CARLA DA SILVA DIAS

PHYSIOLOGICAL AND BIOCHEMICAL CHANGES ON RICE PLANTS SUPPLIED WITH GLUTAMATE IN RESPONSE TO *Pyricularia oryzae* INFECTION

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

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APROVADA: 20 de julho de 2018.

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(Orientador)

I dedicate this thesis to my parents whose examples have guided my steps. They have given me the most valuable legacy that parents can give to a child - love, education, and the encouragement to pursue knowledge.

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BIOGRAFIA

CARLA DA SILVA DIAS, filha de Elaine Ladeira da Silva Dias e Carlos Antônio Dias, nasceu em 22 de outubro de 1990 em Viçosa, Minas Gerais. Iniciou o curso de Agronomia no ano de 2008 na Universidade Federal de Viçosa (UFV) e em março de 2013, graduou-se Engenheira Agrônoma. Durante a graduação, teve oportunidade de trabalhar como bolsista de Iniciação Científica nos Departamento de Microbiologia e de Engenharia Agrícola. Em abril de 2013, iniciou o curso de Mestrado no Programa de Pós-Graduação em Fisiologia Vegetal da UFV, atuando na área da Interação Patógeno-Hospedeiro, sob orientação do Prof. Fabrício Ávila Rodrigues, defendendo a dissertação em fevereiro de 2014. Neste mesmo ano, iniciou o curso de Doutorado no Programa de Pós-Graduação em Fisiologia Vegetal da UFV, também sob orientação do Prof. Fabrício A. Rodrigues e submetendo-se a defesa de tese em julho de 2018.

SUMÁRIO

ABSTRACT

DIAS, Carla da Silva, D.Sc., Universidade Federal de Viçosa, July, 2018. **Physiological and biochemical changes on rice plants supplied with glutamate in response to** *Pyricularia oryzae* **infection.** Adviser: Fabrício Ávila Rodrigues. Co-adviser: Renata Sousa Resende.

Considering the importance of blast, caused by *Pyricularia oryzae*, to decrease rice yield worldwide, this study aimed to assess the photosynthetic performance (leaf gas exchange and chlorophyll (Chl) *a* fluorescence as well as the photosynthetic pigments concentration), the activities of antioxidant enzymes (ascorbate peroxidase (APX), catalase (CAT), peroxidase (POX), superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione-*S*-transferase (GST)) and concentrations of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) in leaves of plants non-supplied (-Glu) or supplied (+Glu) with glutamate (Glu). Blast severity and the number of lesions per cm^2 of leaf were significantly lower for +Glu plants in comparison to -Glu plants. On the infected leaves of +Glu plants, the values for internal CO_2 concentration were lower while the values for net carbon assimilation rate, stomatal conductance as well as for the concentrations of Chl *a,* Chl *b*, and carotenoids were higher in comparison to infected leaves of -Glu plants. The functionality of the photosynthetic apparatus was preserved on the infected leaves of +Glu plants. The activities of CAT, GPX, GR, POX, and SOD increased on the infected leaves of both -Glu and +Glu plants compared to their non-inoculated counterparts, but their activities were lower for +Glu plants. The lower activities of these antioxidative enzymes was triggered by the reduced hydrogen peroxide concentration on the infected leaves of +Glu plants that resulted in lower malondialdehyde concentration. For inoculated +Glu plants, the activities of *β*-1-3-glucanase, chitinase, phenylalanine ammonia-lyase, and polyphenoloxidases as well as the concentrations of total soluble phenolics and lignin-thioglycolic acid derivatives were significantly higher for inoculated +Glu plants in comparison to inoculated -Glu ones. Based on the data from the present study, it can be concluded that photosynthesis was less impaired on plants supplied with glutamate and infected by *P. oryzae* due to the lower biochemical constraints for CO_2 fixation. There was a need for lower activities of reactive oxygen species scavenging enzymes on the infected leaves of plants supplied with glutamate due to the lower oxidative stress as

a result of *P. oryzae* infection. Moreover, blast severity was reduced on Glu supplied plants due to an increase on the activities of defense enzymes and high concentrations of phenolics and lignin.

RESUMO

DIAS, Carla da Silva, D.Sc., Universidade Federal de Viçosa, julho de 2018. **Alterações fisiológicas e bioquímicas em plantas de arroz supridas com glutamato em resposta à infecção por** *Pyricularia oryzae.* Orientador: Fabricio Ávila Rodrigues. Coorientadora: Renata Sousa Resende.

Considerando a importância da brusone, causada pelo fungo *Pyricularia oryzae*, em reduzir a produção de grãos de arroz em todo o mundo, este trabalho teve como objetivo avaliar o desempenho fotossintético (trocas gasosas, fluorescência da clorofila (Cl) *a* e a concentração de pigmentos fotossintéticos), as atividades de enzimas do sistema antioxidativo (ascorbato peroxidases (APX), catalase (CAT), peroxidases (POX), superóxido dismutase (SOD), glutationa peroxidase (GPX), glutationa redutase (GR), glutationa-S-transferase (GST)) e as concentrações de peróxido de hidrogênio (H_2O_2) e aldeído malônico (MDA) em folhas de arroz supridas ou não com glutamato (+Glu e -Glu, respectivamente). O presente estudo também objetivou investigar o potencial do Glu em aumentar a resistência do arroz à brusone avaliando um possível aumento nas atividades de enzimas de defesa (β-1-3-glucanase (GLU), quitinase (QUI), fenilalanina amônia-liase (FAL) e polifenoloxidases (PFO)) e nas concentrações de compostos fenólicos e lignina. A severidade da brusone e o número de lesões por cm^2 nas folhas foram significativamente reduzidas nas plantas +Glu em comparação com as plantas -Glu. As folhas infectadas por *P. oryzae* das plantas +Glu apresentaram menores valores da concentração interna de CO_2 quando comparadas com as folhas das plantas -Glu. Por outro lado, a taxa de assimilação líquida de CO_2, a condutância estomática e as concentrações de pigmentos fotossintéticos foram maiores nas folhas infectadas das plantas +Glu em comparação às folhas infectadas das plantas -Glu. A funcionalidade do aparato fotoquímico foi preservada nas folhas infectadas das plantas +Glu em comparação as plantas -Glu. Comparado às plantas não inoculadas, as atividades da CAT, GPX, GR, POX e SOD aumentaram nas folhas inoculadas das plantas independente do suprimento com Glu. Contudo, menores atividades foram observadas nas folhas inoculadas das plantas +Glu quando comparadas com as folhas das plantas -Glu. As concentrações de H_2O_2 e MDA foram menores nas folhas infectadas das plantas +Glu quando comparado com as folhas infectadas das plantas -Glu. Para as plantas inoculadas, as atividades da GLU, QUI, FAL e PFO, bem como

as concentrações de compostos fenólicos e lignina, foram significativamente maiores para as plantas +Glu em comparação com às plantas -Glu. Os dados do presente estudo fornecem as primeiras evidências de que as trocas gasosas e a eficiência fotoquímica foram preservadas nas plantas supridas com Glu e infectadas por *P. oryzae*. Além disso, as folhas infectadas das plantas supridas com Glu apresentaram menores atividades das enzimas do sistema antioxidativo. Por outro lado, foi evidenciado nesse estudo que o suprimento de glutamato às plantas proporcionou maiores atividades das enzimas de defesa e altas concentrações de compostos fenólicos e lignina.

GENERAL INTRODUCTION

Rice (*Oryza sativa* L.) is one of the world's most important food crops and blast disease, caused by the hemibiotrophic fungus *Magnaporthe grisea* (T. T. Hebert) Yaegashi & Udagawa) Barr (anamorph *Pyricularia grisea* (Cooke) Sacc.), is a major constraint to decrease yield worldwide (TeBeest *et al*., 2007). On leaves, lesions start as small specks that enlarge into spindle-shaped ones with reddish brown margins, chlorotic halo, and gray centers followed by coalesce (TeBeest *et al*., 2007). The use of resistant cultivars and fungicides spray are the methods currently used for blast control (Pooja & Katoch, 2014). However, more environmental-friendly control strategies need to be investigated aiming to reduce blast´s effect to decrease rice yield and the use of glutamate may become an interesting option (Pooja & Katoch, 2014).

Amino acids, such as glutamate, can be of high value to induce mechanisms of defense in plants upon challenged by pathogens (Seifi *et al*. 2013; Kadotani *et al*., 2016). The glutamate metabolism plays an important role in the amino acids metabolism including ammonia assimilation and dissimilation as well as amino acid transamination and biosynthesis (Galili *et al*., 2001, Forde & Lea 2007). Once perceived by receptor or sensor proteins located on the cell surface of plants, glutamate acts as a signal to regulate the expression of defense-related genes (Kan *et al*., 2017). For instance, several wall-associated kinases are involved in the basal defense of rice to blast (Seifi *et al*., 2013; Kan *et al*., 2017). Several defense genes were rapidly expressed in several plants infected by pathogens the presence of glutamate (Seifi *et al*., 2013, Kadotani *et al*., 2016, Kan *et al*., 2017). Exogenous glutamate supply caused a rapid expression of genes encoding for transcriptional activator involved in hypersensitive response, lipoxigenases, trypsin inhibitor,

xylanase inhibitor, aspartic proteinase, chitinase, phenylalanine ammonia-lyase, and disease-related receptor-like protein kinases (Kan *et al*., 2017). The salicylic acid-dependent defense response in rice against *P. oryzae* infection was up-regulated by glutamate (Kadotani *et al*., 2016). The glutamate metabolism undergoes expressive change on diseased plants due to its participation on two distinct defense mechanisms depending on the pathogen´s lifestyle (Seifi *et al*., 2013). The nitrogen remobilization or reutilization, the death of host tissue, and replenishment or exhaustion of the tricarboxylic acid cycle are among the possible glutamate metabolism pathways altered on plants infected by pathogens (Seifi *et al*., 2013).

Photosynthesis, the main driver to achieve high crop yield, is the physiological process of plants most affected by the infection of pathogens of different lifestyles (Bassanezi *et al*., 2002; Dallagnol *et al*., 2011; Rios *et al*., 2018; Viecelli *et al*., 2018). The assessment of the photosynthetic performance of plants infected by pathogens provides insights regarding the mechanisms underlying their infection process and help to find novel strategies for disease control (Rolfe & Scholes, 2010). The chlorophyll *a* (Chl *a*) fluorescence is a non-invasive and non-destructive method that, combined with the leaf gas exchange analysis, allow a better understanding of the changes occurring on photosynthesis during pathogens infection (Rolfe & Scholes, 2010; Dias *et al*., 2018). Changes in the values of the Chl *a* fluorescence parameters or in their ratio has been reported to occur for several host-pathogen interactions such as wheat-*Pyricularia oryzae,* -*Puccinia triticina*, and -*Blumeria graminis* f. sp. *tritici* (Kuckenberg & Tartachnyk, 2009; Aucique-Pérez *et al*., 2014), rice-*Monographella albescens* (Tatagiba *et al*., 2016) and soybean-*Colletotrichum truncatum* (Dias *et al*., 2018).

The production of reactive oxygen species (ROS) by the pathogens at their infection site cause oxidative stress to the leaf tissue (Hafez *et al*., 2012, Sharma *et al*., 2012). The ROS include both free radicals (*e.g.*, superoxide anion (O_2^-), hydroxyl radical (OH^-) and non-radical molecules (*e.g.*, hydrogen peroxide (H_2O_2) and singlet oxygen (O_2^-)) (Giannopolitis & Ries, 1977, Sharma *et al*., 2012). The ROS should be effectively scavenged from the infection sites to avoid the irreversible oxidative damage to the plant cells as the result of lipid peroxidation and destruction of macromolecules such as lipids, nucleic acids, pigments, and proteins (Apel & Hirt, 2004). In order to remove the excess of ROS generated during a certain host-pathogen interaction, plants have evolved a protective antioxidant system that includes small molecules such as ascorbate, glutathione, carotenoids, and flavonoids as well as the action of the antioxidant enzymes ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione-*S*-transferase (GST), peroxidase (POX), and superoxide dismutase (SOD) (Apel & Hirt, 2004; Mittler, 2002; Sharma & Dubey, 2007, Sharma *et al*., 2012).

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Chapter 1

Changes in leaf gas exchange, chlorophyll *a* fluorescence and antioxidant metabolism in rice plants supplied with glutamate and infected by *Pyricularia oryzae*

Abstract

Considering the importance of blast, caused by *Pyricularia oryzae*, to decrease rice yield worldwide, this study aimed to assess the photosynthetic performance (leaf gas exchange and chlorophyll (Chl) *a* fluorescence as well as the photosynthetic pigments concentration), the activities of antioxidant enzymes (ascorbate peroxidase (APX), catalase (CAT), peroxidase (POX), superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione-*S*-transferase (GST)) and concentrations of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) in leaves of plants non-supplied (-Glu) or supplied (+Glu) with glutamate (Glu). Blast severity was reduced on the leaves of +Glu plants. On the infected leaves of +Glu plants, the values for internal CO_2 concentration were lower while the values for net carbon assimilation rate, stomatal conductance as well as for the concentrations of Chl *a,* Chl *b* and carotenoids were higher in comparison to infected leaves of -Glu plants. The functionality of the photosynthetic apparatus was preserved on the infected leaves of +Glu plants. The activities of CAT, GPX, GR, POX, and SOD increased on the infected leaves of both -Glu and +Glu plants compared to their non-inoculated counterparts, but their activities were lower for +Glu plants. The lower activities of these antioxidative enzymes was triggered by the reduced hydrogen peroxide concentration on the infected leaves of +Glu plants that resulted in lower malondialdehyde concentration. Based on the data from the present study, it can be concluded that photosynthesis was less impaired on plants supplied with glutamate and infected by *P. oryzae* due to the lower biochemical constraints for CO_2 fixation. Moreover, there was a need for lower activities of ROS scavenging enzymes on the infected leaves of plants supplied with glutamate due to the lower oxidative stress because of *P. oryzae* infection.

Keywords: antioxidant enzymes, host defense, photosynthesis, rice blast.

Introduction

Rice (*Oryza sativa* L.) is one of the worlds most important food crops and blast disease, caused by the hemibiotrophic fungus *Pyricularia oryzae* (T. T. Hebert) Yaegashi & Udagawa) Barr, is a major constraint to decrease yield worldwide (TeBeest *et al*., 2007). On leaves, lesions start as small specks that enlarge into spindle-shaped ones with reddish brown margins, chlorotic halo, and gray centers followed by coalesce (TeBeest *et al*., 2007). The use of resistant cultivars and fungicides spray are the methods currently used for blast control (Pooja & Katoch, 2014). However, more environmental-friendly control strategies need to be investigated aiming to reduce blast´s effect to decrease rice yield and the use of glutamate may become an interesting option (Pooja & Katoch, 2014).

Photosynthesis, the main driver to achieve high crop yield, is the physiological process of plants most affected by the infection of pathogens of different lifestyles (Bassanezi *et al*., 2002; Dallagnol *et al*., 2011; Rios *et al*., 2018; Viecelli *et al*., 2018). The assessment of the photosynthetic performance of plants infected by pathogens provides insights regarding the mechanisms underlying their infection process and help to find novel strategies for disease control (Rolfe & Scholes, 2010). The chlorophyll *a* (Chl *a*) fluorescence is a non-invasive and non-destructive method that, combined with the leaf gas exchange analysis, allow a better understanding of the changes occurring on photosynthesis during pathogens infection (Baker *et al*., 2001; Rolfe & Scholes, 2010; Dias *et al*., 2018). Changes in the values of the Chl *a* fluorescence parameters or in their ratio has been reported to occur for several host-pathogen interactions such as wheat-*Pyricularia oryzae, -Puccinia triticina*, and -*Blumeria graminis* f. sp. *tritici* (Aucique-Pérez *et al*., 2014; Kuckenberg & Tartachnyk, 2009), rice-*Monographella albescens* (Tatagiba *et al*., 2015), maize-

Stenocarpella macrospora (Bermúdez-Cardona *et al*., 2015), and soybean-*Colletotrichum truncatum* (Dias *et al*., 2018). In general, an initial reduction in the effective PSII quantum yield, an increase in the quantum yield of regulated energy dissipation, and a decline in the maximal photosystem II quantum yield occur on the leaves of plants infected by pathogens (Scholes & Rolfe, 2009).

