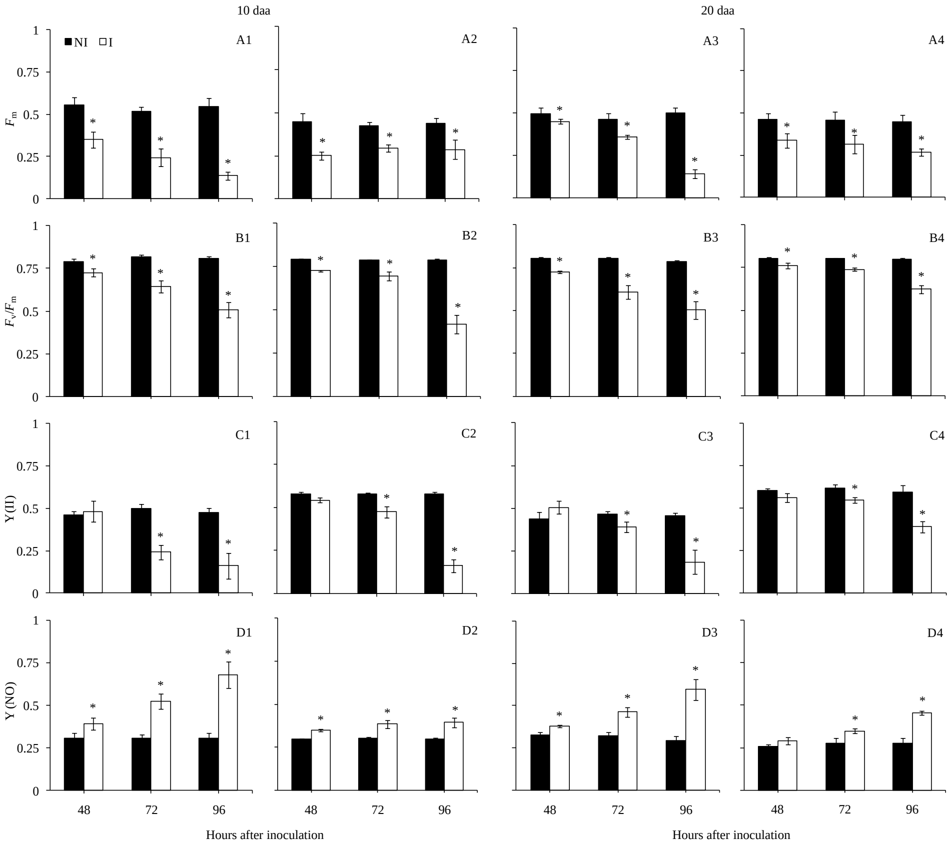

Fig. 3. Parameters of chlorophyll *a* fluorescence: maximal photosystem II quantum efficiency (F_v/F_m), maximal fluorescence (F_m), effective PSII quantum yield (Y(II)) and quantum yield of non-regulated energy dissipation (Y(NO)) determined in the flag leaves and spikes of wheat plants at 10 and 20 days after anthesis that were non-inoculated (NI) or inoculated (I) (48, 72 and 96 hours after inoculation (hai)) with *Pyricularia oryzae*.

