CARLOS EDUARDO AUCIQUE PÉREZ

WHEAT RESISTANCE TO BLAST USING A NON-HOST SELECTIVE TOXIN AND HOST METABOLIC REPROGRAMMING THROUGH A SUCCESSFUL INFECTION BY *Pyricularia oryzae*

Thesis submitted to Federal University of Viçosa, as part of the requirements for obtaining of *Doctor Scientiae* degree in Plant Physiology.

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APPROVED: August 25 2016.

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(Advisor)

The victories are built with effort and dedication. Victories need time. They feed victories of love and tears. The victories are enjoyed with your loved ones and victories never forget.

To my parents Dilza Maria and Luis Eduardo. I dedicate with love.

To my eternal love and traveling companion. Sarita, I dedicate fondly

To my family and friends who supported me

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BIOGRAPHY

CARLOS EDUARDO AUCIQUE-PÉREZ was born in Bogotá D.C-, Colombia, on December 22th, 1982. In 2007, he concluded his graduation in Agronomic Engineering at the National University of Colombia, Bogotá D.C. Between 2007 to 2011; he was a research assistant for biology program at the Center of Research in Oil Palm (CENIPALMA). On March 2013, he obtained his *Master Scientiae* degree in Plant Physiology at "Universidade Federal de Viçosa" (UFV), Viçosa, Minas Gerais State, Brazil, under the supervision of Prof. Fabrício Á. Rodrigues. On April 2013, he begins his doctoral studies in the Plant Physiology Program at UFV under supervision of Prof. Fabrício Á. Rodrigues.

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ABSTRACT

AUCIQUEPÉREZ, Carlos Eduardo. D.Sc., Universidade Federal de Viçosa, August, 2016. **Wheat resistance to blast using a non-host selective toxin and host metabolic reprogramming through a successful infection by *Pyricularia oryzae*.** Adviser: Fabrício Ávila Rodrigues.

Blast, caused by *Pyricularia oryzae*, has become an economically important disease in wheat in South America. One of the management strategies for minimizing the losses caused by blast includes the use of resistant cultivars. Alternatively, the use of inducers of resistance showed the potentiation to increase wheat resistance to blast. This study aimed: *i*) to determine the physiological and biochemical alterations in wheat plants sprayed with a non-phytotoxic concentration of α-picolinic acid (PA), which is a non-host selective toxin produced by *P. oryzae* and *ii*) to establish the degree of metabolic manipulation exerted during the infection by *P. oryzae* on plants from cultivars with different levels of basal resistance to blast. The spray of leaves of plants with a non-phytotoxic concentration of PA (0.1 mg mL^{-1}) resulted in less blast symptoms in association with a better photosynthetic performance, an improvement on the antioxidant metabolism and reduced concentrations of H_2O_2, $O_2^{\bullet-}$ and malondialdehyde. The cultivars BR-18 and EMBRAPA-16 were more resistant to blast in comparison to cultivar BRS-Guamirim. The photosynthetic performance of the infected plants was altered due to diffusional and biochemical limitations for CO_2 fixation. During the asymptomatic phase of *P. oryzae* infection, drastic changes in the carbohydrates metabolism and on the levels of amino acids, intermediates compounds of Krebs cycle and polyamines occurred on plants from the three cultivars suggesting a metabolic manipulation exerted by the pathogen. However, amore efficient antioxidant metabolism was able to help the wheat plants to counteract against the deleterious effects of *P. oryzae* infection in association with great phenylalanine ammonia lyases and polyphenoloxidases activities and high concentrations of phenolics and lignin. Based on this information, it is possible to conclude that a non-phytotoxic concentration of PA elicited the activation of host defense mechanisms that reduced blast severity. Likewise, the infection of leaves by *P. oryzae* induced remarkable disturbances in the primary metabolism and some of them were conserved among the cultivars regardless of their basal level of resistance to blast.

RESUMO

AUCIQUE PÉREZ, Carlos Eduardo. D.Sc., Universidade Federal de Viçosa, agosto de 2016. **Resistencia do trigo à brusone usando uma toxina não selectiva e o sucesso da infecção por *Pyricularia oryzae* através da reprogramação metabólica do hospedeiro.** Orientador: Fabricio Ávila Rodrigues.

A brusone, causada por *Pyricularia oryzae*, é considerada uma doença economicamente importante para trigo na América do Sul. Uma das estratégias de manejo para minimizar as perdas causadas por essa doença é o uso de cultivares resistentes. Alternativamente, pode-se utilizar indutores de resistência para aumentar o nível basal de resistência do trigo à brusone. O presente estudo teve como objetivos: *i*) determinar as alterações fisiológicas e bioquímicas em plantas de trigo pulverizadas com uma concentração não fitotóxica do ácido α-picolinico (AP), o qual é uma toxina não seletiva produzida por *P. oryzae* e *ii*) verificar a manipulação metabólica exercido por *P. oryzae* quando infectando cultivares de trigo com diferentes níveis de resistência basal à brusone. Nas folhas de trigo pulverizadas com uma concentração não fitotóxica de AP (0.1 mg mL^{-1}), os sintomas da brusone desenvolveram menos em associação com um melhor desempenho fotossintético, melhoria do metabolismo antioxidante e redução nas concentrações de H_2O_2, $O_2^{\bullet -}$ e MDA. As cultivares BR-18 e EMBRAPA-16 foram mais resistentes à brusone do que a cultivar BRS-Guamirim. O desempenho fotossintético das plantas infectadas foi alterado devido a limitações de natureza difusiva e bioquímica para uma eficiente fixação do CO_2. Durante a fase assintomática da infecção por *P. oryzae*, mudanças drásticas no metabolismo de carboidratos e nos níveis de aminoácidos, compostos intermediários do ciclo de Krebs e poliaminas ocorreram nas plantas das três cultivares sugerindo, assim, uma manipulação metabólica exercida por *P. oryzae*. No entanto, um metabolismo antioxidativo mais eficiente foi importante para neutralizar os efeitos deletérios da infecção por *P. oryzae* em associação com maiores atividades da fenilalanina amônia liase e polifenoloxidase e maiores concentrações de compostos fenólicos e lignina. Com base nesses resultados e possível concluir que a concentração não fitotóxica de AP foi capaz de potencializar a defesa das plantas de trigo e reduzir a severidade da brusone. A infecção do trigo por *P. oryzae* ocasionou distúrbios no metabolismo primário das plantas e alguns deles foram semelhantes entre as cultivares independentemente do nível basal de resistência delas.