The production of reactive oxygen species (ROS) by the pathogens at their infection site cause the oxidative stress to the leaf tissue (Hafez *et al*., 2012, Sharma *et al*., 2012). The ROS include both free radicals (*e.g.*, superoxide anion (O_2^-), hydroxyl radical (OH^-) and non-radical molecules (*e.g.*, hydrogen peroxide (H_2O_2) and singlet oxygen (O_2^-)) (Giannopolitis & Ries, 1977, Sharma *et al*., 2012). The ROS should be effectively scavenged from the infection sites to avoid the irreversible oxidative damage to the plant cells as the result of lipid peroxidation and destruction of macromolecules such as lipids, nucleic acids, pigments, and proteins (Verma & Dubey, 2003, Apel & Hirt, 2004). In order to remove the excess of ROS generated during a certain host-pathogen interaction, plants have evolved a protective antioxidant system that includes small molecules such as ascorbate, glutathione, carotenoids, and flavonoids as well as the action of the

antioxidant enzymes ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione-*S*-transferase (GST), peroxidase (POX), and superoxide dismutase (SOD) (Apel & Hirt, 2004; Mittler, 2002; Sharma & Dubey, 2007, Sharma *et al*., 2012; Rios *et al*., 2017).

Amino acids, such as glutamate, can be of high value to induce mechanisms of defense in plants upon challenged by pathogens (Seifi *et al*. 2013; Kadotani *et al*., 2016). The glutamate metabolism plays an important role in the amino acids metabolisms including ammonia assimilation and dissimilation as well as amino acid

transamination and biosynthesis (Galili *et al*., 2001, Forde & Lea 2007). The amino acids arginine, proline, and γ-aminobutyric acid (GABA) are involved in the resistance of some plant species against diseases (Forde & Lea 2007; Galili *et al*., 2001). The glutamate metabolism undergoes expressive change on diseased plants due to its participation on two distinct defense mechanisms depending on the pathogen´s lifestyle (Seifi *et al*., 2013). Rice plants supplied with glutamate through the roots showed a reduction on blast severity due to an increase on the expression of genes related to defense (Kadotani *et al*., 2016). The nitrogen remobilization or reutilization, the death of host tissue, and replenishment or exhaustion of the tricarboxylic acid cycle are among the possible glutamate metabolism pathways altered on plants infected by pathogens (Seifi *et al*., 2013).

Considering the lack of information in the literature regarding the physiological and biochemical changes occurring in rice plants supplied with glutamate in response to *P. oryzae* infection, the present study aimed to fill this gap by examining the effect of this amino acid on the photosynthetic performance (*e.g.*, leaf gas exchange and Chl *a* fluorescence parameters as well as the photosynthetic pigments pool) and on the antioxidative metabolism of non-infected and infected plants.

Materials and Methods

Nutrient solution preparation and plant growth

Rice seeds from cultivar Metica-1, susceptible to blast, were surface sterilized in sodium hypochlorite 1% (v/v) for 3 min, washed in sterilized water for 3 min, and germinated in trays containing sand previously autoclaved, at 25^{o}C for 10 days. Five seedlings were transferred to 5 L plastic pots (30-cm diameter) (Ecovaso, Jaguariúna, São Paulo, Brazil) with one-half-strength nutrient solution. The nutrient solution was prepared according to Hoagland & Arnon (1950) with some modifications as follow: 1.0 mM KNO_3, 0.25 mM $NH_4H_2PO_4$, 0.1 mM NH_4Cl, 0.5 mM $MgSO_4.7H_2O$, 1.0 mM $(CaNO_3)_2$, 0.3 μM $CuSO_4.5H_2O$, 0.33 μM $ZnSO_4.7H_2O$, 11.5 μM H_3BO_3, 3.5 μM $MnCl_2.4H_2O$, 0.1 μM $(NH_4)_6Mo_7O_{24}.4H_2O$, 25 μM $FeSO_4.7H_2O$, and 25 μM ethylenediaminetetraacetic (EDTA) disodium. The nutrient solution was changed every four days. The pH was checked daily and maintained between 5.5-5.8 using NaOH or HCl (1 M) solutions.

Glutamate application

Plants were grown in nutrient solution for 30 days and then supplied with the same nutrient solution containing 10 mM of glutamate (Sigma-Aldrich, São Paulo, Brazil) for 24 h. Plants non-supllied with glutamate served as the control treatment. After this period, the roots of each plant were carefully washed with deionized water and plants returned to their respective plastic pots containing the original nutrient solution.

Inoculation procedure

The isolate UFV-DFP *Po*22 of *P. oryzae* was used to inoculate the plants. This isolate was preserved on disks of filter paper placed inside of plastic microtubes containing silica gel and stored at 4°C. Disks of filter paper containing fungal mycelia were transferred to Petri dishes containing oat-agar medium. After five days, plugs of the oat-agar medium containing fungal mycelia were transferred to new Petri dishes containing the same medium. The Petri dishes were incubated in a growth chamber at 25°C under continuous light for 12 days. After this period, conidia were carefully removed from the media using a soft bristle brush and with water-containing gelatin (1% wt/vol). The conidial suspension was calibrated with a hemocytometer to obtain a concentration of 1×10^5 conidia mL^{-1}. The conidial suspension was sprayed on the adaxial surface of the leaves of each plant (50 mL per pot) with an atomizer (Paasche Airbrush Co., Chicago, IL, USA). After inoculation, plants were kept in a mist chamber (temperature of 25 ± 2°C and relative humidity of 90 ± 5%) at the dark during 24 h. The plants were then transferred to a plastic mist growth chamber inside (temperature of 24 ± 1°C (day) and of 18 ± 2°C (night) a greenhouse for the duration of the experiment. A misting system with nozzles (model NEB-100; KGF Company, São Paulo, Brazil) spraying mist every 30 min above the plant canopy kept the relative humidity of 90 ± 5%. Plant canopy received a natural photon flux density of ≈ 900 μmol m^{-2} s^{-1}.

Blast severity assessments

The second and third leaves, from base to top, of each plant per replication of each treatment were used to evaluate blast severity. These leaves were collected at 48, 72, and 96 hours after inoculation (hai), scanned at the resolution of 300 dpi, and the

images obtained were used to quantify blast severity using the QUANT software (Resende *et al*., 2012). The area under blast progress curve (AUBPC) was calculated using the trapezoidal integration of the blast progress curve over time according to Shaner & Finney (1997).

Determination of the leaf gas exchange parameters

Leaf gas exchange parameters were determined on the third leaf, from base to top, of each plant per replication of each treatment at 48, 72, and 96 hai. The net carbon assimilation rate (A), stomatal conductance to water vapor (g_s), transpiration rate (E), and internal CO_2 concentration (C_i) were estimated from 09:00 to 12:00 h under artificial and saturating photon irradiance (1000 μmol m^{-2} s^{-1}), and an external CO_2 concentration of 400 μmol mol^{-1} using a portable open-system infrared gas analyzer (LI-6400, LI-COR Inc., Lincoln, NE, USA). All measurements were performed by setting the block temperature at 25°C.

Imaging of the chlorophyll (Chl) *a* fluorescence parameters

Images and parameters of Chl *a* fluorescence were determined on the third leaf, from base to top, of each plant per replication of each treatment 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 leaf. Initially, the leaves were dark-adapted for 60 min, after which they were individually fixed in a support at a distance of 18.5 cm from the CCD camera. The leaves 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 PSII reaction centers were "open". Next, a saturating white light pulse of 2,400 μmol m^{-2} s^{-1} (10 Hz) was applied for 0.8 s to ensure the maximum fluorescence emission (F_m) when all the PSII reaction centers were "closed". From these initial measurements, the maximum PSII photochemical efficiency of the dark-adapted leaves was estimated through the variable-to-maximum Chl fluorescence ratio [$F_v/F_m = (F_m - F_0)/F_m$)]. The leaves were subsequently exposed to actinic photon irradiance (185 μmol m^{-2} s^{-1}) for 300 s to obtain the steady-state fluorescence yield (F_s), after which a saturating white light pulse (2,400 μmol m^{-2} s^{-1}; 0.8 s) was applied to achieve the light-adapted maximum fluorescence (F_m'). Following the calculations proposed by Kramer *et al*. (2004), the energy absorbed by the 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 Chl *a*, Chl *b*, and carotenoids were determined using dimethyl sulfoxide (DMSO) as an extractor (Santos *et al*., 2008). Five disks (1 cm in diameter) of the third leaf were immersed in glass tubes containing 5 mL of saturated DMSO solution with calcium carbonate ($CaCO_3$) (5 g/L) (Wellburn, 1994) and kept in the dark at room temperature for 24 h. The absorbances of the extracts were read at 480, 649, and 665 nm using a saturated solution of DMSO with $CaCO_3$ as a blank.

Biochemical assays

For all biochemical assays, the fourth, fifth and sixth leaves, from base to top, of each plant per replication of each treatment were collected at 48, 72, and 96 hai. Leaves collected from non-inoculated plants at these same sampling times served as the control treatment. Leaf samples were kept in liquid nitrogen during sampling and stored at -80°C.

Determination of antioxidative enzymes

A total of 200 mg of homogenized leaf tissue was ground into a fine powder using a mortar and pestle with liquid nitrogen. The fine powder was immediately homogenized in 2 mL of a solution containing 50 mM of potassium phosphate buffer (pH 6.8), 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 12,000 *g* for 15 min at 4°C and the supernatant was used as a crude enzyme extract to determine the activities of ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), glutathione-*S*-transferase (GST), glutathione reductase (GR), peroxidase (POX), and superoxide dismutase (SOD).

The SOD activity was determined using the method described by Del Longo *et al*. (1993) which measures the capacity of SOD to photochemically reduce the *p*-nitrotetrazolium blue (NTB). The reaction was initiated by adding 40 µL of the crude enzyme extract to 960 µl of a mixture containing 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NTB, 0.1 mM EDTA, and 2 µM riboflavin. The reaction was carried out at 25°C under 15-W lamp light per 10 min. Following light exposure, it was turned-off and the production of blue formazan, which resulted from the photoreduction of NTB, was measured at 560 nm with a spectrophotometer

(Giannopolitis & Ries, 1977). For the control samples, the reaction mixture was maintained in darkness for 10 min and the absorbance was measured at 560 nm. The values obtained from the experimental samples (light) were subtracted from the values obtained from the control samples to determine SOD activity. The amount of enzyme necessary to inhibit NBT photoreduction by 50% was defined as one unit of SOD (Beauchamp & Fridovich, 1971).

The CAT activity was determined according to Cakmak & Marschner (1992). The reaction was initiated following the addition of 25 µL of the crude enzyme extract to 975 µL of a reaction mixture consisting of 50 mM potassium phosphate buffer (pH 6.8) and 20 mM hydrogen peroxide (H_2O_2). The CAT activity was determined based on the rate of H_2O_2 decomposition measured in spectrophotometer at 240 nm for 1 min at 25°C. The extinction coefficient of 36 M^{-1} cm^{-1} was used to calculate CAT activity (Anderson *et al*., 1995).

The POX activity was assayed following the colorimetric determination of pyrogallol oxidation according to Kar & Mishra (1976). The reaction was started after the addition of 15 µL of the crude enzyme extract to 985 µL of a reaction mixture containing 25 mM potassium phosphate (pH 6.8), 20 mM pyrogallol, and 20 mM H_2O_2. The POX activity was measured by the absorbance of colored purpurogallin at 420 nm for 1 min at 25°C. The extinction coefficient of 2.47 mM^{-1} cm^{-1} was used to calculate POX activity (Chance & Maehley, 1955).

The APX activity was assayed using the method proposed by Nakano & Asada (1981). The reaction was started following the addition of 25 µL of the crude enzyme extract to 975 µL of a reaction mixture containing 50 mM potassium phosphate buffer (pH 6.8), 1 mM H_2O_2, and 0.8 mM ascorbate. The APX activity was measured via the rate of ascorbate oxidation at 290 nm for 1 min at 25°C. The extinction

coefficient of 2.8 mM^{-1} cm^{-1} was used to calculate APX activity (Nakano & Asada, 1981).

For determination of GR activity, the reaction was started after the addition of 100 µl of the crude enzyme extract to a volume of 1.9 mL of a mixture containing 100 mM potassium phosphate (pH 7.5), 1 mM EDTA, 1 mM oxidized glutathione (GSSG), and 0.1 mM NADPH prepared in 0.5 mM Tris-HCl buffer (pH 7.5) (Carlberg & Mannervik, 1985). The decrease in absorbance was determined at 340 nm for 1 min at 30°C. The extinction coefficient of 6.22 mM^{-1} cm^{-1} was used to calculate GR activity (Foyer & Halliwell, 1976), which was expressed as µmol min^{-1} mg^{-1} protein.

The GPX activity was estimated after the addition of 100 µL of the crude enzyme extract to a mixture containing 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 0.114 M NaCl, 1 mM GSH, 0.2 mM NADPH, 0.25 mM H_2O_2, and 1 U of GR (Nagalakshmi & Prasad, 2001). The decrease in absorbance was measured at 340 nm for 1 min at 30°C. The extinction coefficient of 6.22 mM^{-1} cm^{-1} was used to calculate GPX activity (Anderson & Davis, 2004), which was expressed as nmol min^{-1} mg^{-1} protein.

The GST activity was estimated after the addition of 150 µL of the crude enzyme extract to 1.35 mL of a mixture containing 50 mM potassium phosphate buffer (pH 6.5) and 50 mM reduced glutathione (GSH). The reaction was initiated after the addition of 500 µl of 30 mM 1-chloro-2,4-dinitrobenzene and then incubated for 4 min at 25°C. The absorbance was measured at 340 nm over 3 min. The extinction coefficient of 9.6 mM^{-1} cm^{-1} was used to determine GST activity (Habig *et al*., 1974), which was expressed as µmol min^{-1} mg^{-1} protein.

The enzymes activities were expressed on a protein basis. Protein concentration in each sample was determined according to Bradford (1976).

Determination of malondialdehyde (MDA) concentration

Oxidative damage in the leaf cells was estimated as the concentration of total 2-thiobarbituric acid (TBA) reactive substances and expressed as equivalents of MDA according to Cakmak and Horst (1991). A total of 100 mg of leaf tissue was ground into a fine powder using a mortar and pestle with liquid nitrogen. The fine powder obtained was homogenized in 2 mL of 0.1% (w/v) trichloracetic acid (TCA) solution following centrifugation at 12,000 *g* for 15 min at 4°C. A total of 500 µL of the supernatant reacted with 1.5 mL of 2-thiobarbituric acid solution (0.5% in 20% TCA) in a ThermoMixer (Eppendorf, Hamburg, Germany) at 95°C for 30 min. After this period, the reaction was stopped in an ice bath. The samples were centrifuged at 9,000 *g* for 10 min and the specific absorbance was determined at 532 nm. The non-specific absorbance was estimated at 600 nm and subtracted from the specific absorbance value. The extinction coefficient of 155 mM^{-1} cm^{-1} was used to calculate MDA concentration (Heath and Packer, 1968), which was expressed as nmol per gram of fresh weight (FW).

Determination of hydrogen peroxide (H_2O_2) concentration

A total of 100 mg of homogenized leaf tissue was ground into a fine powder using a mortar and pestle with liquid nitrogen. The fine powder was homogenized with an extraction solution containing 50 mM potassium phosphate buffer (pH 6.5) and 1 mM hydroxylamine in a volume of 2 mL. The homogenate was centrifuged at 10,000 *g* for 15 min at 4°C. The reaction was initiated with the addition of 100 µL of the

supernatant to a reaction mixture containing 100 μM ferric ammonium sulfate ($FeNH_4[SO_4]$), 25 mM sulfuric acid, 250 μM xylenol orange, and 100 mM sorbitol in a volume of 2 mL (Gay & Gerbicki, 2000; Kuo & Kao, 2003). Samples were kept in darkness for 30 min and the absorbance was determined at 560 nm. The controls for the reagents and the crude extracts were prepared under the same conditions and subtracted from the sample. The H_2O_2 concentration was estimated based on a standard curve using H_2O_2 (Sigma-Aldrich, São Paulo, Brazil).

Histochemical determination of H_2O_2

Leaf disks (4 cm^2) were places in glass vials containing 5 mL of 3,3-diaminobenzidine (DAB) solution (pH 5.5; 1 mg mL^{-1}) and kept in the darkness for 24 h (Thordal-Christensen *et al*., 1997). In the next step, the leaf disks were immersed in alcohol (96%) for 3 h and then scanned at 600 dpi resolution.

Experimental design and statistical analysis of data

A 2 × 2 factorial experiment consisting of plants non-supplied or supplied with glutamate (refereed as -Glu and +Glu plants, respectively) and non-inoculated or inoculated with *P. oryzae* was arranged in a completely randomized design with five replications. The experiment was repeated once. Each experimental unit consisted of a plastic pot containing five plants. Means from each parameter and variable were compared by F test ($P \leq 0.05$) using SAS (version 6.12; SAS Institute, Inc., Cary, NC). The relationship among the parameters and variables evaluated was determined using the principal components analysis (PCA) technique. Data were analyzed using the Minitab software (version 15; Minitab Corporation).