3

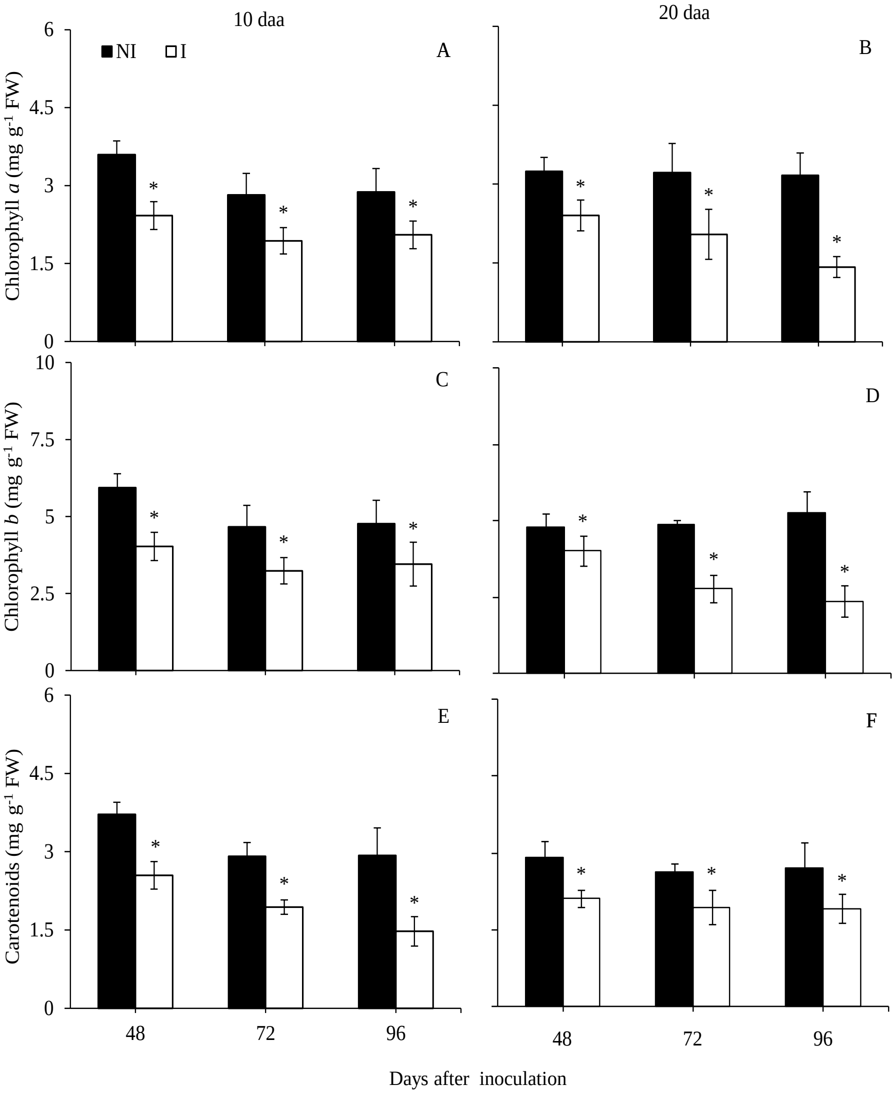

Fig. 4. Concentrations of chlorophyll *a* (A and B), chlorophyll *b* (C and D) and carotenoids (E and F) determined in the flag leaves of wheat plants at 10 and 20 days after anthesis that were non-inoculated (NI) or inoculated (I) (48, 72 and 96 hours after inoculation) with *Pyricularia oryzae*. Means from the NI and I treatments followed by an asterisk (*), within each evaluation time, are significantly different by the *t*-test ($P \leq 0.05$). Bars represent the standard deviations of the means. FW = fresh weigh. $n = 5$.