GENERAL INTRODUCTION

Blast, caused by the hemibiotrophic fungus *Pyricularia oryzae*, has become an economically important disease in wheat (*Triticum aestivum* L.). In Brazil, this disease was first reported on wheat in 1986 in the Northern of Paraná State and since then has negatively affected wheat production in Brazil and in other South American countries such as Bolivia, Argentina and Paraguay (Igarashi et al., 1986; Goulart and Paiva, 1992). In 2016, blast was reported in eight districts of Bangladesh and affected about 15% of the total area used to grow wheat and yield losses reached around 90% due to the use of susceptible cultivars (Malaker et al., 2016). Wheat blast is considered a major quarantine disease and a threat to wheat crops in the United States (Kohli et al., 2011).

The dramatic reductions in grain production are due to fungal infection on spikes and spikelets leading to the reduction on the translocation of nutrients to the grains (Goulart et al., 2007). Gray-green and water-soaked lesions with dark green borders are the symptoms occurring on the leaves (Igarashi et al., 1986). Seedling blight, spike tip death and bright black spots on the rachis are other common symptoms caused by *P. oryzae* (Goulart et al., 2007).

Currently, wheat blast management is based on the use of cultivars with a desirable level of resistance in association with fungicide application (Cruz et al., 2010; Castroagudín et al., 2015; Rios et al., 2016). However, the use of potassium phosphate and the supply of silicon contributed to decrease blast severity (Aucique-Pérez et al., 2014; Cruz et al., 2015). Unfortunately, alterations in the genetic structure of populations of *P. oryzae*, in conjunction with the occurrence of environmental conditions favorable for blast epidemics in most of the wheat growing regions, put the

release of cultivars with high level of partial resistance at increased risk for becoming more susceptible to the disease (Castroagudín et al., 2016; Cruz et al., 2016).

In general, the primary metabolism of plants infected by pathogens greatly increased in order to provide the necessary metabolites to be use in the pathways related to host defense mechanisms (Berger et al., 2007; Torres et al., 2015). However, pathogens through different strategies (*e.g.* effectors, enzymes, non-host selective toxins and others compounds) can manipulate their host's metabolism to acquire the necessary nutrients to infect the tissues successfully (Major et al., 2010). The non-host selective toxins are secondary products originated from the primary metabolism of necrotrophic and hemibiotrophic fungal pathogens and are very important for their aggressiveness towards their hosts. On plants, the non-selective toxins cause the suppression of cell division, physiological disorders associated with changes on the primary metabolism as well as biochemical alterations especially related to the production of reactive oxygen species (ROS), loss of cellular homeostasis and damage of the cell membranes that will ultimately result in cell death (Berestetskiy, 2008; Dong et al., 2012). *P. oryzae* infecting rice leaves produced non-host selective toxins as pyricularin, pyriculol, epipyriculol, tenuazonic acid and picolinic acid (Tamari and Kali, 1954; Iwasaky et al., 1969; Tsurushima et al., 2010). Several studies demostred the importance of tenuazonic acid and picolinic acid in the development of the characteristic symptoms of blast (Umetsu et al., 1972; Iwahashi et al., 1999; Yoder, 1980). However, the non-host selective toxins could be of some value aiming to disease control. The pre-spray of non-host selective toxins at non-toxic concentrations (*e.g.* tenuazonic acid and picolinic acid) on rice leaves, stimulate the production of reative oxygen species (ROS) inhibited *P. oryzae* hyphae growth on the leaf tissues (Pasechnik et al., 1993; Zhang et al., 2004; Aver'yanov et al., 2007), demonstrated

great potential to activate some host defense response against pathogens attack. Zhang et al. (2004), suggested that NADPH oxidase was involved in the oxidative burst and high accumulation of transcripts of *PAL, PIR2* and *RCH10* genes of rice plants sprayed with picolinic acid that exhibited increased resistance to blast.

On the other hand, *P. oryzae* infection collapses the gas exchange of leaves, explained for the lower influx of CO_2, stomatal closure and consequent reduction in net carbon assimilation rate (*A*). Additionally, the maximum rate of carboxylation and the maximum rate of electron transport used in RuBP regeneration, as well as disturbances in the photochemical phase are drastically affected by the advance of lesions caused by *P. oryzae*. Therefore, strong diffusional limitations and biochemical character related to CO_2 fixation are the factors responsible for the reduction of the photosynthetic process product damage caused by *P. oryzae* (Debona et al., 2014; Aucique-Pérez et al., 2014). This research showed partial resistance to blast related to minor damage to the photosynthetic process. On the other hand, wheat resistance to blast was intrinsically associated with an increase in the production of ROS that favored host defense mechanisms against *P. oryzae* infection (Debona et al., 2012). However, although there is no evidence showing the metabolic alterations caused by infection of *P. oryzae* in response to the partial blast resistance.