Results

Blast severity and AUBPC

On leaves of -Glu plants, the number and size of the necrotic lesions were greater and there were much more lesions coalescing in comparison to the leaves of +Glu plants (Fig. 1A-B). Blast severity was significantly lower by 93, 75, and 68% for +Glu plants in comparison to -Glu plants at 48, 72, and 96 hai, respectively (Fig. 1A-B). The AUPBC was significantly lower by 74% for +Glu plants compared to -Glu ones (Fig. 1C).

Leaf gas exchange parameters

At 96 hai, the g_s and E values significantly increased by 30 and 21%, respectively, for non-inoculated +Glu plants in comparison to non-inoculated -Glu ones (Fig. 2). For inoculated -Glu plants, there were significant decreases of 63 and 79% for A, of 57 and 56% for g_s, and of 47 and 53% for E at 72 and 96 hai, respectively, in comparison to inoculated +Glu plants. There were significant increases of 7 and 17% for C_i and of 6 and 16% for C_i/C_a at 72 and 96 hai, respectively, for inoculated -Glu plants compared to inoculated +Glu plants. Compared to non-inoculated plants, there were significant reductions on A values of 40, 73, and 92% for inoculated -Glu plants and of 37, 26, and 62% for inoculated +Glu plants at 48, 72, and 96 hai, respectively. There were significant reductions of 51 and 85% for g_s values and of 47 and 81% for E values, respectively, at 72 and 96 hai, for inoculated -Glu plants relative to their non-inoculated counterparts. For inoculated +Glu plants, there were significant reductions of 74 and 67%, respectively, for g_s and E at 96 hai in comparison to non-inoculated -Glu plants. There were significant increases of 22 and 17% for C_i and of 19 and 14% for C_i/C_a for inoculated -Glu plants in comparison to non-inoculated -

Glu ones. For inoculated +Glu plants, the C_i/C_a values were significantly higher by 10% at 96 hai in comparison to non-inoculated +Glu plants (Fig. 2).

Imaging of Chl *a* fluorescence

The photosynthetic performance of plants, especially those non-supplied with Glu, was dramatically impaired upon *P. oryzae* infection based on the Chl *a* fluorescence analysis (Fig. 3). For both -Glu and +Glu plants, alterations in the images of Chl *a* fluorescence parameters became evident at 48 hai. The blast lesions expansion was accompanied by a progressive loss of the photosynthetic activity as noticed by the black areas on the leaves, mainly from inoculated -Glu plants at 72 and 96 hai. These necrotic areas on the infected leaves reflected serious damage of the photosynthetic apparatus and a complete loss of their optical properties (Fig. 5). For inoculated -Glu plants, there were significant reduction of 37% for F_o at 96 hai, 30 and 62% for F_m at 72 and 96 hai, and 13, 15, and 35% for F_v/F_m at 48, 72, and 96 hai, respectively, in comparison to inoculated +Glu plants (Fig. 3). There were significant decreases of 33% for F_o at 96 hai, of 41, 38, and 65% for F_m, and of 17, 16, and 37% for F_v/F_m at 48, 72, and 96 hai, respectively, for inoculated -Glu plants in comparison to non-inoculated -Glu plants. There were significant reductions of 22, 25, and 16% for F_m, respectively, for inoculated +Glu plants in comparison to non-inoculated +Glu ones. At 96 hai, F_v/F_m was significantly reduced by 44% for inoculated +Glu plants in comparison to non-inoculated +Glu ones (Fig. 3).

For inoculated -Glu plants, there were reductions of 24 and 39% for Y(II) at 72 and 96 hai, respectively, in comparison to inoculated +Glu plants. The Y(NO) values were significantly higher by 31 and 60% for inoculated -Glu plants at 72 and 96 hai, respectively, in comparison to inoculated +Glu ones (Fig. 4). At 48 hai, the Y(NPQ)

significantly decreased by 25% for inoculated +Glu plants in comparison to inoculated -Glu plants. There were significant reductions of 18, 35, and 37% on the Y(II) values for inoculated -Glu plants at 48, 72, and 96 hai, respectively, and of 15% for inoculated +Glu plants at 72 hai in comparison to their non-inoculated counterparts. At 48 hai, Y(NPQ) significantly decreased by 24% for inoculated +Glu plants in comparison to non-inoculated +Glu ones. For inoculated -Glu plants, Y(NO) significantly increased by 32, 54, and 91% at 48, 72, and 96 hai, respectively, in comparison to non-inoculated ones. At 48 hai, Y(NO) significantly increased by 30% for inoculated +Glu plants in comparison to non-inoculated +Glu ones (Fig. 4).

Concentration of photosynthetic pigments

For inoculated +Glu plants, Chl *a* concentration significantly increased by 10 and 32% at 72 and 96 hai, respectively, in comparison to inoculated -Glu plants. At 96 hai, Chl *b* concentration significantly increased by 24% for inoculated +Glu plants in comparison to inoculated -Glu ones. For carotenoids concentration, there were significant increases of 9 and 35% at 72 and 96 hai, respectively, for inoculated +Glu plants in comparison to inoculated -Glu ones (Fig. 6). Compared to non-inoculated -Glu plants, the Chl *a* concentration for inoculated -Glu plants was significantly reduced by 9, 17, and 37% at 48, 72, and 96 hai, respectively. For inoculated +Glu plants, the Chl *a* concentration significantly reduced by 13% at 72 hai when compared to non-inoculated +Glu plants. For Chl *b* concentration, there were significant reductions of 12 and 22% for inoculated -Glu plants at 72 and 96 hai, respectively, and of 17% for inoculated +Glu plants at 72 hai in comparison to their non-inoculated counterparts. There were significant reductions of 24 and 34% at 72

and 96 hai, respectively, for carotenoids concentrations for inoculated -Glu plants in comparison to non-inoculated -Glu plants (Fig. 6).

Enzymes activities

For inoculated -Glu plants, there were significant increases of 21 and 22% for SOD activity, of 19 and 40% for POX activity, and of 18 and 13% for CAT activity at 72 and 96 hai, respectively, in comparison to inoculated +Glu ones. At 96 hai, APX activity significantly increased by 27% for inoculated +Glu plants in comparison to inoculated -Glu plants (Fig. 7). For SOD activity, there were significant increases of 31 and 67% at 72 and 96 hai, respectively, for inoculated -Glu plants in comparison to non-inoculated -Glu ones. At 96 hai, SOD activity significantly increased by 26% for inoculated +Glu plants in comparison to non-inoculated +Glu plants. For POX activity, there were significant increases of 70, 113, and 275% for inoculated -Glu plants and of 133, 116, and 80% for inoculated +Glu plants at 48, 72, and 96 hai, respectively, in comparison to non-inoculated ones. There were significant increases of 44 and 55% for APX activity at 48 and 96 hai, respectively, for inoculated +Glu plants in comparison to non-inoculated +Glu ones. For CAT activity, there was significant reduction of 13% at 96 hai for inoculated +Glu plants in comparison to non-inoculated +Glu ones. There were significant reductions of 35 and 24% for GPX activity and of 46 and 49% for GR activity for inoculated +Glu plants at 72 and 96 hai, respectively, in comparison to inoculated -Glu ones. For inoculated -Glu plants, there were significant increases of 38, 60, and 37% for GPX activity at 48, 72, and 96 hai, respectively, and of 47 and 63% for GR activity at 72 and 96 hai, respectively, in comparison to non-inoculated -Glu ones. At 72 hai, GPX activity was

significantly reduced by 44% for inoculated +Glu plants in comparison to non-inoculated +Glu ones (Fig. 7).

MDA concentration

The MDA concentration significantly increased by 17, 20, and 29% at 48, 72, and 96 hai, respectively, for inoculated -Glu plants in comparison to inoculated +Glu ones (Fig. 8). For inoculated -Glu plants, there were significant increases of 20, 39, and 66% for MDA concentration at 48, 72, and 96 hai, respectively, in comparison to non-inoculated -Glu ones (Fig. 8).

Histochemical detection of H_2O_2 and H_2O_2 concentration

The presence of H_2O_2 on the infection sites of *P. oryzae*, as indicated by the brown color, was less intense on the leaves of +Glu plants in contrast to the leaves of -Glu ones (Fig 9A). For inoculated -Glu plants, H_2O_2 concentration significantly increased by 57 and 55% at 72 and 96 hai, respectively, in comparison to non-inoculated -Glu ones (Fig. 9B). The H_2O_2 concentration was significantly reduced by 13 and 35% for inoculated +Glu plants at 72 and 96 hai, respectively, in comparison to inoculated -Glu ones (Fig. 9C).

Principal components analysis

Changes on the physiological parameters and biochemical variables on the leaves of plants non-supplied or supplied with Glu and infected by *P. oryzae* ere unequivocally demonstrated using the PCA analysis (Figs. 10 and 11). The two principal components (PC) explained 72% of the total variation in the biochemical variables (15 and 57%, respectively, for PC1 and PC2) (Fig. 10A). Similarly, for the

physiological parameters, the two PC explained 84% of the total variation (15 and 69%, respectively, for PC1 and PC2) (Fig. 11A). Based on PCA, the +Glu NI and -Glu NI treatments at 96 hai were grouped close on the center of the bi-plot (physiological and biochemical data) (Figs. 10A and 11A). Similarly, the inoculated +Glu plants treatment (+Glu I) was places exactly over the center (Figs. 10A and 11A). Conversely, the inoculated -Glu plants was observed farther from the center of the bi-plot (Figs. 10A and 11A). In the loading values for the first PC, positive scores were obtained for Sev, H_2O_2, POX, SOD, GR, GPX, APX, and MDA (being all grouped) and negative correlations with GST (Fig. 10B). For the physiological data, the loading values for the first PC resulted in positive scores for *A,* Chl *a*, Chl *b*, carotenoids F_o, F_m, Y(NPQ), Y(II), and F_v/F_m (being all grouped) and negative correlations with Y(NO), Sev, C_i/C_a, and C_i (Fig. 11B).

Discussion

Results from the present study provides, to the best of authors´ knowledge, novel evidence of the potential of glutamate to reduce blast symptoms on rice from a physiological and biochemical perspectives. The photosynthetic performance of rice plants infected by *P. oryzae* was down-regulated through impairments on photosynthesis (*e.g.*, expressive changes on the values of Chl *a* fluorescence parameters and lower photosynthetic pigments concentration) and on the biochemical capacity of these plants to fix CO_2 due to the lower leaf gas exchange parameters values. These findings are well supported by previous studies reporting physiological impairments on plants infected by pathogens of different lifestyles (Resende *et al*., 2012; Debona *et al*., 2012; Tatagiba *et al*., 2015; Bermúdez-Cardona *et al*., 2015; Domiciano *et al*., 2015, Rios *et al*., 2017, Dias *et al*., 2018). It is important to stress out that the reduced blast symptoms on the leaves of plants supplied with glutamate resulted in a preservation of the functionality of their photosynthetic apparatus based on the great A and g_s values in association with a reduced oxidative stress that culminated in lower activities of the ROS scavenging enzymes.

The photosynthetic capacity of the leaves infected by *P. oryzae* was impaired based on the lower efficiency of PSII. Dysfunctions on the photochemical level caused by blast were greater for plants non-supplied with glutamate. The F_v/F_m values were close to 0.80 for the infected leaves of plants supplied with glutamate suggesting the absence of chronic photoinhibition to photosynthesis. Additionally, high Y(II) values obtained from infected leaves of plants supplied with glutamate reflected great apparent electron transport activity (Krause & Weis, 1991). It is known that great F_v/F_m and Y(II) values indicate a better photoprotection capacity of the infected plants due to reduction on the photooxidative damage (Rolfe & Scholes,

2010) as noticed for plants supplied with glutamate. Indeed, plants supplied with glutamate suffered from lower loss of photosynthetic pigments, especially the Chl *a* that is preferentially photobleached in comparison to Chl *b* (Murchie & Horton, 1997). These findings indicate that the photochemical performance of plants supplied with glutamate was, to some extent, preserved during the infection process of *P. oryzae*. Conversely, lower Y(NO) values reflected the higher capacity of the infected plants supplied with glutamate to regulate their mechanisms of photoprotection (Rolfe & Scholes, 2010) resulting in lower photooxidative damage to the infected leaves. These lower dysfunctions at the photochemical level for infected leaves from plants supplied with glutamate suggest a higher provision of ATP and, consequently, higher power for CO_2 assimilation (Rolfe & Scholes, 2010).

Progressive decreases in A and g_s values were attenuated for infected plants supplied with glutamate. According to Bastiaans & Kropff (1993), decreases in photosynthesis on rice leaves infected by *P. oryzae* were proportional to the level of blast severity. It is plausible that the action of non-host selective toxins produced by *P. oryzae* may have potentiated the deleterious effect of this fungus on photosynthesis. According to Yoshii (1937), the cell wall and cellular inclusions in the necrotic tissue of rice leaves infected by *P. oryzae* disintegrated and compromised the transport of water and photoassimilates to the foliar tissue. A reduced water content on rice leaves may affect photosynthesis either directly (*e.g.* inhibition of chloroplasts) or indirectly (*e.g.* stomata closure) (Kaiser, 1987). However, despite the reduced stomatal aperture, which should reduce the CO_2 influx, the C_i values increased for infected leaves of plants non-supplied with glutamate throughout the time course evaluated. Therefore, decreases in the A values during the infection process of *P. oryzae* may be associated with non-stomatal factors either due

to a decreased on the mesophyll conductance or dysfunctions at the biochemical level involving CO_2 fixation. For the wheat-*P. oryzae* interaction, the effect of fungal infection on photosynthesis was linked to biochemical constraints related to a lowered Rubisco activity (Aucique-Pérez *et al*., 2014; Debona *et al*., 2014).

The ROS play a key role in host defense against pathogens attack (Doke *et al*., 1996). In order to prevent the ROS-induced cellular damage, plants have a broad range of antioxidant mechanisms to regulate the balance between their production and scavenging during infection by pathogens of different lifestyles (Del Río *et al*., 2015). In the present study, infected leaves of plants supplied with glutamate were less prone to the chronic state of hyperexcitation compared to the leaves of non-supplied plants mainly because they displayed lower activities of ROS scavenging enzymes. The SOD acts as the first line of defense against ROS by dismutating O_2^- to H_2O_2 (Apel & Hirt, 2004). The SOD activity increased on leaves as blast symptoms developed while on the leaves of non-inoculated plants its activity was kept lower during the time course evaluated. Moreover, SOD activity increased in lower magnitude on infected leaves of plants supplied with glutamate suggesting the occurrence of lower O_2^- concentration. According to Govrin & Levine (2000), the ROS can benefit some pathogens due to an increase in the availability of nutrients on the infection sites that maximizes the colonization of the host tissue. Thus, lower SOD activity on infected leaves of plants supplied with glutamate may be a reflection of a restriction for *P. oryzae* to colonize the leaf tissue and, consequently, a need to keep a lower ROS concentration.

The H_2O_2 homeostasis in plant cells is maintained by the CAT, GPX, GR, and POX enzymes in a tentative to remove the ROS generated in response to pathogens attack (Magbanua *et al*., 2007). In the present study, infected leaves of plants non-

supplied with glutamate displayed prominent increases on the activities of CAT, GPX, GR, and POX compared to plants supplied with this amino acid. The high H_2O_2 concentration on the leaves of plants non-supplied with glutamate is an indicative of loss of the cellular control over the oxidative processes suggesting a positive association between the amount of H_2O_2 produced at the infection sites with an enhanced rice susceptibility to blast. In the present study, high H_2O_2 concentration in infected leaves of plants non-supplied with glutamate resulted in lipid peroxidation based on the great MDA concentration. Thus, high H_2O_2 concentration may be associated with the oxidative damage caused by blast on rice leaves of plants non-supplied with glutamate in a scenario where the CAT and POX activities were kept high.

The removal of H_2O_2 is the first step of the ascorbate-glutathione cycle, which comprises a series of redox reactions involving, in addition to APX, the enzymes dehydroascorbate reductase, glutathione reductase, and monodehydroascorbate reductase (Foyer & Halliwell, 1976, Nakano & Asada, 1981). The APX is responsible for H_2O_2 removal in the chloroplasts, mitochondria, and peroxisomes using the ascorbate as a specific electron donor to reduce H_2O_2 to water (Asada, 1992; Quan *et al*., 2008). High *apx* transcripts and great APX activity occurring for the *P. oryzae*-rice and *Erysiphe graminis* f. sp. *hordei*-barley interactions (Agrawal *et al.,* 2002; El-Zahabi *et al*., 1995). The APX activity was kept high during the infection process of *P. oryzae* on the leaves of plants supplied with glutamate in comparison to plants non-supplied with this amino acid suggesting a pivotal role played by this enzyme in maintaining a lowered H_2O_2 concentration in the infected leaf tissue.