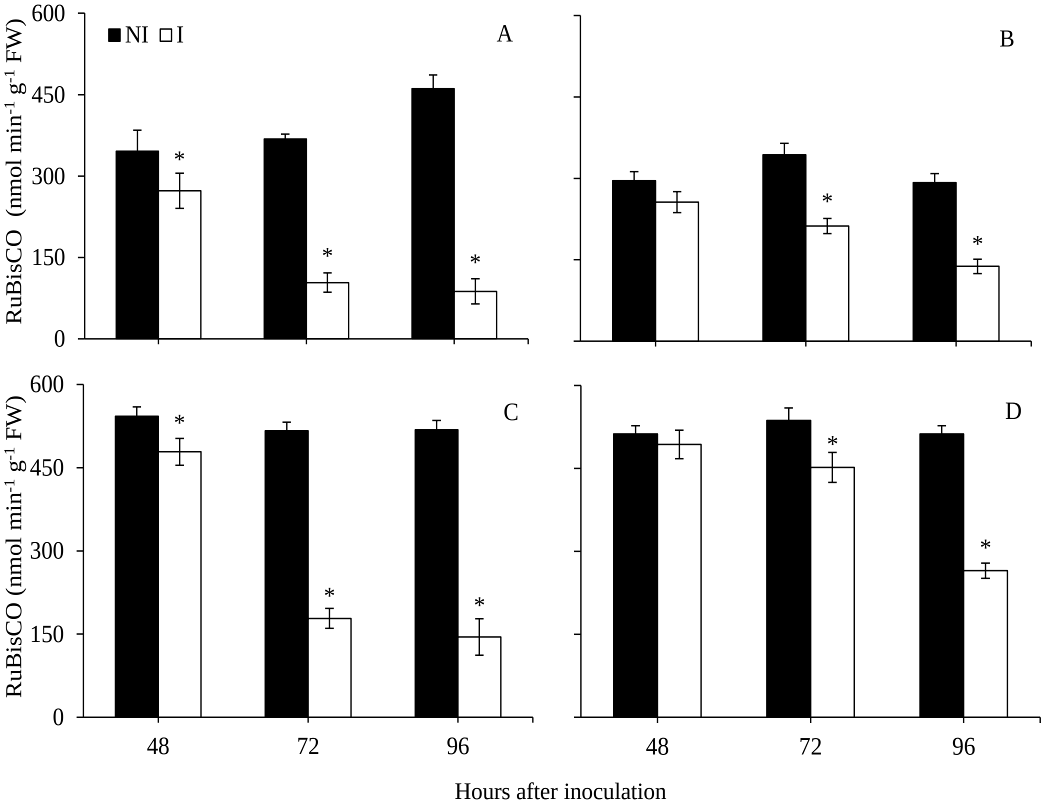

Fig. 5. Initial (A and B) and final (C and D) activity of Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) determined in the flag leaves of wheat plants at 10 (A and C) and 20 (B and D) days after anthesis that were non-inoculated (NI) or inoculated (I) (48, 72 and 96 hours after inoculation) with *Pyricularia oryzae*. Means from NI and I treatments followed by an asterisk (*), within each evaluation time, are significantly different ($P \leq 0.05$) by the *t*-test. Bars represent the standard deviations of the means. $n = 4$. FW = fresh weight.