Considering the current need to find alternatives to wheat blast control, this study aimed to investigate the potential of using a non-toxic concentration of a non-host selective toxin produced by *P. oryzae* as an activator of defense mechanisms. On the other hand, using metabolomics, biochemical and physiological analysis determine the level of abundance of metabolites differentially produced in a certain wheat-*P. oryzae* interaction, specially in cultivars with different levels of basal resistance to blast.

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Chapter 1

Physiological and Biochemical Alterations on Wheat Leaves Pre-Treated with Picolinic Acid and Infected with *Pyricularia oryzae*

Abstract

The α-picolinic acid (PA), a non-host selective toxin produced by *Pyricularia oryzae*, plays an important role on the development of blast symptoms on wheat leaves. In this study, it was investigated the effect of PA at different concentrations on mycelial growth of *P. oryzae*, conidia production and their germination *in vitro* as well as on the photosynthetic performance of treated wheat plants. Mycelia growth, conidia production and their germination significantly reduced at PA concentrations higher than 0.1 mg mL^{-1}. There were drastic reductions on gas exchange parameters including net CO_2 assimilation rate, stomatal conductance and transpiration rate, as well as on chlorophyll *a* fluorescence parameters sunch as maximum PSII quantum efficiency, quantum yield of regulated energy dissipation and photochemical quenching coeficient. Besides high hydrogen peroxide (H_2O_2) and superoxide anion radical ($O_2^{\bullet-}$) concentrations on the leaves of wheat plants sprayed with PA at concentrations greater than 0.3 mg mL^{-1}. The spray of leaves with a non-phytotoxicconcentration of 0.1 mg PA mL^{-1} resulted in less blast symptoms in association with a better photosynthetic performance, an improvement on the antioxidant metabolism and reduced production of H_2O_2 and $O_2^{\bullet-}$ as well as lower concentration of malondialdehyde reducing cell damage as well as a lower impact on the photosynthetic capacity. Based on these findings, concluded that non-phytotoxicconcentrations of PA contributes significantly to the reduction of blast severity, through the early stimulation of the antioxidant system to prevent damage associated with the production of ROS during infection of *P. oryzae*.

Key words: elicitor, foliar disease, host defense responses, photosynthesis, non-host selective toxin, wheat blast

Introduction

Blast, caused by the hemibiotrophic fungus *Pyricularia oryzae* Cavara (teleomorph *Magnaporthe grisea* (T. T. Hebert) M. E. Barr) (Igarashi et al., 1986; Goulart and Paiva, 1992), has caused significant yield losses in wheat (*Triticum aestivum* L.) cultivated in Bangladesh as well as in Argentina, Bolivia, Brazil and Paraguay (Goulart et al., 1992; Malaker et al. 2016). Upon infection by *P. oryzae*, gray-green and water-soaked lesions with dark green borders that become light tan in color with necrotic borders appear on leaves and gray-brown lesions are noticed on both spikes and spikelets and bright black spots are found on the rachis (Reis and Casa, 2005; Cruz et al., 2015; Kohli et al., 2011; Igarashi et al., 1986; Goulart et al., 2007; Debona et al., 2012). Yield losses became of great concern when gray-brown lesions are found on rachis, spikes and spikelets because of the reduction on the translocation of nutrients to the grains and may explain the great production losses attributed to this disease due to the underweight grains obtained (Goulart et al., 2007). Treatment of seeds with fungicides, foliar application of systemic fungicides, potassium phosphate, the supply of silicon to plants either by soil of foliar spray and the use of resistant cultivars have been the main strategies for blast control (Castroagudín et al. 2015; Cruz et al. 2010, 2011, 2015; Maciel et al., 2014; Pagani et al., 2014; Urashima et al., 2004).

The infection process of pathogens is favored by the intensive production of both hydrolytic enzymes and non-host selective toxins at the infection sites (Willis et al., 1991; Howlett, 2006). The non-host selective toxins are secondary products originated from the primary metabolism of necrotrophic and hemibiotrophic fungal pathogens and are very important for their aggressiveness towards their hosts. On plants, the non-selective toxins cause the suppression of cell division, physiological disorders associated with changes on the primary metabolism as well as biochemical alterations

especially related to the production of reactive oxygen species (ROS), loss of cellular homeostasis and damage of the cell membranes that will ultimately result in cell death (Berestetskiy, 2008; Dong et al., 2012). The non-host selective toxins pyricularin, pyriculol, epipyriculol, tenuazonic acid and picolinic acid were produced by *P. oryzae* infecting rice leaves (Tamari and Kali, 1954; Iwasaky et al., 1969; Tsurushima et al., 2010), and both tenuazonic acid and picolinic acid were of importance for the development of the characteristic symptoms of blast (Umetsu et al., 1972; Iwahashi et al., 1999; Yoder, 1980). The non-host selective toxins can be of some value aiming to disease control. The spray of non-host selective toxins at non-phytotoxic concentrations showed great potential to activate some host defense response against pathogens attack. Rice leaves sprayed with both tenuazonic acid and picolinic acid produced ROS that inhibited *P. oryzae* hyphae growth on the leaf tissues (Pasechnik et al., 1993; Zhang et al., 2004; Aver'yanov et al., 2007). The mechanism of rice resistance against *P. oryzae* infection when plants were either sprayed with picolinic acid or with a suspension culture of this fungus was associated with an increase on NADPH oxidase activity, a rapid increase in the concentration of hydrogen peroxide and the accumulation of transcripts of defense-related genes (Zhang et al., 2004).