In the present study, the MDA concentration was lower on leaves of plants supplied with glutamate and reflected the reduced blast symptoms on them. It is known that the amount of MDA generated during lipid peroxidation in cell membranes is a very trustable indicative of the level of cellular damage caused by the infection of necrotrophic and hemibiotrophic pathogens (Dallagnol *et al*., 2011).

Based on the data from the present study, it can be concluded that photosynthesis was less impaired on plants supplied with glutamate and infected by *P. oryzae* due to the lower biochemical constraints for CO_2 fixation. Moreover, there was a lower need for activities of ROS scavenging enzymes on the infected leaves of plants supplied with glutamate due to the lower oxidative stress as a result of *P. oryzae* infection.

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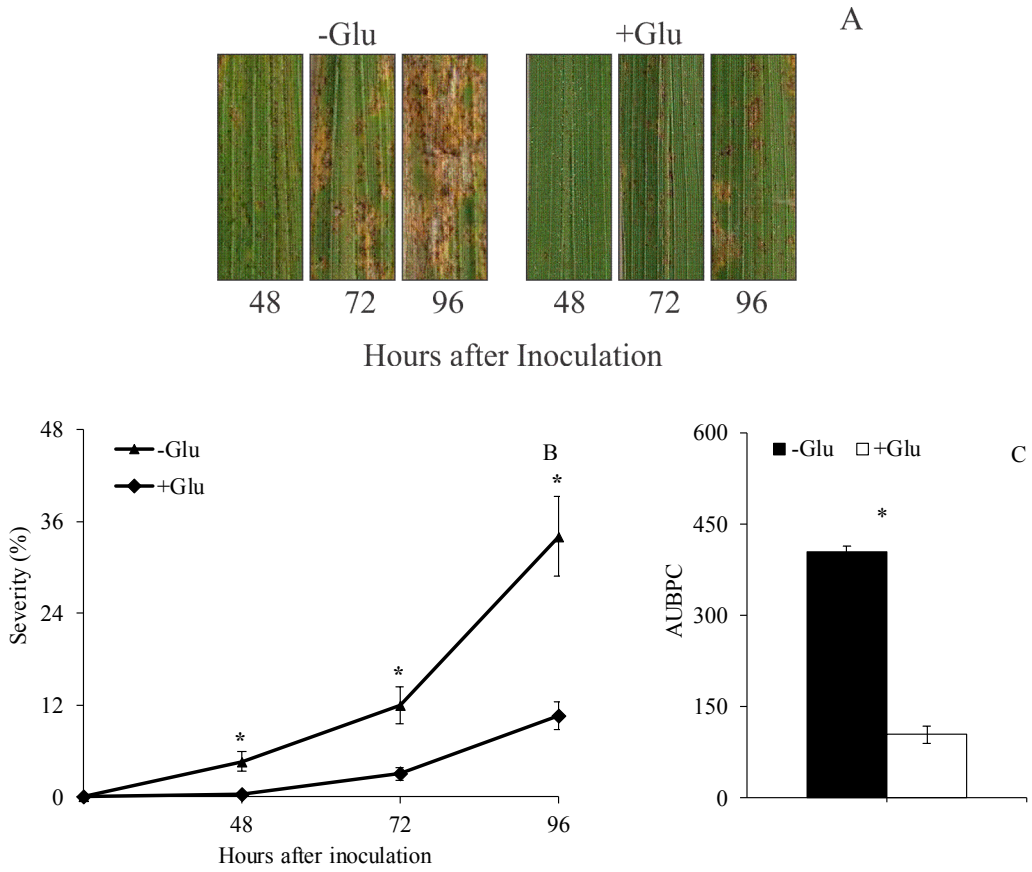

Figure 1. Blast severity (A and B) and area under blast progress curve (AUBPC) (C) for rice plants non-supplied or supplied with 10 mM of glutamate (-Glu and +Glu plants, respectively). Means from -Glu and +Glu treatments followed by an asterisk (*), at each evaluation time, are significantly different by F test ($P \leq 0.05$). Bars represent the standard deviation of the means. $n = 10$.

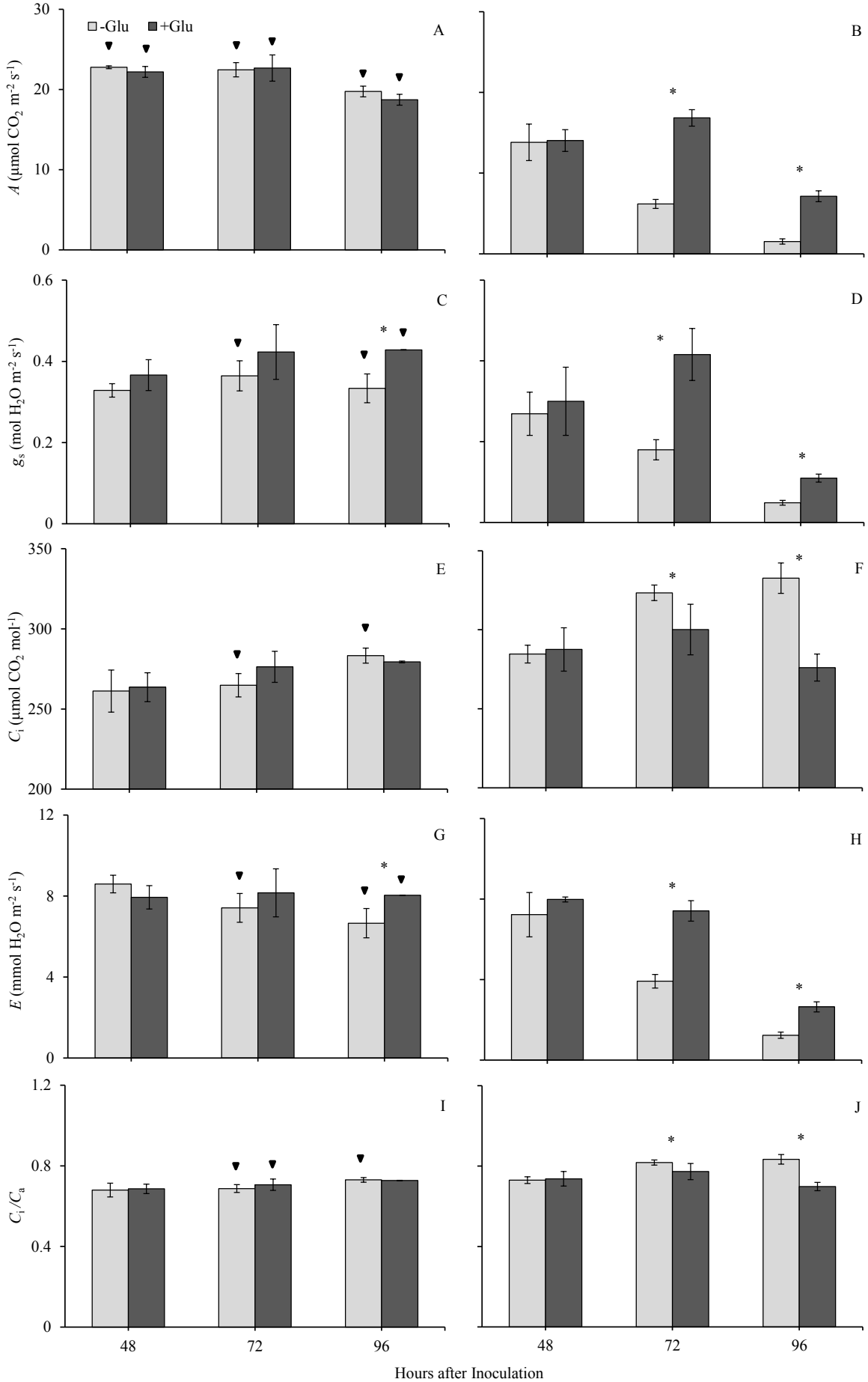
-Glu
+Glu
A
B
C
D
E
F
G
H
I
J
A (μmol CO2 m-2 s-1)
gs (mol H2O m-2 s-1)
Ci (μmol CO2 mol-1)
E (mmol H2O m-2 s-1)
Ci/Ca
30
20
10
0
0.6
0.4
0.2
0
350
300
250
200
12
8
4
0
1.2
0.8
0.4
0
48
72
96
Hours after Inoculation

Figure 2. Leaf gas exchange parameters net carbon assimilation rate (*A*) (A and B), stomatal conductance to water vapor (g_s) (C and D), internal CO_2 concentration (C_i) (E and F), transpiration rate (*E*) (G and H), and ratio of internal to atmospheric CO_2 concentration (C_i/C_a) (I and J) determined in the leaves of rice plants non-supplied or supplied with 10 mM of glutamate (-Glu and +Glu plants, respectively) and non-inoculated (NI) (A, C, E, G, and I) or inoculated (I) (B, D, F, H, and J) with *Pyricularia oryzae*. For each parameter, means from -Glu and +Glu treatments, within each evaluation time, that are followed by an asterisk (*) and from NI and I treatments followed by an inverted triangle (▼), at each evaluation time, are significantly different by F test ($P \leq 0.05$). Bars represent the standard deviation of the means. $n = 5$.

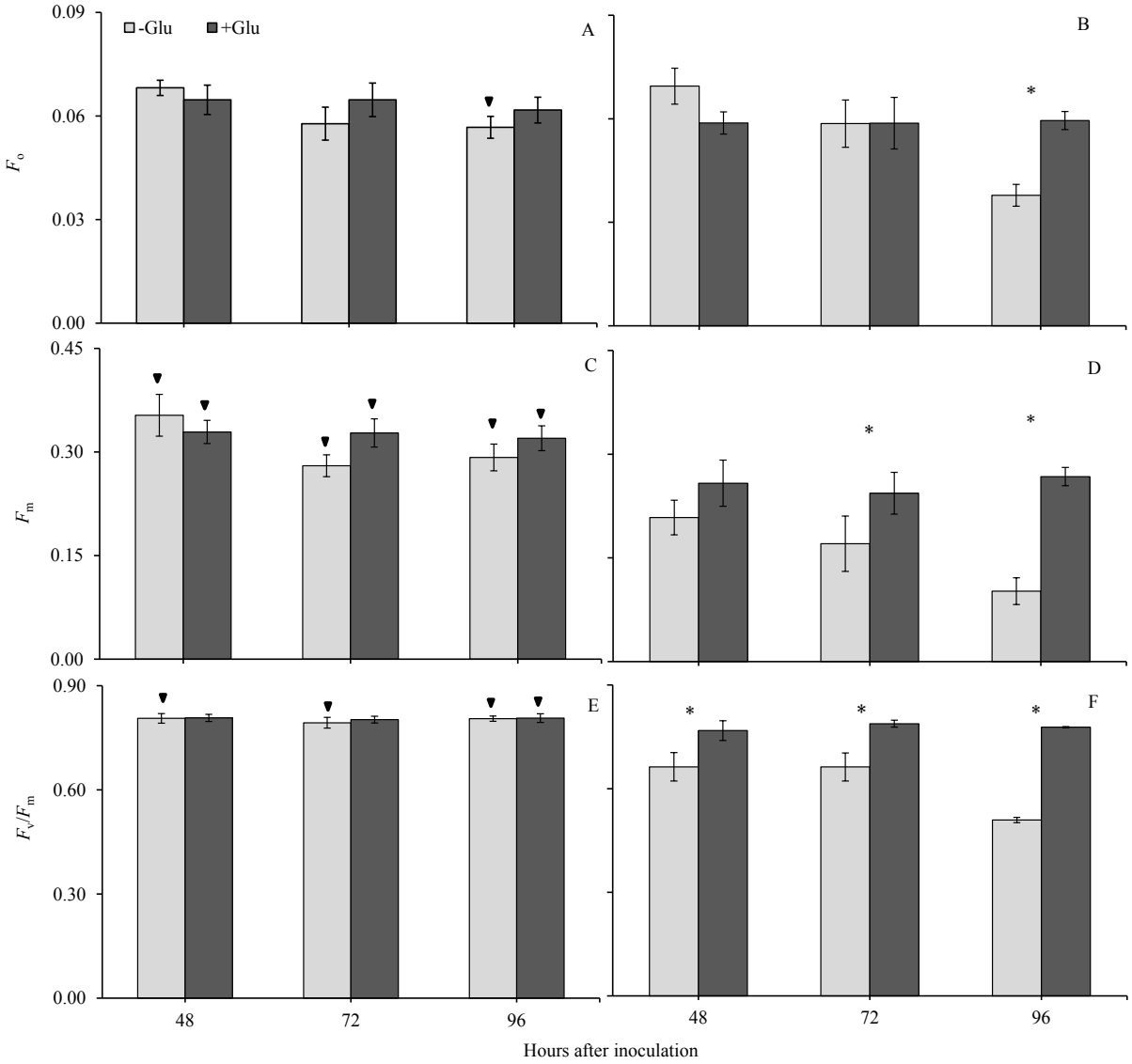

Figure 3. Minimal fluorescence (F_0) (A and B), maximal fluorescence (F_m) (C and D), and maximal photosystem II quantum yield (F_v/F_m) (E and F) determined in the leaves of rice plants non-supplied or supplied with 10 mM of glutamate (-Glu and +Glu plants, respectively) and non-inoculated (NI) or inoculated (I) with *Pyricularia oryzae*. For each parameter, means from the -Glu and +Glu treatments, within each evaluation time, that are followed by an asterisk (*) and from NI and I treatments followed by an inverted triangle (▼), at each evaluation time, are significantly different by F test ($P \leq 0.05$). Bars represent the standard deviation of the means. n = 10.

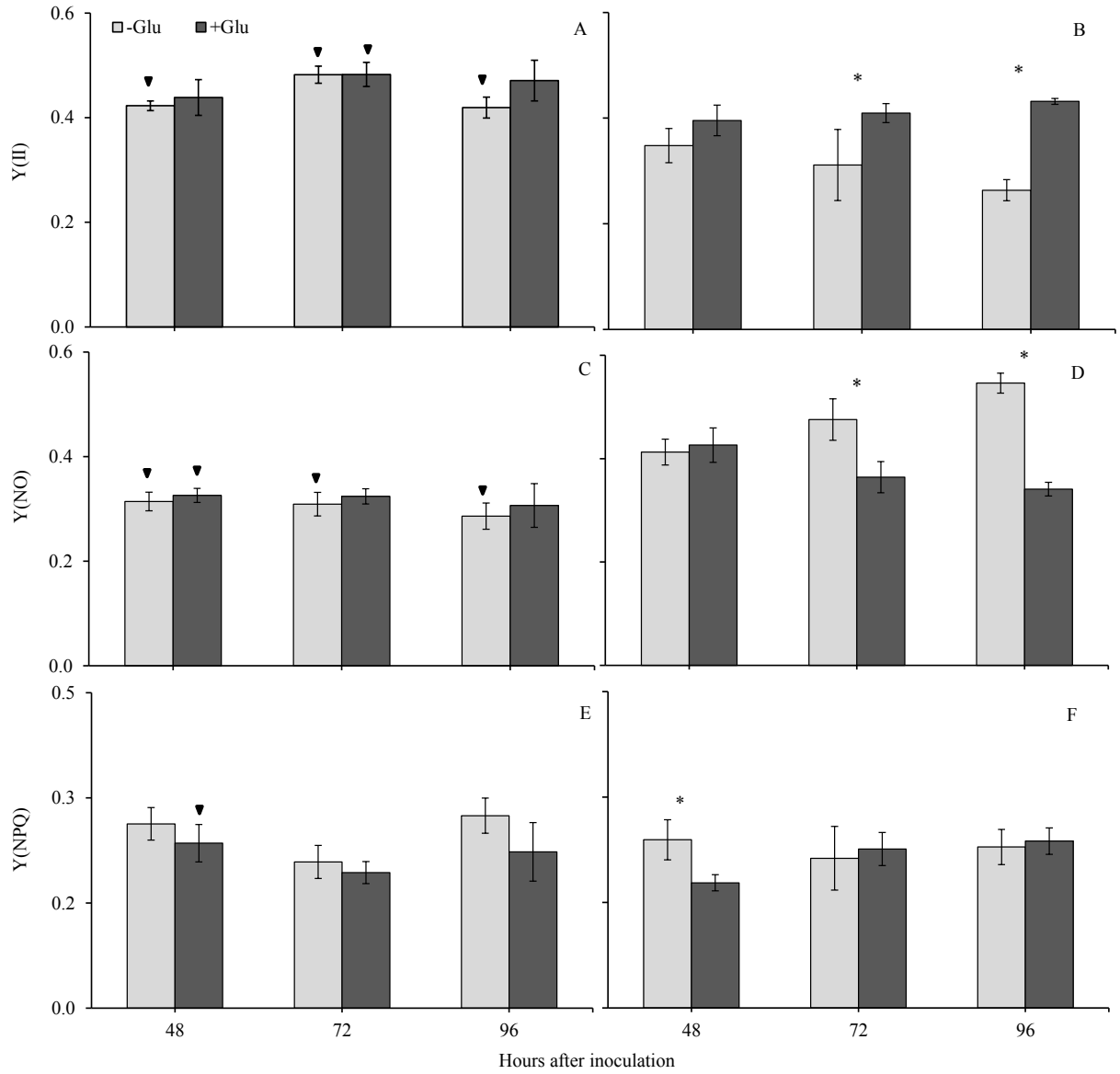

Figure 4. Effective PS II quantum yield (Y(II)) (A and B), quantum yield of non-regulated energy dissipation (Y(NO)) (C and D), and quantum yield of regulated energy dissipation (Y(NPQ)) (I and J) determined in the leaves of rice plants non-supplied or supplied with 10 mM of glutamate (-Glu and +Glu plants, respectively) and non-inoculated (NI) or inoculated (I) with *Pyricularia oryzae*. For each parameter, means from the -Glu and +Glu treatments, within each evaluation time, that are followed by an asterisk (*) and from NI and I treatments followed by an inverted triangle (▼), at each evaluation time, are significantly different by F test ($P \leq 0.05$). Bars represent the standard deviation of the means. $n = 10$.