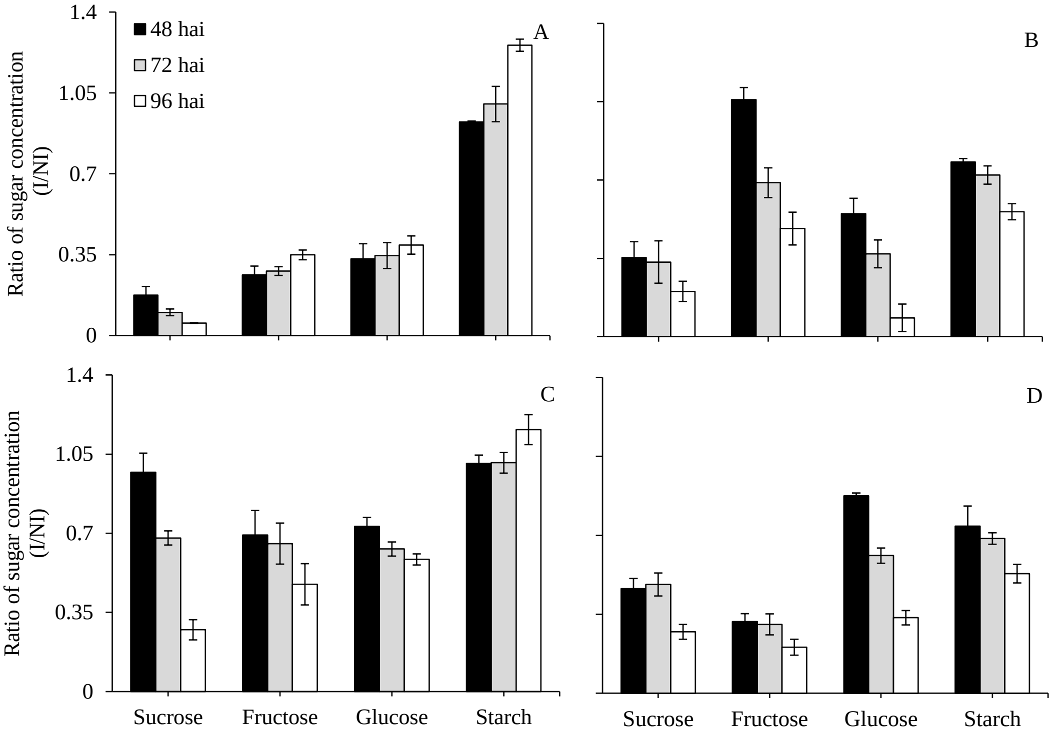

Fig. 6. Ratio of sucrose, fructose, glucose and starch concentrations determined in the flag leaves (A and C) and spikes (B and D) of wheat plants at 10 (A and B) and 20 (C and D) days after anthesis that were non-inoculated or inoculated (48, 72 and 96 hours after inoculation) with *Pyricularia oryzae*. The temporal analysis comparing the up or down changes on the concentration of carbohydrates was performed using the confidence intervals (bars) of the means ($P \leq 0.05$). $n = 4$.

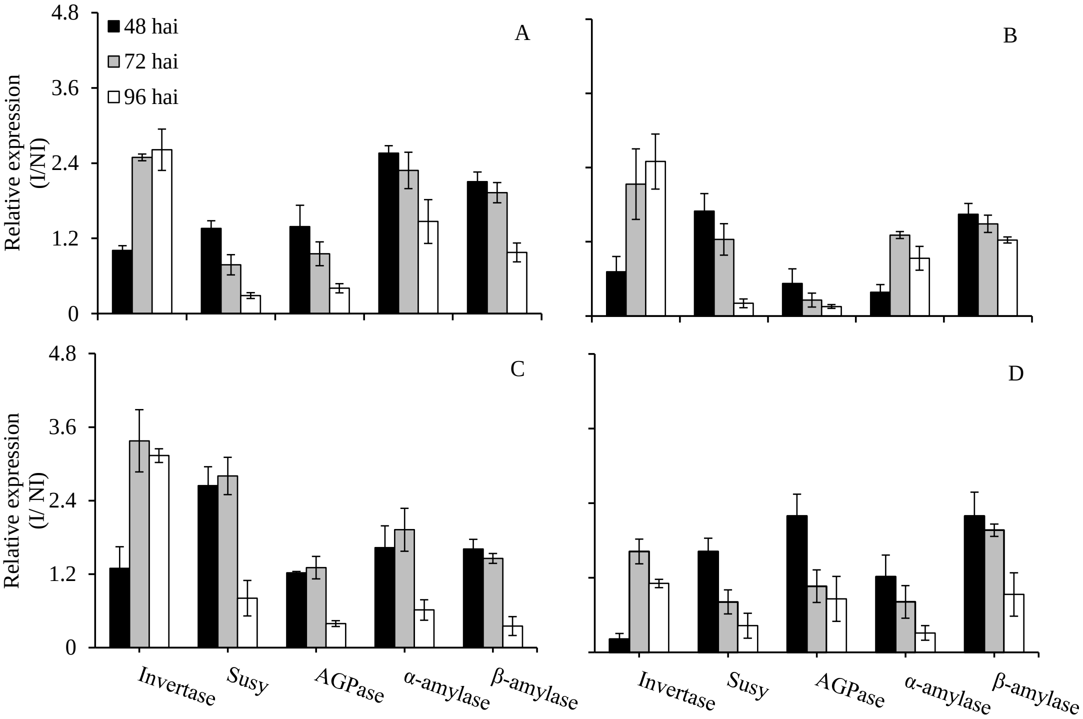

Fig. 7. Relative expression of genes encoding for *invertases*, *sucrose synthase* (*Susy*), *ADP-glucose pyrophosphorylase* (*AGPase*) and *α-amylase* and *β-amylase*, as determined by quantitative reverse transcription qRT-PCR, on flag leaves (A and C) and spikelets (B and D) of wheat plants at 10 (A and B) and 20 (C and D) days after anthesis that were non-inoculated (NI) or inoculated (I) (48, 72 and 96 hours after inoculation) with *Pyricularia oryzae*. Genes expression levels on flag leaves and spikelets of inoculated plants were normalized using the expression of the constitutive genes *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and *ubiquitin* (*UBIQ*) and related to the corresponding values obtained from flag leaves spikelets of non-inoculated plants. The temporal analysis comparing the up or down genes expression was performed using the confidence intervals (bars) of the means ($P \leq 0.05$). $n = 4$.