Considering the potential of using non-host selective toxins as efficient elicitors to activate host defense mechanisms and the lack of information about their effect on the wheat-*P. oryzae* interaction, the present study was carried out to investigate the potential of picolinic acid, at non-phytotoxicconcentration, to increase wheat resistance to blast by performing an analysis of the photosynthetic performance and antioxidant metabolism of the plants.

Material and Methods

Effect *in vitro* of the α-picolinic acid (PA) on mycelial growth, conidia production and conidia germination

The sensitivity of *P. oryzae* to PA was evaluated *in vitro* using different concentrations of PA as follows: 0, 0.1, 0.3, 0.5, 1.0, 1.5, 3.0 and 5.0 mg mL^{-1} on both potato-dextrose-agar (PDA) and oat-agar (OA) medium. The PA, at the eight concentrations, incorporated into the PDA or OA medium and then poured into Petri dishes (20 mL per plate). After 4 h, one PDA plug (5 mm in diameter), containing fungal mycelia obtained from the edge of a ten-days old *P. oryzae* colony, was placed in the central region of each Petri dish, which were kept in a growth chamber (25°C and 12 h photoperiod). At 24 h, fungal colony in each Petri dish measured in two orthogonal directions using a digital paquimeter in order to obtain its diameter. At 5 days, conidia production in each Petri dish containing only OA medium with the concentrations of 0, 0.1 and 0.3 mg PA mL^{-1} was determined. A total of 20 ml of sterile distilled water was added to each Petri dish and conidia were carefully disrupted using a camel-hair brush in a laminar flow chamber. The concentration of conidia was quantified in 500 µL of suspension per each treatment using a hemacytometer.

In order to determine the effect of PA on conidia germination, conidia obtained from ten-days old *P. oryzae* colony in Petri dishes containing OA medium were removed using a camel-hair brush to obtain a conidial suspension at the concentration of 1×10^5 conidia mL^{-1}. 10 µL of conidial suspension was transferred to five Petri dishes containing OA with the concentrations of 0, 0.1 and 0.3 mg PA mL^{-1} and was homogeneously distributed on each dish using a Drigalski glass stick. Petri dishes were transferred to a growth chamber at 25°C. After 4 h, lactophenol was added to the plates to stop conidia germination. Two hundred conidia were randomly examined from each

Petri dish under a microscope (Carl Zeiss AxioImager A1) at 40×Bmagnification. A conidium was considered germinated when the germ tube was longer than its diameter. The percentage of germination was calculated.

Plant growth

Wheat seeds (cultivar BRS-Guamirim; susceptible to blast) (Cruz et al., 2010) were surface-sterilized in 10% (v v^{-1}) NaOCl for 2 min, rinsed in sterilized water for 3 min and germinated on plastic pots (20-cm diameter) filled with 1 kg of substrate made from a 1:1:1 mixture of pine bark, peat and expanded vermiculite (Tropstrato, Vida Verde, Mogi Mirim, SP, Brazil). A total of 1.63 g of calcium phosphate monobasic was added to each plastic pot. A total of nine seeds were sown per pot and at eight days after seedlings emergence, each pot was thinned to six seedlings. Substrate in each pot was fertilized with a nutrient solution containing, in g L^{-1}, 6.4 KCl, 3.48 K_2SO_4, 5.01 $MgSO_4 \cdot 7H_2O$, 2.03 $(NH_2)_2CO$, 0.009 $NH_4MO_7O_{24} \cdot 4H_2O$, 0.054 H_3BO_3, 0.222 $ZnSO_4 \cdot 7H_2O$, 0.058 $CuSO_4 \cdot 5H_2O$ and 0.137 $MnCl_2 \cdot 4H_2O$ (Xavier-Filha et al., 2011). A volume of 15 mL of nutrient solution containing 0.27 g of $FeSO_4 \cdot 7H_2O$ and 0.37 g of EDTA bisodic L^{-1} was also applied after seedlings emergence. The nutrient solution was prepared using deionized water and applied weekly. Plants were watered with deionized water as needed.

Effect of PA spray on the photosynthetic performance of wheat plants

Plants (35 days after emergence; growth stage 39) (Lancashire et al., 1991) were sprayed with PA solutions at the concentrations of 0, 0.1, 0.3, 0.5, 1.0, 1.5, 3.0 and 5.0 mg mL^{-1}. The leaf gas exchange parameters net CO_2 assimilation rate (A), stomatal conductance to water vapor (g_s), internal CO_2 concentration (C_i) and transpiration rate (E) were measured by using a portable open-flow gas exchange system (LI-6400XT; Li-Cor Inc., Lincoln, NE) at the attached fourth leaf, from the top to the base, of each

plant per replication of each treatment from 0900 to 1100 h (solar time) at 24 and 48 hours after spray the eight PA solutions. At those times, *A* was at its maximum under artificial photosynthetically active radiation (i.e., 1000 µmol photons m^{-2} s^{-1} at the leaf level and 400 µmol atmospheric CO_2 mol^{-1}). All measurements were performed at 25°C and the vapor pressure deficit was maintained at approximately 1.0 kPa and the amount of blue light was set to 10% of the photosynthetic photon flux density to optimize the stomatal.