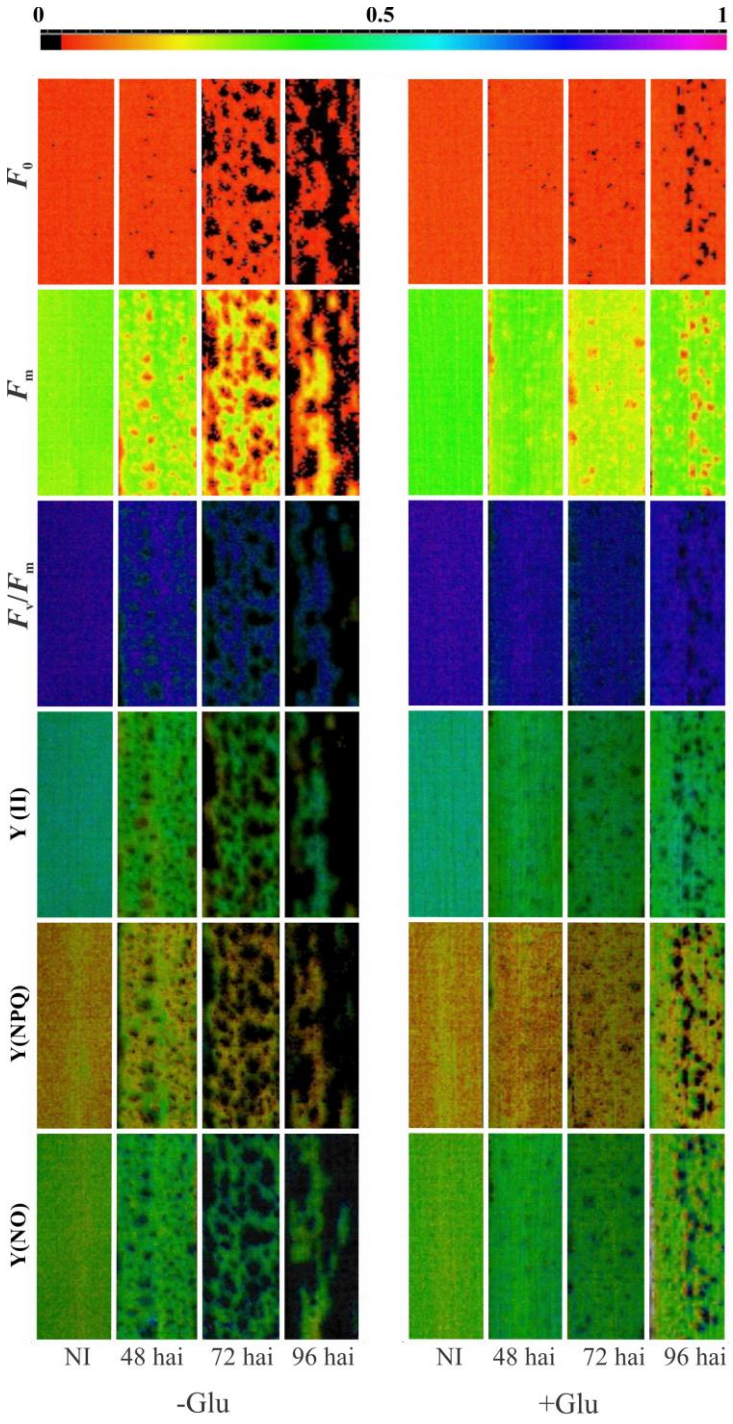

Figure 5. Images of the parameters of chlorophyll *a* fluorescence: minimal fluorescence (F_0), maximal fluorescence (F_m), maximal photosystem II quantum yield (F_v/F_m), effective PS II quantum yield (Y(II)), quantum yield of non-regulated energy dissipation (Y(NO)), and quantum yield of regulated energy dissipation (Y(NPQ)) determined in the leaves of rice plants non-supplied or supplied with 10 mM of glutamate (-Glu and +Glu plants, respectively) and non-inoculated (NI) or at 48, 72, and 96 hours after inoculation (hai) with *Pyricularia oryzae*.

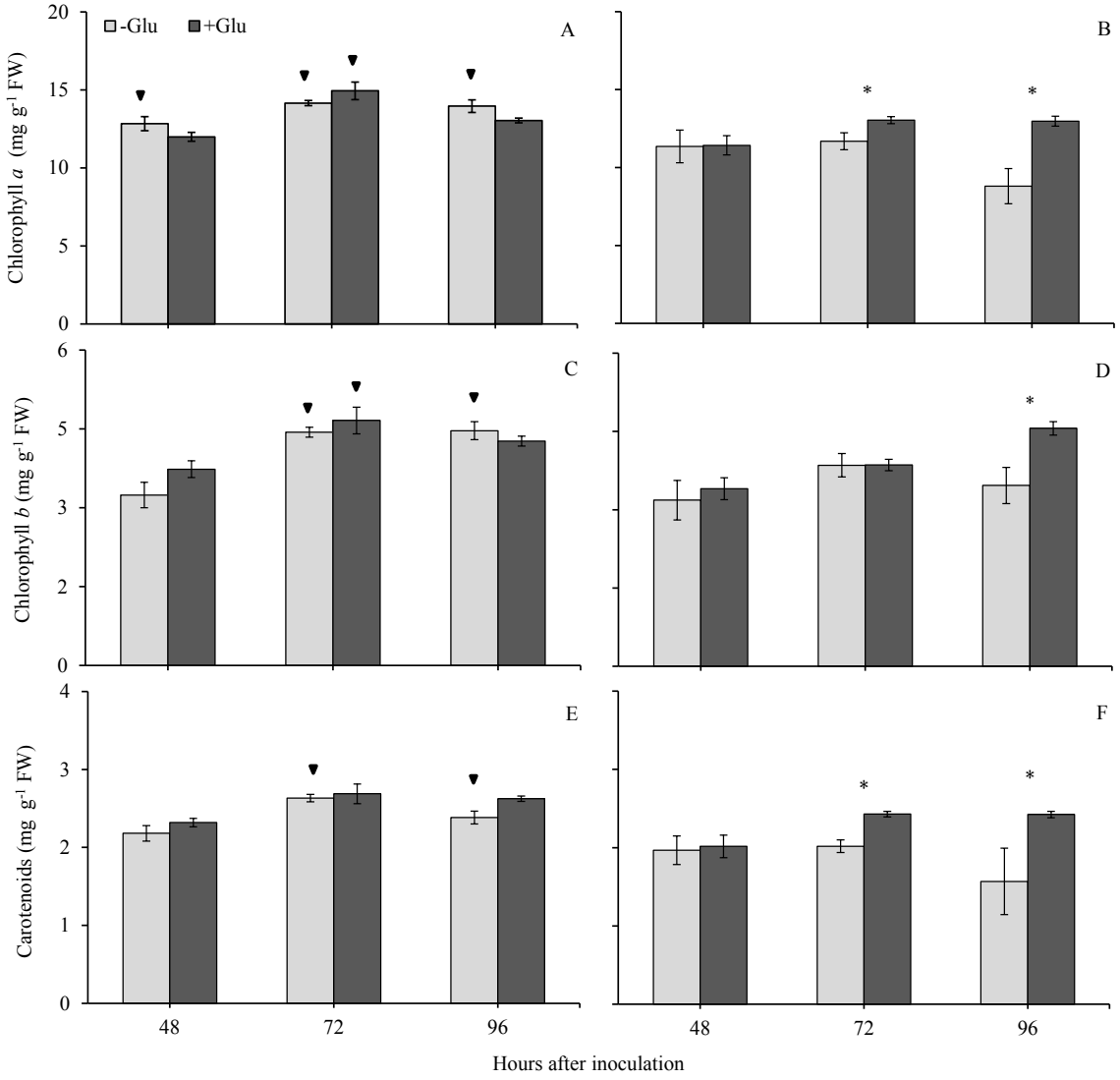

Figure 6. Concentration of chlorophyll *a* (Chl *a*) (A and B), chlorophyll *b* (Chl *b*) (B and C), and carotenoids (D and E) in the leaves of rice plants non-supplied or supplied with 10 mM of glutamate (-Glu and +Glu plants, respectively) and non-inoculated (NI) or inoculated (I) with *Pyricularia oryzae*. Means from the -Glu and +Glu treatments, within each sampling time, that are followed by an asterisk (*) and from NI and I treatments followed by an inverted triangle (▼), at each sampling time, are significantly different by F test ($P \leq 0.05$). Bars represent the standard deviation of the means. $n = 5$.

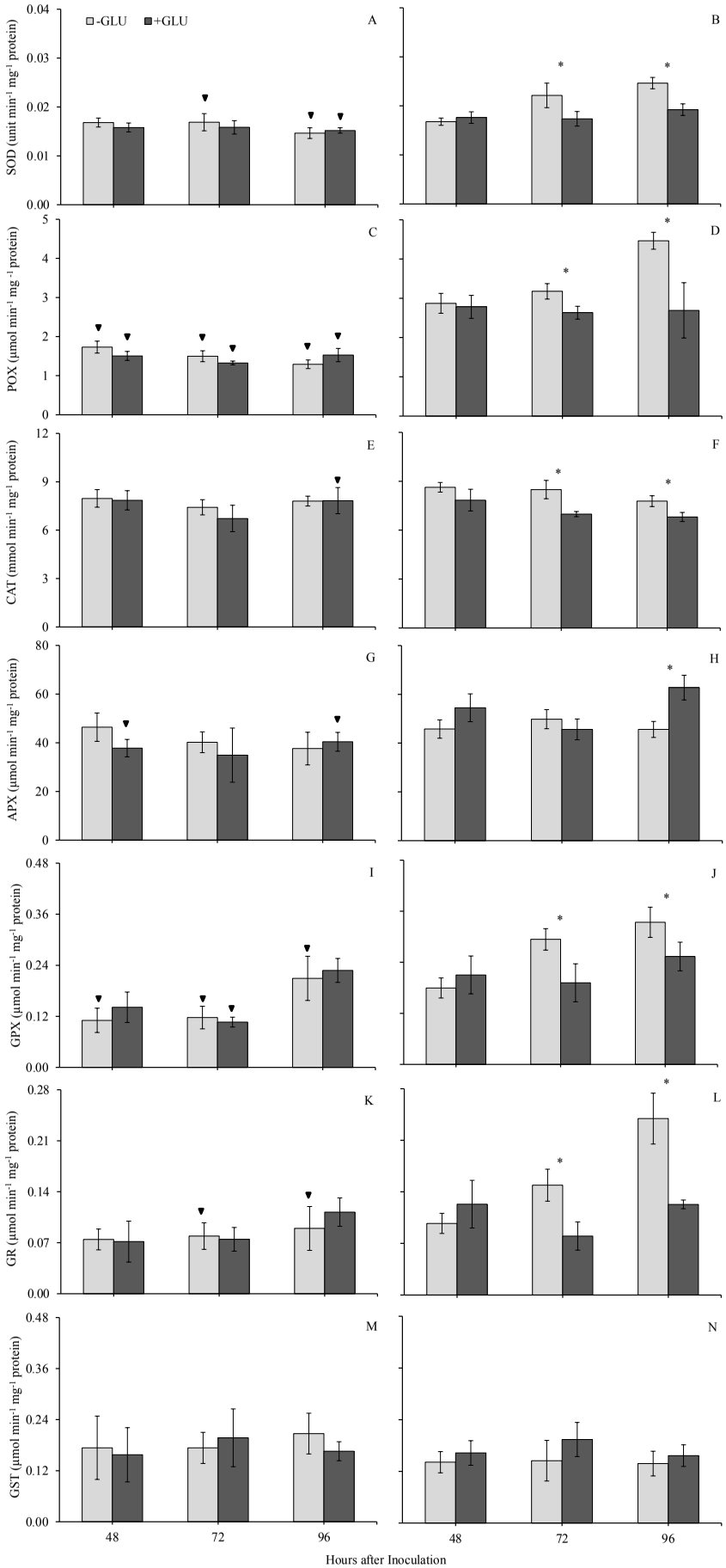
-GLU
+GLU
A
B
C
D
E
F
G
H
I
J
K
L
M
N
SOD (unit min-1 mg-1 protein)
POX (µmol min-1 mg-1 protein)
CAT (mmol min-1 mg-1 protein)
APX (µmol min-1 mg-1 protein)
GPX (µmol min-1 mg-1 protein)
GR (µmol min-1 mg-1 protein)
GST (µmol min-1 mg-1 protein)
0.04
0.03
0.02
0.01
0.00
5
4
3
2
1
0
12
9
6
3
0
80
60
40
20
0
0.48
0.36
0.24
0.12
0.00
0.28
0.21
0.14
0.07
0.00
48
72
96
Hours after Inoculation

Figure 7. Activities of superoxide dismutase (SOD) (A and B), peroxidase (POX) (C and D), catalase (CAT) (E and F), and ascorbate peroxidase (APX) (G and H) in the leaves of rice plants non-supplied or supplied with 10 mM of glutamate (-Glu and +Glu plants, respectively) and non-inoculated (NI) or inoculated (I) with *Pyricularia oryzae*. Means from the -Glu and +Glu treatments, within each sampling time, that are followed by an asterisk (*) and from NI and I treatments followed by an inverted triangle (▼), at each sampling time, are significantly different by F test ($P \leq 0.05$). Bars represent the standard deviation of the means. $n = 5$.

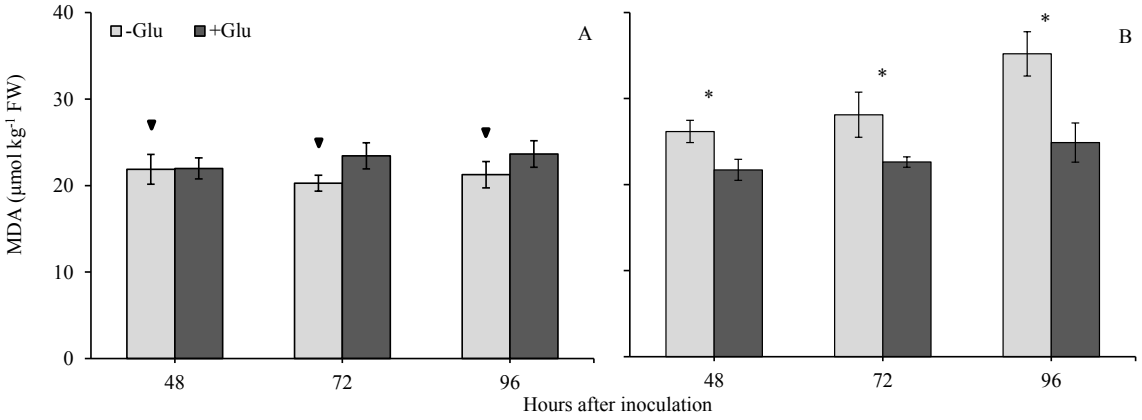

Figure 8. Concentration of malondialdehyde (MDA) in the leaves of rice plants non-supplied or supplied with 10 mM of glutamate (-Glu and +Glu plants, respectively) and non-inoculated (A) or inoculated (B) with *Pyricularia oryzae*. Means from the -Glu and +Glu treatments, within each sampling time, that are followed by an asterisk (*) and from NI and I treatments followed by an inverted triangle (▼), at each sampling time, are significantly different by F test ($P \leq 0.05$). Bars represent the standard deviation of the means. FW = fresh weight. $n = 5$.