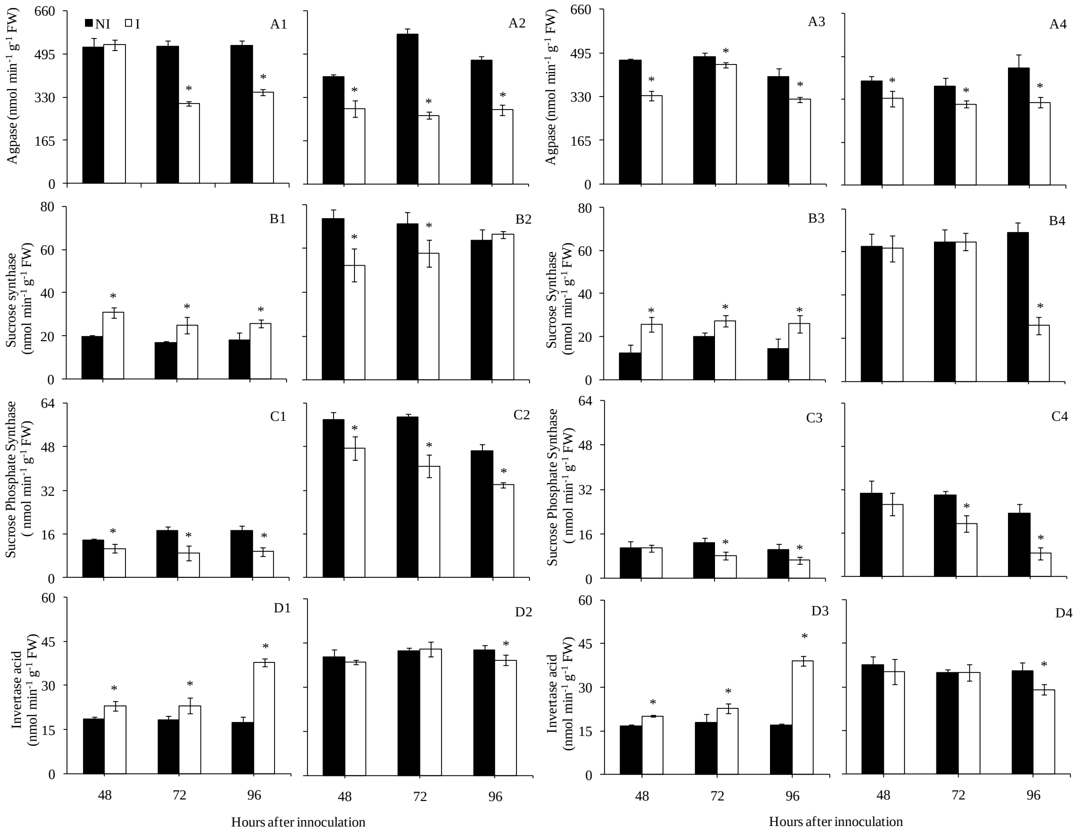

Fig. 8. Activities of ADP-glucose pyrophosphorylase (AGPase) (A), sucrose synthase (B), sucrose phosphate synthase (C) and invertases acid (D) in the flag leaves (A1-D1, A3-D3) and spikelets (A2-D2, A4-D4) of wheat plants at 10 (A1-D1, A2–D2) and 20 (A3-D3, A4-D4) days after anthesis that were non-inoculated (NI) or inoculated (I) (48, 72 and 96 hours after inoculation) with *Pyricularia oryzae*. Means from NI and I treatments followed by an asterisk (*), at each sampling time, are significantly different ($P \leq 0.05$) by the *t*-test. Bars represent the standard deviations of the means. $n = 4$. FW = fresh weight.