The imaging of the chlorophyll (Chl) *a* fluorescence parameters were determined by using the Imaging-PAM MAXI chlorophyll fluorometer and the software ImagingWIN (version 2.32) (Heinz Walz GmbH, Effeltrich, Germany). The sensor system consisted of 44 LED's lamp high power (450 nm) required to apply fluorescence excitation, actinic illumination and saturation pulses. These LEDs were arranged in pairs, with each pair featuring a red (660 nm) and a near-infrared (780 nm) LED. A CCD camera with 640 × 480 resolution pixels was located above the plants canopies at a distance of 13.5 cm to give an image area of 10 × 13 cm. Plants from the different experiments were adapted to darkness for 45 min (Baker, 2008) and the minimum fluorescence image (F_0) was obtained from the fourth leaf, from the top to the base, of each plant per replication of treatment after being exposed to a light pulse intensity of 0.5 µmol m^{-2} s^{-1} (1Hz) at 24 and 48 hours after spray the eight PA solutions. Next, a saturating pulse of blue light (470 nm) of 2400 µmol m^{-2} s^{-1} intensity (10 Hz) was delivered at 0.8 s in order to obtain the maximum fluorescence image (F_m). The software performed the calculation and image of the fluorescent quantum efficiency ($F_v/F_m = (F_m-F_0)/F_m$) (Baker, 2008). Leaves were subsequently exposed to actinic photon irradiance (110 µmol m^{-2} s^{-1}) for 300 s to obtain the steady-state fluorescence yield (F_s), after which a saturating white light pulse (2400 µmol m^{-2} s^{-1}; 0.8 s) was

applied to achieve the light-adapted maximum fluorescence (F_m'). The light-adapted initial fluorescence (F_0') was estimated according to Oxborough and Baker (1997). Following the calculations of Kramer et al. (2004), the energy absorbed by PSII for the following three yield components for dissipative processes was determined: the effective PSII quantum yield [Y(II) = (F_m'-F_s)/F_m')], the quantum yield of regulated energy dissipation [Y(NPQ) = (F_s/F_m') - (F_s/F_m)] and the quantum yield of non-regulated energy dissipation [Y(NO) = F_s/F_m]. For semi-quantitative analyses of Chl *a* fluorescence parameters, a total of five areas of interest (1 cm^2 each) were randomly selected in each leaf of plant per replication of each treatment at 24 and 48 hours after spray the eight PA solutions.

Histochemical localization of hydrogen peroxide (H_2O_2) and superoxide anion radical ($O_2^{\bullet-}$) on wheat leaves sprayed with PA

In order to examine the presence of H_2O_2, five fragments of the fourth and fifth leaves (≈ 10 cm of length), from the top to the base, of each plant per replication of each treatment were collected at 24 hours after spray the PA solutions, placed in glass vials containing a solution of 3,3´-diaminobenzidine tetrahydrochloride (1 mg mL^{-1}) (Sigma-Aldrich, São Paulo, Brazil) and kept in the dark at 25°C for 12 h. After this period, the leaf fragments were cleared in boiling ethanol for 60 min and then stored in glycerol solution (70%). For detection of the presence of $O_2^{\bullet-}$, five fragments obtained from of the fourth and fifth leaves (≈ 10 cm of length), from the top to the base, of each plant per replication of each treatment were collected at 24 hours after spray the eight PA solutions, placed in glass vials and infiltrated with a solution of 0.1% nitroblue tetrazolium (Sigma-Aldrich, São Paulo, Brazil) in 10 mM potassium phosphate buffer (pH 6.8) for 40 min. Thereafter, leaf fragments were cleared in boiling 95% ethanol for 60 min and then stored in glycerol solution (70%).

Inoculum production, PA application and plant inoculation with *P. oryzae*

A pathogenic isolate of *P. oryzae* (UFV/DFP *Po*-01), obtained from the spikes of wheat plants (cultivar BR-18), was used to inoculate the plants (Debona et al., 2012). Disks of filter paper containing fungal mycelia were transferred to Petri dishes containing oat-agar medium. After growing the disks containing mycelia, the media with the fungus were transferred to new Petri dishes containing the same medium. The dishes were incubated in a growth chamber at 25°C with a 24 h photoperiod for 10 days. After this period, conidia were carefully removed from the Petri dishes with a soft bristle brush using water containing gelatin (1% w v^{-1}). The conidial suspension was calibrated with a hemacytometer to obtain a concentration of 1×10^5 conidia mL^{-1}. At 24 h after plant inoculation, they were sprayed with a solution of PA at the concentration of 0.1 mg mL^{-1} (35 mL per plant). This concentration was used based on data obtained from a preliminary experiment aimed to determine the effect of the different PA concentrations on the photosynthetic performance of the plants. The conidial suspension was sprayed with an atomizer (Paasche Airbrush Co., Chicago, IL, USA) on the adaxial surface of the leaves of wheat plants at growth stage 39. After inoculation, plants were kept in a mist chamber under darkness at 25°C during 24 h. After this period, plants were transferred to a greenhouse with relative humidity of 80 ± 5% and temperature of 25 ± 3°C and natural photosynthetically active radiation (PAR) of 900 µmol photons m^{-2} s^{-1}that was measured at midday.

Assessment of blast severity

The fourth and fifth leaves, from the top to the base, of each plant per replication of each treatment were marked and used to evaluate blast severity at 48, 72 and 96 hours after inoculation (hai) using the scale proposed by Rios et al. (2013).