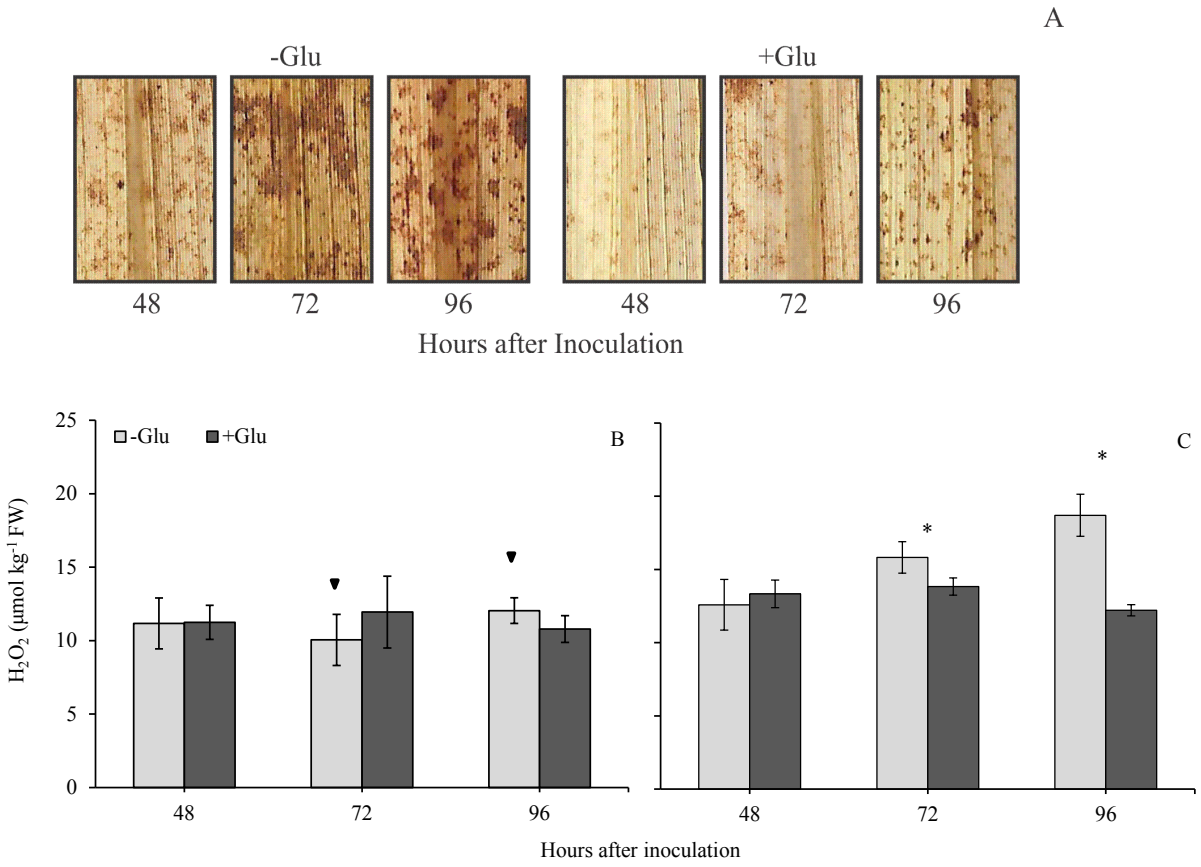

Figure 9. Histochemical detection of hydrogen peroxide (H_2O_2) (A) and concentration of hydrogen peroxide (H_2O_2) (B and C) in the leaves of rice plants non-supplied or supplied with 10 mM of glutamate (-Glu and +Glu plants, respectively) and non-inoculated (B) or inoculated (C) with *Pyricularia oryzae*. Means from the -Glu and +Glu treatments, within each sampling time, that are followed by an asterisk (*) and from NI and I treatments followed by an inverted an inverted triangle (▼) at each sampling time, are significantly different by F test ($P \leq 0.05$). Bars represent the standard deviation of the means. FW = fresh weight. $n = 5$.

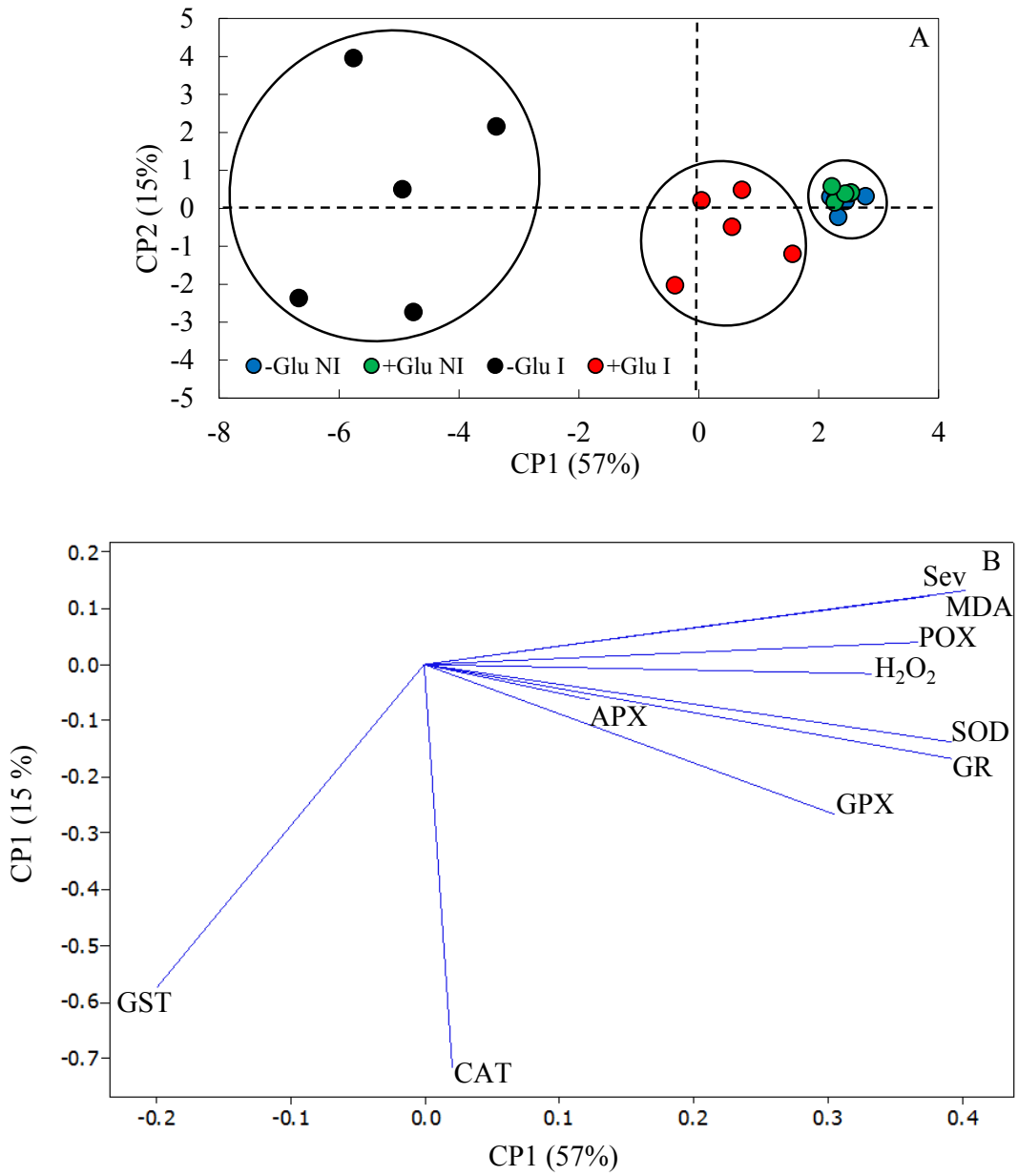

Figure 10. Score plot (A) and loading values (B) of principal components analysis comparing blast severity (Sev), enzymes of the antioxidant system (superoxide dismutases [SOD], glutathione reductases [GR], ascorbate peroxidases [APX], catalases [CAT], peroxidases [POX], glutathione peroxidases [GPX], and glutathione S-transferases [GST]) as well as the concentrations of malondialdehyde [MDA] and hydrogen peroxide [H_2O_2] in the leaves of rice plants supplied with 0 and 10 mM of glutamate (-Glu and +Glu, respectively) and non-inoculated (NI) or at 96 hours after inoculation (I) with *Pyricularia oryzae*.

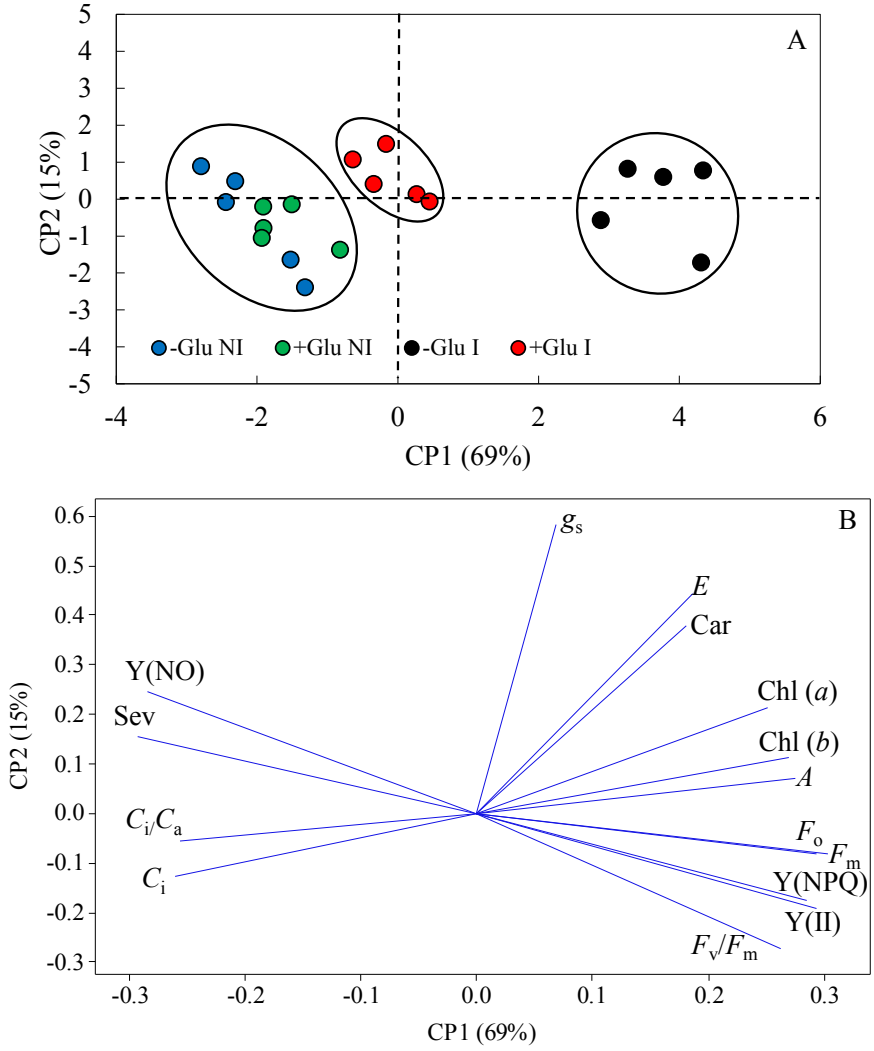

Figure 11. Score plot (A) and loading values (B) of principal components analysis comparing blast severity (Sev), the leaf gas exchange parameters (net CO_2 assimilation rate [A], stomatal conductance to water vapor [g_s], transpiration rate [E], internal CO_2 concentration [C_i] and ratio of internal to atmospheric CO_2 concentration [C_i/C_a]), chlorophyll *a* fluorescence imaging parameters [minimal fluorescence (F_0), maximal fluorescence (F_m), maximum quantum quenching [F_v/F_m)], effective PSII quantum yield [Y(II)], quantum yield of regulated energy dissipation [Y(NPQ)], and quantum yield of non-regulated energy dissipation [Y(NO)]), the concentration of chlorophyll (Chl) *a*, Chl *b* and carotenoids (Car) determined in leaves of rice plants non-supplied or supplied with 10 mM of glutamate (-Glu and +Glu, respectively) and non-inoculated (NI) or at 96 hours after inoculation (I) with *Pyricularia oryzae*.

Chapter 2

Glutamate-Potentiated Rice Resistance to Blast

Abstract

Blast, caused by the hemibiotrophic fungus *Pyricularia oryzae*, is the most important disease affecting rice production worldwide. The present study aimed to investigate the potential of glutamate (GLU) to increase rice resistance to blast. Rice plants (cultivar Metica-1) were non-supplied (-Glu) or supplied (10 mM) (+Glu) with GLU and non-inoculated or inoculated with *P. oryzae*. Blast severity and the number of lesions per cm^2 of leaf were significantly lower by 55 and 50%, respectively, for +Glu plants in comparison to -Glu plants at 96 hours after inoculation (hai). The area under blast progress curve was significantly lower by 70% for +Glu plants in comparison to -Glu plants. For inoculated +Glu plants, the activities of *β*-1-3-glucanase, chitinase, phenylalanine ammonia-lyase, and polyphenoloxidases as well as the concentrations of total soluble phenolics and lignin-thioglycolic acid derivatives were significantly higher for inoculated +GLU plants in comparison to inoculated -Glu ones. In conclusion, the results of the present study indicated that blast symptoms were reduced on the leaves of +GLU plants due to great activities of defense enzymes and concentrations of phenolics and lignin.

Keywords: *Pyricularia oryzae,* host defense responses, lignin, phenylpropanoid pathway, phenolic acids.

Introduction

Blast, caused by the hemibiotrophic fungus *Pyricularia oryzae* (T. T. Hebert) Yaegashi and Udagawa) Barr, is a destructive disease affecting rice (*Oryza sativa*) production worldwide (TeBeest *et al*., 2007). On leaves, lesions initially are of gray-green color with darker green border and with water-soaked appearance (TeBeest *et al*., 2007). Currently, a considerable emphasis on integrated rice blast management strategies for minimizing yield losses include the use of resistant cultivars and fungicides spray (Nasrudddin and Amin 2013; Miah *et al*., 2017). However, more environmental-friendly control strategies need to be investigated aiming to reduce blast´s effect on yield and the use of glutamate may become an interesting option (Pooja and Katoch, 2014).

Glutamate plays a pivotal role in amino acids metabolism and orchestrates crucial metabolic functions on plants mainly involved with host defense responses against pathogens infection (Seifi *et al.*, 2013). Once perceived by receptor or sensor proteins located on the cell surface of plants, glutamate acts as a signal to regulate the expression of defense-related genes (Kan *et al*., 2017). For instance, several wall-associated kinases are involved in the basal defense of rice to blast (Seifi *et al*., 2013; Kan *et al*., 2017). Several defense genes were rapidly expressed in several plants infected by pathogens the presence of glutamate (Seifi *et al*., 2013, Kadotani *et al*., 2016, Kan *et al*., 2017). Exogenous glutamate supply caused a rapid expression of genes encoding for transcriptional activator involved in hypersensitive response, lipoxigenases, trypsin inhibitor, xylanase inhibitor, aspartic proteinase, chitinase, phenylalanine ammonia-lyase, and disease-related receptor-like protein kinases (Kan *et al*., 2017). The salicylic acid-dependent defense response in rice against *P. oryzae* infection was up-regulated by glutamate (Kadotani *et al*., 2016).

Plants respond against pathogens infection through many different defense mechanisms, which include the expression of several defense-related genes, the synthesis of antimicrobial compounds such as phenolics and phytoalexins, the production of reactive oxygen species, and tissue lignification (Lozovaya *et al.*, 2004; Upchurch and Ramirez 2010). The plants' ability to reduce fungal colonization on their tissues is most likely dependent on the combination of different mechanisms of defense, which are quite different depending on the host-pathogen interaction (Hammond-Kosack and Jones, 1996; Hammond-Kosack and Parker, 2003). High phenylalanine ammonia-lyase (PAL), polyphenoloxidase (PPO), chitinase (CHI), and *β*-1,3-glucanases (GLU) activities on infected tissues is one of the most common defense mechanism of plants to counteract the infection by pathogens (Loon *et al.,* 2006).

The present study aimed to investigate the effect of glutamate on the potentiation of rice defense responses against *P. oryzae* infection.