Photosynthesis measurements and histochemical localization of H_2O_2 and $O_2^{\bullet-}$

The leaf gas exchange and the Chl *a* fluorescence parameters were measured on the attached fourth leaf, from the top to the base, of each plant per replication of each treatment as described above at 48, 72 and 96 hai. The histochemical localization of H_2O_2 and $O_2^{\bullet-}$ was performed at 24, 48 and 72 hai as previously described.

Determination of enzymes activities

The fourth and fifth leaves, from the base to the top, of each plant per replication of each treatment were collected at 48, 72 and 96 hai. Leaf samples were kept in liquid nitrogen during sampling and then stored at -80ºC until further analysis. To determine the activities of superoxide dismutases (SOD, EC 1.15.1.1), catalases (CAT, EC 1.11.1.6), peroxidases (POX, EC 1.11.1.7), glutathione-*S*-transferases (GST, EC 2.5.1.18), ascorbate peroxidases (APX, EC 1.11.1.11) and glutathione peroxidases (GPX, EC 1.11.1.9), a total of 0.3 g of leaf tissue was ground into a fine powder in a mortar and pestle with liquid nitrogen. The fine powder was homogenized in an ice bath in 2 mL of a solution containing 100 mM potassium phosphate buffer (pH 6.8), 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2% (wt vol^{-1}) polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 12000 × *g* for 15 min at 4°C and the supernatant was used as a crude enzyme extract. To determine the glutathione reductases (GR, EC 1.8.1.7) activity, a total of 0.3 g of leaf tissue was ground as described above. The fine powder was homogenized in an ice bath in 2 mL of a solution containing 100 mM potassium phosphate buffer (pH 7.5), 0.1 mM EDTA, 1 mM DL-dithiothreitol, 1 mM PMSF and 2% (wt vol^{-1}) PVPP. The homogenate was centrifuged as described above.

The SOD activity was determined by measuring its ability to photochemically reduce the *p*-nitrotetrazole blue (NTB) (Del Longo et al., 1993). The reaction was

started after the addition of 60 µL of the crude enzyme extract to 1.94 mL of a mixture containing 100 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NTB, 0.1 mM EDTA and 2 µM riboflavin. The reaction occurred at 25°C under a 15W lamp light. After 10 min of light exposure, the light was interrupted and the production of formazan blue, which resulted from the photoreduction of NTB, was monitored by the increase in absorbance at 560 nm in spectrophotometer (Giannopolitis and Ries, 1977). The reaction mixture for the control samples was kept in darkness for 10 min and the absorbance measured at 560 nm. The values obtained were subtracted from the values obtained from the samples of the replications of each treatment exposed to light. One unit of SOD was defined as the amount of enzyme necessary to inhibit NBT photoreduction by 50% (Beauchamp and Fridovich, 1971).

The CAT activity was determined following the method of Cakmak and Marschner (1992). The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 6.8) and 20 mM H_2O_2 in a volume of 1.95 mL. The reaction was initiated after the addition of 50 µL of the crude enzyme extract and the CAT activity was determined by the rate of H_2O_2 decomposition at 240 nm for 1 min at 25°C. An extinction coefficient of 36 M^{-1} cm^{-1} (Anderson et al., 1995) was used to calculate CAT activity, which was expressed as µmol min^{-1} mg^{-1} of protein.

The POX activity was assayed following the colorimetric determination of pyrogallol oxidation according to Kar and Mishra (1976). The reaction mixture contained 50 mM potassium phosphate (pH 6.8), 20 mM pyrogallol and 20 mM H_2O_2 in a volume of 1.98 mL. The reaction was started after the addition of 15 µL of the crude enzyme extract and the POX activity was determined through the absorbance of colored purpurogallin recorded at 420 nm for 1 min at 25°C. An extinction coefficient

of 2.47 mM^{-1} cm^{-1} (Chance and Maehley, 1995) was used to calculate POX activity, which was expressed as mmol of purpurogallin produced min^{-1} mg^{-1} of protein.

The GST activity was determined using the methodology proposed by Habig et al. (1974). A total of 150 µL of the crude enzyme extract was added to 1.35 mL of the 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. An extinction coefficient of 9.6 mM^{-1} cm^{-1} (Habig et al., 1974) was used to determine GST activity, which was expressed as µmol min^{-1} mg^{-1} of protein.

The APX activity was determined according to method of Nakano and Asada (1981). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 6.8), 1 mM H_2O_2 and 0.8 mM ascorbate in a volume of 1.95 mL. The reaction was started after the addition of 50 µL of the crude enzyme extract. The APX activity was measured by the rate of ascorbate oxidation at 290 nm for 1 min at 25°C. An extinction coefficient of 2.8 mM^{-1} cm^{-1} (Nakano and Asada, 1981) was used to calculate APX activity, which was expressed as µmol min^{-1} mg^{-1} of protein.

To determine the 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) according to Carlberg and Mannervik (1985). The decrease in absorbance was determined at 340 nm for 1 min at 30°C. An extinction coefficient of 6.22 mM^{-1} cm^{-1} (Foyer and Halliwell, 1976) was used to calculate GR activity, which was expressed as µmol min^{-1} mg^{-1} of 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 and Prasad, 2004). The decrease in absorbance was measured at 340 nm for 1 min at 30°C. An extinction coefficient of 6.22 mM^{-1} cm^{-1} (Anderson and Davis, 2004) was used to calculate GPX activity, which was expressed as µmol min^{-1} mg^{-1} of protein.