Materials and Methods

Nutrient solution preparation and plant growth

Rice seeds from cultivar Metica-1, susceptible to blast, were surface sterilized in sodium hypochlorite 1% (v/v) for 3 min, washed in sterilized water for 3 min, and germinated in trays containing sand previously autoclaved at 25^{o}C for 10 days. Five seedlings were transferred to 5 L plastic pots (30-cm diameter) (Ecovaso, Jaguariúna, São Paulo, Brazil) with one-half-strength nutrient solution. The nutrient solution was prepared according to Hoagland and Arnon (1950) with some modifications as follow: 1.0 mM KNO_3, 0.25 mM $NH_4H_2PO_4$, 0.1 mM NH_4Cl, 0.5 mM $MgSO_4.7H_2O$, 1.0 mM $(CaNO_3)_2$, 0.3 μM $CuSO_4.5H_2O$, 0.33 μM $ZnSO_4.7H_2O$, 11.5

μM H_3BO_3, 3.5 μM $MnCl_2.4H_2O$, 0.1 μM $(NH_4)_6Mo_7O_{24}.4H_2O$, 25 μM $FeSO_4.7H_2O$, and 25 μM ethylenediaminetetraacetic (EDTA) disodium. The nutrient solution was changed every four days. The pH was checked daily and maintained between 5.5-5.8 using NaOH or HCl (1 M) solutions.

Glutamate application

Plants were grown in nutrient solution for 30 days and then supplied with the same nutrient solution containing 10 mM of glutamate (Sigma-Aldrich, São Paulo, Brazil) for 24 h. Plants non-supplied with glutamate served as the control treatment. After this period, the roots of each plant were carefully washed with deionized water and plants were returned to their respective pots containing the original nutrient solution.

Inoculation procedure

The isolate UFV-DFP *Po*22 of *P. oryzae* was used to inoculate the plants. This isolate was preserved on disks of filter paper placed inside of plastic microtubes containing silica gel and stored at 4°C. Disks of filter paper containing fungal mycelia were transferred to Petri dishes containing oat-agar medium. After five days, plugs of the oat-agar medium containing fungal mycelia were transferred to new Petri dishes containing the same medium. The Petri dishes were incubated in a growth chamber at 25°C under continuous light for 12 days. After this period, conidia were carefully removed from the media using a soft bristle brush and with water-containing gelatin (1% wt/vol). The conidial suspension was calibrated with a hemocytometer to obtain a concentration of 1×10^5 conidia mL^{-1}. The conidial suspension was sprayed on the adaxial surface of the leaves of each plant (50 mL per plant) at 45 days after emergence with an atomizer (Paasche Airbrush Co., Chicago, IL, USA). After inoculation, plants were kept in a mist chamber (temperature of 25 ± 2°C and relative humidity of 90 ± 5%) during 24 h at darkness. Plants were then transferred to a plastic mist growth chamber (temperature of 24 ± 1°C (day) and of 18 ± 2°C (night)) inside a greenhouse for the duration of the experiment. A misting system with nozzles (model NEB-100; KGF Company, São Paulo, Brazil) spraying

mist every 30 min above the plant canopy kept the relative humidity of 90 ± 5%. Plant canopy received a natural photon flux density of ≈ 900 μmol m^{-2} s^{-1}.

Blast severity assessments

The second and third leaves, from base to top, of each plant per replication of each treatment were used to evaluate blast severity. These leaves were collected at 48, 72, and 96 hours after inoculation (hai), scanned at the resolution of 300 dpi, and the images obtained were used to quantify blast severity using the QUANT software (Resende *et al.*, 2012). The area under blast progress curve (AUBPC) was calculated using the trapezoidal integration of the blast progress curve over time according to Shaner and Finney (1977). The number of lesions per cm^2 of leaf was counted at three places randomly chosen on the third leaf, from base to top, of each plant per replication of each treatment at 96 hai.

Biochemical assays

For all biochemical assays, the fourth, fifth, and sixth leaves, from base to top, of each plant per replication of each treatment were collected at 24, 48, 72, and 96 hai. Leaves collected from non-inoculated plants at these same sampling times served as the control treatment. Leaf samples were kept in liquid nitrogen during sampling and stored at -80°C.

Determination of defense enzymes activities

A total of 0.2 g of leaf tissue was ground into a fine powder with liquid nitrogen using a mortar and pestle to obtain the powder to determine the activities of *β*-1,3-glucanase (GLU) (EC 3.2.1.39), chitinase (CHI) (EC 3.2.1.14), phenylalanine ammonia-lyase (PAL) (EC 4.3.1.5), and polyphenoloxidase (PPO) (EC 1.10.3.1).

The fine powder was homogenized in 2 mL of a solution containing 50 mM potassium phosphate buffer (pH 6.8), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2% (w/v) polyvinylpyrrolidone (PVP). Then, the homogenate was centrifuged at 12,000 *g* for 15 min at 4°C and the supernatant was collected and used to determine the GLU, CHI, PAL, and PPO activities. The GLU activity was determined according to the method of Lever (1972). First, 20 µL of the crude enzyme extract was added to a reaction mixture containing 50 mM sodium acetate buffer (pH 5.0) and laminarin (1 mg/mL). Next, the reaction mixture was incubated in a ThermoMixer (Eppendorf, Hamburg, Germany) at 45°C for 1 h. Then, 500 µL of the reaction mixture was added to 1.5 mL of dinitrosalicylic acid (DNS) and incubated at 100°C for 15 min. The reaction was stopped using an ice bath until the solution reached 25°C. The amount of reducing sugars released was calculated with a calibration curve using glucose (Sigma-Aldrich, São Paulo, Brazil) as a standard, and the absorbance was measured at 540 nm (Miller 1959). A similar procedure was used for the control samples, but the first incubation was excluded. The CHI activity was determined according to the method of Harman *et al*. (1993). The reaction was started with the addition of 20 µL of the crude enzyme extract to 1980 µL of a reaction mixture containing 50 mM sodium acetate buffer (pH 5.0) and 0.1 mM *p*-nitrophenyl-*β*-*D*-*N*-*N*'-diacetylchitobiose. Next, the reaction mixture was incubated at 37°C for 2 h and the reaction was stopped by adding 500 µL of 0.2 M sodium carbonate. The control samples received 500 µL of 0.2 M sodium carbonate immediately after the addition of the crude enzyme extract to the reaction mixture. The final product released by CHI was measured at 410 nm and the CHI activity was calculated using the extinction coefficient of 70 $mM^{-1}cm^{-1}$. The PAL activity was assayed following the method proposed by Guo *et al*. (2007) with some

modifications. First, the reaction was started by adding 100 µL of crude enzyme extract to 0.9 mL of a reaction mixture containing 40 mM sodium borate buffer (pH 8.8) and 20 mM *L*-phenylalanine. The reaction mixture was incubated at 30°C for 1 h. For the control samples, the extract was replaced by borate buffer. The reaction was stopped by adding 50 µL of 6 N HCl. The absorbance of the trans-cinnamic acid derivatives was recorded at 290 nm. The PAL activity was estimated using an extinction coefficient of 10 $M^{-1}cm^{-1}$ (Zucker, 1965). The PPO activity was assayed following the colorimetric determination of pyrogallol oxidation according to the method of Kar and Mishra (1976) with some modifications. The reaction was started after the addition of 15 µL of the crude enzyme extract to 985 µL of a reaction mixture containing 25 mM potassium phosphate buffer (pH 6.8) and 20 mM pyrogallol. Immediately after the reaction was initiated, the absorbance was determined at 420 nm for 1 min at 25°C. The PPO activity was calculated using the extinction coefficient of 2.47 $mM^{-1}cm^{-1}$ according to the PPO ionically bound to the cell wall (Chance and Maehley 1955). The enzyme activity was expressed based on protein concentration determined in each sample according to the method of Bradford (1976).

Determination of the concentrations of total soluble phenolics (TSP) and lignin-thioglycolic acid (LTGA) derivatives

A total of 100 mg of leaf tissue was ground into a fine powder with liquid nitrogen using a mortar and pestle and homogenized in 1 mL of a solution containing 80% (v/v) methanol. Next, the crude extract was shaken at 300 rpm at 25°C for 2 h. Subsequently, the mixture was centrifuged at 17,000 *g* for 30 min. The methanolic extract was collected and used to determine the TSP concentration and the pellet was

maintained at 20°C to further determine the LTGA derivatives concentration. The TSP concentration was assayed following the methodology proposed by Zieslin and Ben-Zaken (1993) adapted by Rodrigues *et al*. (2005). The reaction was started after the addition of 150 µL of methanolic extract to 750 µL of 0.2 M Folin-Ciocalteu phenol reagent and incubated at 25°C for 5 min. The next step was the addition of 0.1 M sodium carbonate to the solution, which was maintained at 25°C for 10 min. Afterwards, 1 mL of deionized water was also added to the mixture and the solution was incubated at 25°C for 1 h. The TSP concentration was calculated based on a calibration curve using catechol (Sigma-Aldrich, São Paulo, Brazil) as a standard and the absorbance was read at 725 nm. For the determination of the LTGA derivatives concentration, the pellet was resuspended in 1.5 mL of deionized water and homogenized and centrifuged at 12,000 *g* for 15 min. Afterwards, the supernatant was discarded and the pellet was dried at 65°C for 12 h. The alcohol-insoluble dry residue was used to determine the concentration of LTGA derivatives as described by Barber and Ride (1988). The absorbance of the LTGA derivatives supernatant was read at 280 nm and the concentration was determined from a calibration curve using lignin alkali and 2-hydroxypropyl ether (Sigma-Aldrich, São Paulo, Brazil) as a standard.

Experimental design and statistical analysis of data

A 2 × 2 factorial experiment consisting of plants non-supplied or supplied with glutamate (referred as -Glu and +Glu plants, respectively) and non-inoculated or inoculated with *P. oryzae* was arranged in a completely randomized design with five replications. The experiment was repeated once. Each experimental unit consisted of a plastic pot containing five plants. Means from each variable were compared by F

test ($P \leq 0.05$) using SAS (version 6.12; SAS Institute, Inc., Cary, NC). The relationship among the variables evaluated was determined using the principal components analysis (PCA) technique. Data were analyzed using the Minitab software (version 15; Minitab Corporation).

Results

Blast severity, AUBPC, and NL

Blast severity was significantly lower by 88, 77, and 55% for +Glu plants in comparison to -Glu plants at 48, 72, and 96 hai, respectively (Fig. 1A). The AUPBC was significantly lower by 70% for +Glu plants in comparison to -Glu ones (Fig. 1B). The NL was significantly lower by 50% for +Glu plants in comparison to -Glu ones at 96 hai (Fig. 1C).

Defense enzymes activities

There were significantly increases of 23 and 27% for PAL activity at 0 and 24 hai, respectively, for non-inoculated -Glu plants in comparison to non-inoculated -Glu ones (Fig. 2A). For inoculated +Glu plants, there were significantly increases of 24, 20, 17, and 19% for PAL activity at 0, 24, 72, and 96 hai, respectively, in comparison to inoculated -Glu ones (Fig. 2B). There were significant increases of 20 and 22% for PAL activity at 72 and 96 hai, respectively, for inoculated +Glu plants in comparison to non-inoculated +Glu ones (Fig. 2A and 2B).

For inoculated +Glu plants, there were significant increases of 24 and 21% for PPO activity at 72 and 96 hai, respectively, in comparison to inoculated -Glu ones (Fig. 2D). There were significantly increases of 39, 27, and 63% for PPO activity at 48, 72, and 96 hai, respectively, for inoculated +Glu plants in comparison to non-inoculated +Glu ones (Fig. 2C and 2D). There were significantly increases of 16 and 45% for PPO activity at 72 and 96 hai, respectively, for inoculated -Glu plants in comparison to non-inoculated -Glu plants (Fig. 2C and 2D). At 0 hai, CHI and GLU activities significantly increased by 46 and 25%, respectively, for non-inoculated +Glu plants in comparison to non-inoculated -Glu ones (Fig. 2E and 2G). There were

significantly increases of 39 and 21% for CHI activity for inoculated +Glu plants at 0 and 24 hai, respectively, in comparison to inoculated -Glu ones. At 96 hai, CHI activity was significantly reduced by 32% for inoculated +Glu plants in comparison to inoculated -Glu plants. (Fig. 2F). For CHI activity, there were significantly increases of 46 and 73% for inoculated +Glu plants and of 63 and 67% for inoculated -Glu plants at 72 and 96 hai, respectively, in comparison to non-inoculated ones (Fig.2F and 2G). For inoculated +Glu plants, there were significantly increases of 24 and 38% for GLU activity at 72 and 96 hai, respectively, in comparison to inoculated -Glu ones (Fig.2H). There were significantly increases of 41, 63, and 49% for GLU activity at 48, 72, and 96 hai, respectively for inoculated +Glu plants in comparison to non-inoculated +Glu ones (Fig. 2G-2H). At 72 hai, GLU activity was significantly increased by 34% for inoculated -Glu plants in comparison to non-inoculated -Glu plants ((Fig. 2G and 2H).

Concentrations of TSP and LTGA derivatives

For non-inoculated +Glu plants, there were significantly increases of 23 and 14% for TSP concentration at 0 and 48 hai, respectively, in comparison to non-inoculated -Glu ones (Fig. 3A). The TSP concentration significantly increased by 24, 15, 21, and 16% at 0, 24, 72, and 96 hai, respectively, for inoculated +Glu plants in comparison to inoculated -Glu ones (Fig. 3B). For inoculated +Glu plants, there were significantly increases of 41, 19, and 18% for TSP concentration at 24, 72, and 96 hai, respectively, in comparison to non-inoculated +Glu ones (Fig. 3A and 3B). For inoculated +Glu plants, there were significantly increases of 15, 16, and 11% for LTGA derivatives concentration at 24, 72, and 96 hai, respectively, in comparison to inoculated -Glu ones. (Fig. 3D). The LTGA derivatives concentration significantly

increased by 17, 13, and 18% at 24, 72, and 96 hai, respectively, for inoculated +Glu plants in comparison to non-inoculated +Glu ones (Fig. 3C and 3D).

Principal components analysis

Changes on the biochemical variables on leaves of plants non-supplied or supplied with Glu and infected by *P. oryzae* were unequivocally demonstrated using the PCA analysis (Fig. 4). The two principal components (PC) explained 77% of the total variation in the biochemical variables (61 and 17%, respectively, for PC1 and PC2) (Fig. 4A and B). Based on PCA, the +Glu NI, -Glu NI, and +Glu I treatments at 96 hai were grouped close on the center of the bi-plot. Conversely, the -Glu I treatment was placed farther from the center of the bi-plot (Figs. 4A). In the loading values for the first PC, positive scores were obtained for Sev, TSP, LTGA derivatives and for GLU, PAL, PPO, and CHI activities which were all grouped (Fig. 4B).

Discussion

The present study provides, to the best of the authors' knowledge, the first biochemical evidences of the potential of glutamate to increase rice resistance against *P. oryzae* infection. The high activities of the defense enzymes PAL, PPO, GLU, and CHI as well as great concentrations of TSP and LTGA derivatives on the leaves of plants supplied with glutamate resulted in lower blast symptoms. It is well recognized the importance of the defense enzymes GLU, CHI, PAL, and PPO for the resistance of many plant species in response to pathogens infection (Lozovaya *et al*., 2004; Sahoo *et al*., 2009; Gnanamangai *et al*., 2011; Leite *et al*., 2014; Nascimento *et al*., 2016). The potential of glutamate to control blast on rice has been reported in others studies (Seifi *et al*., 2013; Kan *et al*., 2017). According to Kadotani *et al*. (2016), the supply of glutamate to the roots of rice plants reduced blast severity on leaves due to a systemic induction of salicylic acid-dependent defense responses. The transcriptome analysis revealed that many genes related to rice defense response against blast were also rapidly induced by glutamate (Kan *et al*., 2017).

In the present study, PAL activity was high for plants supplied with Glu indicating the importance of the phenylpropanoid pathway for rice resistance against blast. The high PAL activity potentiated by glutamate was noticed at early stages of fungal infection. The expression of PAL gene was rapidly induced in rice plants supplied with glutamate (Kan *et al*., 2017). A high PAL activity was of great importance for rice resistance to blast because it converts *L*-phenylalanine into *trans*-cinnamic acid, which is the precursor of various phenolics and flavonoids with antimicrobial action (Dixon *et al*., 2002; Hao *et al.*, 2011; Borges *et al*., 2012). PAL is also a key enzyme for the biosynthesis of salicylic acid that is involved in the systemic acquired resistance in plants (Gruner *et al*., 2013; Malamy *et al*., 2014). Our findings are

consistent with previously reports showing the importance of PAL for the resistance of several crops to pathogens of different lifestyles (Sana *et al*., 2010; Giberti *et al*., 2012; Gupta *et al*., 2012; Li *et al*., 2013; Zhang *et al*., 2013; Kim and Hwang 2014; Riaz *et al*., 2014; Bhat *et al*., 2014, Tatagiba *et al*., 2016). In the present study, it is plausible to hypothesize that high PAL activity for plants supplied with glutamate resulted in a greater production of phenolics and lignin. It is known that lignin deposition can reinforce the plant cell wall providing a structural barrier to delay or avoid pathogen colonization and pathogen growth can be inhibited by phenolics precursors of lignin (Venere, 1980; Ride, 1983; Reimers and Leach 1991; Naoumkina *et al*., 2010). Additionally, the covalently bound of lignin to polysaccharides and hemicelluloses in the cell wall increase their mechanical strength that may affect the diffusion of hydrolytic enzymes and non-host selective toxins released by pathogens during their infection process (Chabannes *et al*., 2001). Thus, a high LTGA derivatives concentration on rice plants supplied with glutamate contributed to reduce the deleterious effects of hydrolytic enzymes and non-host selective toxins produced during *P. oryzae* on the infected leaf tissues. Phenolics and lignin precursors were toxic to *P. oryzae* in rice due to their interaction with lipids and phospholipids present in the fungal cell wall (Rodrigues *et al*., 2003).