For each enzyme, four separate extractions were performed for samples from each treatment. Each extraction was read three times. A boiled extract was used as the blank treatment during the determination of the activity of each enzyme. The soluble protein concentration of each extract was measured according to (Bradford, 1976) using bovine serum albumin as the standard protein.

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 was homogenized in 2 mL of 0.1% (wt vol^{-1}) trichloracetic acid (TCA) solution in an ice bath. The homogenate was centrifuged at 12000 × g for 15 min at 4°C. After centrifugation, a total of 0.5 ml of the supernatant was reacted with 1.5 mL of TBA solution (0.5% in 20% TCA) for 30 min in a boiling water bath at 95°C. After this period, the reaction was stopped in an ice bath. The samples were centrifuged at 9000 × g for 10 min and the specific absorbance was determined at 532 nm. The nonspecific absorbance was estimated at 600 nm and subtracted from the specific absorbance value. An extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer, 1968) was used

to calculate the MDA concentration, which was expressed as nmol g^{-1} of fresh weight (FW).

Experimental design and data analysis

A 2 × 8 factorial experiment (Experiment 1), consisting of two media (PDA and OA) and eight PA concentrations, was arranged in a completely randomized design with eight replications to evaluate the effect of the different PA concentrations on mycelial growth and conidia production. An experiment (Experiment 2), consisting of three PA concentrations, was arranged in a completely randomized design with eight replications to evaluate the effect of PA concentrations on conidia germination. Another experiment (Experiment 3) aiming to determine the effect of PA on the parameters of leaf gas exchange and Chl *a* fluorescence was arranged in a completely randomized design with eight treatments (corresponding to the eight PA concentrations) and eight replications. Another experiment (Experiment 4) was performed to determine the effect of PA on H_2O_2 and O_2^- production in the leaf tissue and was arranged in a completely randomized design with eight treatments (corresponding to the eight PA concentrations) and eight replications. An experiment (Experiment 5) aiming to determine the effect of PA on the parameters of leaf gas exchange and Chl *a* fluorescence was arranged in a completely randomized design with four treatments [plants sprayed with water (control), plants sprayed with PA (PA), plants sprayed with PA and inoculated with *P. oryzae* (PA/*Po*) and plants inoculated with *P. oryzae* (*Po*)] and eight replications. An experiment (Experiment 6) aiming to determine the effect of PA on H_2O_2 and $O_2^{\bullet-}$ production in the leaf tissue was arranged in a completely randomized design with four treatments (control, PA, PA/*Po* and *Po*) and eight replications. Another experiment (Experiment 7) was performed to obtain the leaf samples for the biochemical analysis and consisted of four treatments (control,

PA, PA/*Po* and *Po*) and was arranged in a completely randomized design with four replications. All experiments were repeated twice. Each experimental unit corresponded to a plastic pot containing six plants. Data obtained from Experiments 1 to 7 and their repetitions were analyzed using the MIXED procedure of the SAS software (Release 8.02 Level 02M0 for Windows, SAS Institute, Inc., 1989, Cary, NC, USA) to determine if data from the repeated experiments could be combined (Moore and Dixon, 2015). Data from all variables and parameters were analyzed by ANOVA and means from the treatments were compared either with *F*, Dunnet or Tukey tests ($P \leq 0.05$) using SAS (version 6.12; SAS Institute, Inc., Cary, NC). Data from each parameter and variables obtained from the treatments PA and PA/*Po* of Experiment 5, and its repetition, were correlated among them using Pearson correlation.

Results

In vitro effect of the PA on mycelia growth, conidia production and conidia germination

Mycelial growth was reduced by 70 and 22%, respectively, on PDA and OA media amended with 0.3 mg PA mL^{-1} in comparison to the control treatment (Fig. 1A). Mycelial growth was visually reduced on Petri dishes containing both PDA and OA media amended with 0.1 mg PA mL^{-1} in comparison to the control treatment (Fig 1B). Indeed, mycelial growth was completely inhibited on Petri dished containing PDA and OA media amended with PA at the concentrations ranging from 0.5 to 5.0 mg mL^{-1}(data not shown). Conidia production and germination were significantly reduced by 66 and 58%, respectively, on PDA and OA media amended with0.3 mg PA mL^{-1} in comparison to the control treatment (Fig. 1C and D).

Effect of PA at different concentrations on leaf gas exchange

There were significant reductions of 13, 25, 26, 33, 41 and 52% for *A*atthe concentrations of 0.3, 0.5, 1.0, 1.5, 3.0 and 5.0 mg PA mL^{-1}, respectively, compared to the concentrations of 0 and 0.1 mg PA mL^{-1} (Fig. 2A). The g_s significantly decreased by 26, 23, 37, 34 and 53% at the concentrations of 0.5, 1.0, 1.5, 3.0 and 5.0 mg PA mL^{-1}, respectively, compared to the concentrations of 0, 0.1 and 0.3 mg PA mL^{-1}(Fig. 2B). There were significant reductions of 26, 23, 37, 34 and 53% for *E* at the concentrations of 0.5, 1.0, 1.5, 3.0 and 5.0 mg PA mL^{-1}, respectively, compared to the concentrations of 0, 0.1 and 0.3 mg PA mL^{-1} (Fig. 2A).