It can be postulated that changes in the diversity and concentrations of phenolics during the infection process of *P. oryzae*, possibly modulated by glutamate, may be also associated with high PPO activity. PPO activity was kept high at advanced stages of *P. oryzae* infection on leaves of plants supplied with glutamate. A high concentration of phenolics is important as the substrate for the oxidative reactions catalyzed by PPO which consume oxygen and produce fungitoxic quinones on plant tissues that will delay the colonization of pathogens (Lattanzio *et al*., 2006). PPO is

associated with the production of monolignol radicals which are subsequently cross-linked to generate lignin subunits through oxidative coupling during pathogens infection (Huttermann *et al*., 2001; Mohammadi and Kazemi, 2002; Parveen *et al*., 2010). The high PPO activity on leaves of plants supplied with glutamate and infected by *P. oryzae* was also important in the scenario of gluatamate potentiated rice resistance to blast.

The CHI and GLU are often linked with the resistance of plants to either abiotic and biotic stresses and their importance against fungal pathogens is related to their capacity to hydrolyse chitin and *β*-1,3-glucan, respectively (Wu and Bradford 2003; Lattanzio *et al*., 2006; Gnanamangai *et al*., 2011). Moreover, the release of elicitors (*e.g.*, glucan and chitin) upon the degradation of fungal cell walls by CHI and GLU amplify other mechanisms of host defense (Wu and Bradford 2003). The high CHI and GLU activities at early and advanced stages of *P. oryzae* infection, respectively, on leave of plants supplied with glutamate also explain the increase of rice resistance to blast potentiated by this amino acid. High CHI and GLU activities were important for the resistance of rice against foliar pathogens (Nascimento *et al*., 2016; Tatagiba *et al*., 2016).

In conclusion, the results of the present study indicated that the colonization of rice leaf tissues by *P. oryzae* was reduced on gluatamate supplied plants due to an increase on the activities of defense enzymes and high concentrations of phenolics and lignin.

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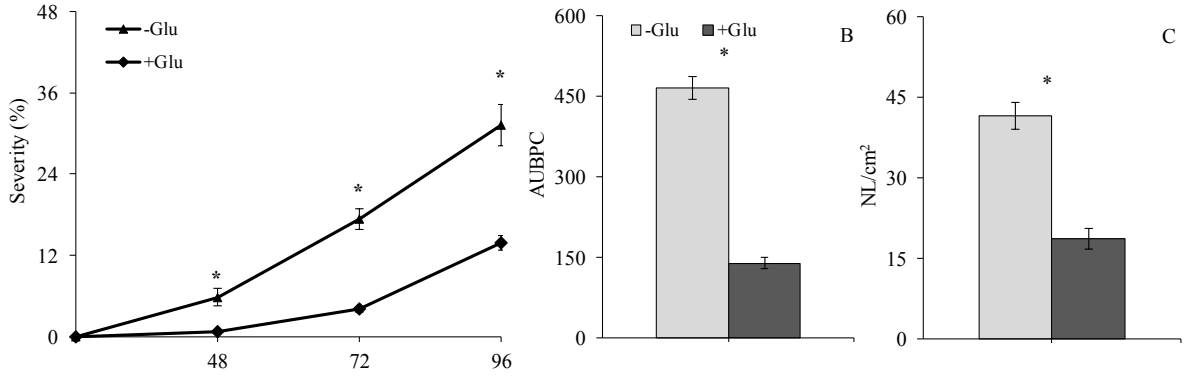

Figure 1. Blast severity (A), area under blast progress curve (AUBPC) (B), and the number of lesions per cm² of leaf (NL/cm²) (C) evaluated on the leaves of rice plants non-supplied (-Glu) or supplied (+Glu) with glutamate (Glu). Means from -Glu and +Glu treatments followed by an asterisk (*) are significantly different based on F test ($P \leq 0.05$). Bars represent the standard deviation of the means. $n = 5$.

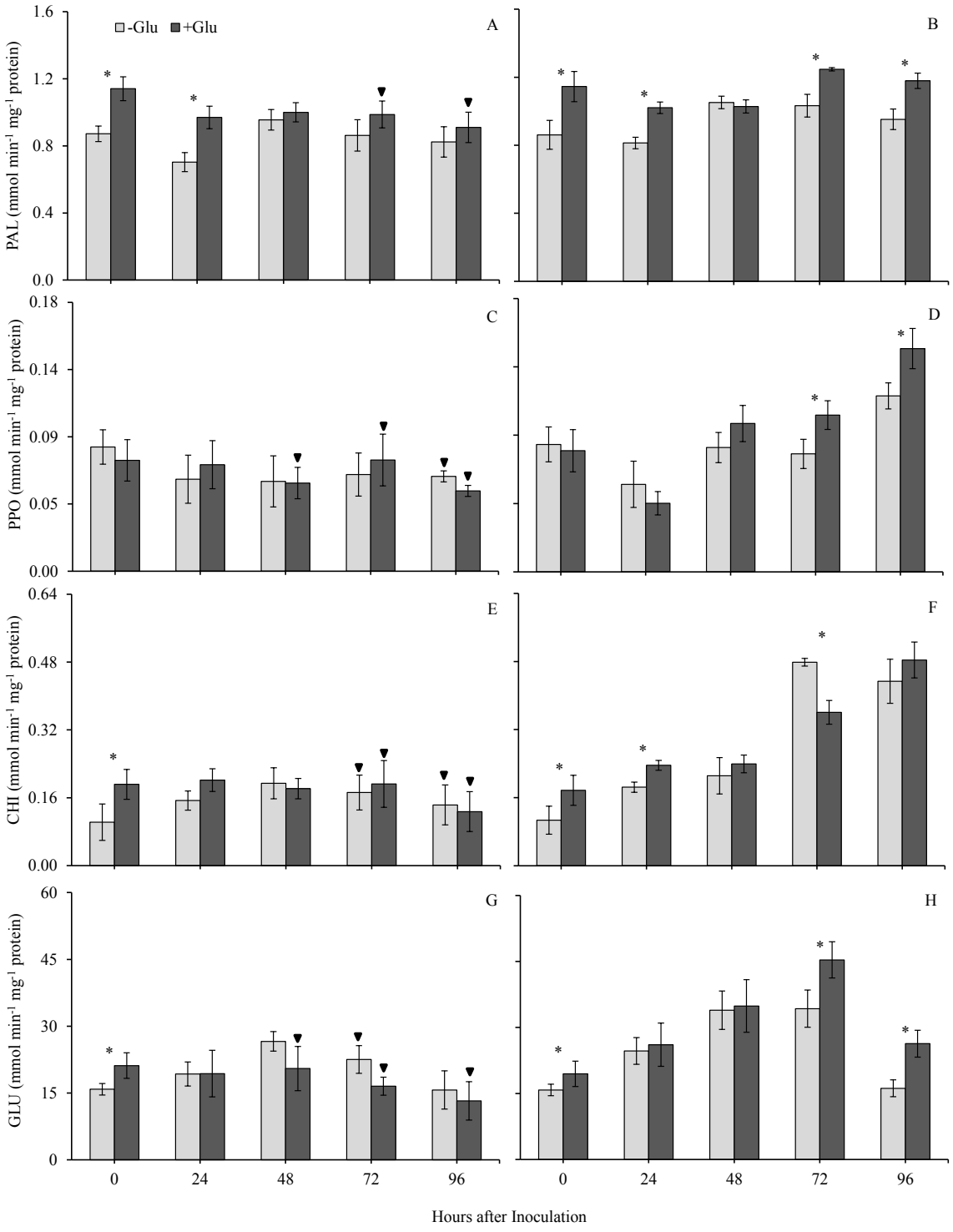
-Glu
+Glu
A
B
C
D
E
F
G
H
PAL (mmol min-1 mg-1 protein)
PPO (mmol min-1 mg-1 protein)
CHI (mmol min-1 mg-1 protein)
GLU (mmol min-1 mg-1 protein)
0.0
0.4
0.8
1.2
1.6
0.00
0.05
0.09
0.14
0.18
0.00
0.16
0.32
0.48
0.64
0
15
30
45
60
0
24
48
72
96
Hours after Inoculation

Figure 2. Activities of phenylalanine ammonia-lyase (PAL) (A and B), polyphenoloxidase (PPO) (C and D), chitinase (CHI) (E and F), and *β*-1-3-glucanase (GLU) (G and H) in the leaves of rice plants non-supplied (-Glu) or supplied (+Glu) with glutamate (Glu) and non-inoculated (A, C, E, and G) or inoculated (B, D, F, and H) with *Pyricularia oryzae*. Means from -Glu and +Glu treatments, at each evaluation time, followed by an asterisk (*) and from non-inoculated and inoculated treatments followed by an inverted triangle (▼), at each evaluation time, are significantly different based on F test ($P \leq 0.05$). Bars represent the standard deviation of the means. $n = 5$.

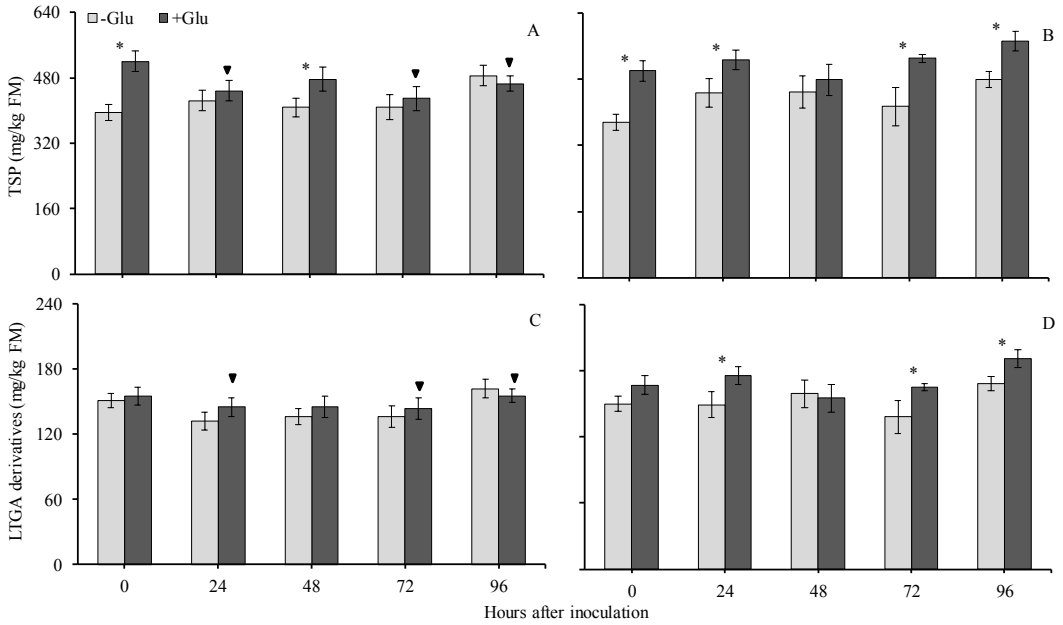

Figure 3. Concentrations of total soluble phenolics (TSP) (A and B) and lignin-thioglycolic acid (LTGA) derivatives (C and D) in the leaves of rice plants non-supplied (-Glu) or supplied (+Glu) with glutamate (Glu) and non-inoculated (A and C) or inoculated (B and D) with *Pyricularia oryzae*. Means from -Glu and +Glu treatments, at each evaluation time, followed by an asterisk (*) and from non-inoculated and inoculated treatments followed by an inverted triangle (▼), at each evaluation time, are significantly different based on F test ($P \leq 0.05$). Bars represent the standard deviation of the means. FM = fresh matter. $n = 5$.

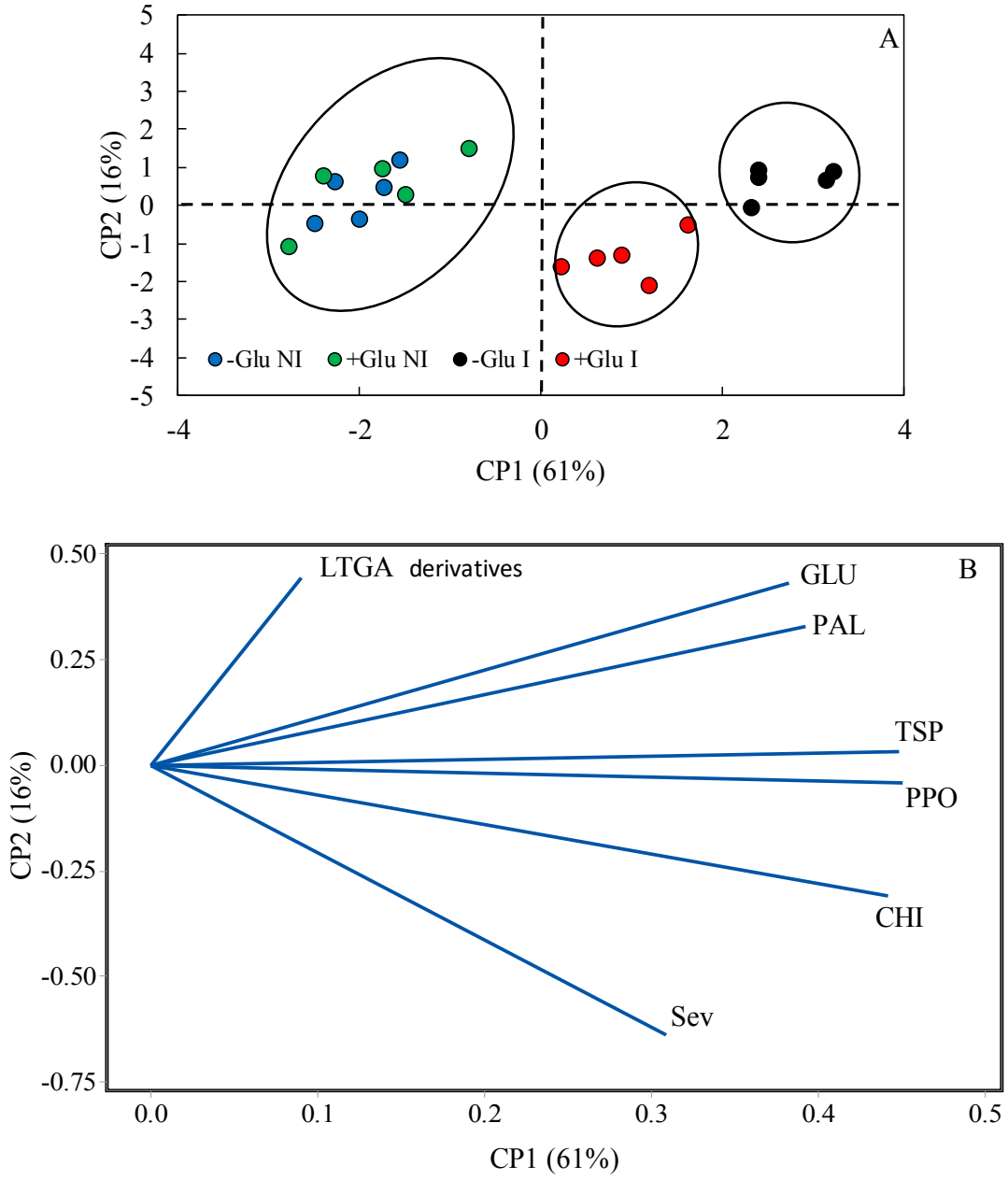

Figure 4. Score plot (A) and loading values (B) of principal components analysis comparing blast severity (Sev), activities of *β*-1-3-glucanase (GLU), chitinase (CHI), phenylalanine ammonia-lyase (PAL), and polyphenoloxidase (PPO) as well as concentrations of total soluble phenolics (TSP) and lignin-thioglycolic acid (LTGA) derivatives determined in the leaves of rice plants non-supplied or supplied with 10 mM of glutamate (-Glu and +Glu plants, respectively) and non-inoculated (NI) or inoculated (I) with *Pyricularia oryzae* (data from 96 hours after inoculation).