Effect of PA at different concentrations on imaging and parameters of Chl *a* fluorescence

At 48 hai, necrotic lesions were noticed on the leaves of plants sprayed with PA solutionsat concentrations ranging from 0.3 to 5.0 mg mL^{-1} while on the leaves of

plants sprayed with 0.1 mg PA mL^{-1} they were imperceptible (Fig. 3). Alterations in the imaging of the parameters F_v/F_m, Y(NO) and q_P became more pronounced as the concentrations of PA increased from 0.3 to 5.0 mg mL^{-1} due to the bigger necrotic lesions. By contrast, these alterations were less discernible on the leaves of plants sprayed with PA at the concentration of 0.1 mg mL^{-1}. At the concentrations of 3.0 and 5.0 mg PA mL^{-1}, there were significant decreases of 9 and 11% for F_v/F_m and of 10 and 18% for q_P, respectively, in comparison to the other PA concentrations (Fig. 2B and F). The Y(NO) significantly increased by 28 and 34% at the concentrations of 3.0 and 5.0 mg PA mL^{-1}, respectively, in comparison to the other PA concentrations (Fig. 2D).

Histochemical localization of H_2O_2 and $O_2^{\bullet-}$

Effect of different PA concentrations

At 24 hai, H_2O_2 and O_2^- production, as indicated by the brown color, increased as the concentrations of PA increased from 0.3 to 5.0 mg mL^{-1}. By contrast, on the leaves of plants sprayed with 0.1 mg PA mL^{-1}, the brown color was less remarkable resembling the leaves from plants from the control treatment (Figs. 4 and 5).

Effect of a non-phytotoxic PA concentration and plant inoculation with *P. oryzae*

The production of H_2O_2 and $O_2^{\bullet-}$, as indicated by brown color, was more noticeable on the leaves of plants from the *Po* treatment in comparison to the PA/*Po* treatment, especially at 72 hai (Figs. 6 and 7).

Wheat blast severity

Blast severity significantly decreased by 59 and 23% at 72 and 96 hai, respectively, for plants sprayed with PA in comparison to the non-sprayed plants (Fig. 8A). On leaves of plants non-sprayed with PA, many necrotic lesions with intense chlorosis and coalescence were noticed as the disease developed. By contrast, on leaves of plants

sprayed with PA, the number and size of the necrotic lesions were reduced and there was less lesion coalescence and much less chlorosis (Fig. 8B).

Effect of a non-phytotoxic PA concentration and plant inoculation with *P. oryzae* on leaf gas exchange

A significantly decreased by 32, 60 and 82% for the *Po* treatment and by 20, 50 and 57% for the PA/*Po* treatment, respectively, at 48, 72 and 96 hai in comparison to the control treatment. At 96 hai, *A* significantly decreased by 58% for the treatment *Po* in comparison to the PA/*Po* treatment (Fig. 9A). At 72 and 96 hai, g_s was significantly reduced by 60 and 67% for the *Po* treatment and by 37 and 39% for the PA/*Po* treatment, respectively, in comparison to the control treatment.$g_s$significantly decreased by 84% for the treatment *Po* in comparison to the PA/*Po* treatment (Fig. 9B).

At 48 hai, C_i significantly increased by 13% for the *Po* treatment in comparison to the control treatment. C_i significantly increased by 7% for the PA/*Po* treatment in comparison to the control treatment. There were significant increases in C_i of 23 and 18% for the PA/*Po* and PA treatments, respectively, in comparison to the control treatment. For the *Po* treatment, C_i significantly increased by 10% in comparison to the PA treatment (Fig. 9C). There were significant decreases in C_i of 3 and 5% for the PA/*Po* and *Po* treatments, respectively, in comparison to the PA treatment at 72 hai. For the *Po* treatment, *E* significantly increased by 49% in comparison to the control treatment. At 96 hai, *E* was significantly reduced by 29 and 63% for the PA/*Po* and *Po* treatments, respectively, in comparison to the control treatment (Fig. 9B). At 96 hai, *E* significantly decreased by 49% for the *Po* treatmentin comparison to the PA/*Po* treatment (Fig. 9D).

Effect of a non-phytotoxicPA concentration and plant inoculation with *P. oryzae*on imaging and parameters of Chl *a* fluorescence

F_v/F_m significantly decreased by 9, 7 and 10% for the PA/*Po* treatment and by 8, 8 and 24% for the *Po* treatment, respectively, at 48, 72 and 96 hai in comparison to the control treatment. At 96 hai, F_v/F_m significantly decreased by 15% for the treatment *Po* in comparison to the PA/*Po* treatment (Fig. 9E). At 96 hai, Y(II) significantly decreased for the *Po* treatment in comparison to the other treatments. There were significant decreases of 17 and 47% for the PA/*Po* and *Po* treatments, respectively, in comparison to the PA treatment at 96 hai. At 96 hai, Y(NO) significantly increased by 61 and 98% for the PA/*Po* and *Po* treatments in comparison to the control treatment. Y(NO) significantly increased by 23% for the *Po* treatment in comparison to the PA/*Po* treatment at 96 hai.

The first alterations on the parameters F_v/F_m, Y(II), Y(NPQ) and Y(NO), based on the images of Chl *a* fluorescence, were noticed at 72 hai and were more evident on the leaves of plants from the *Po* treatment in comparison to leaves of plants from the PA/*Po* treatment. At 96 hai, decreases for the parameters F_v/F_m, Y(II) and Y(NPQ) along with increase for Y(NO) parameter were closely related with reductions in the light energy dissipated for the photosynthetic process as indicated by the black areas in the images, especially on the leaves of plants from the *Po* treatment in comparison to leaves of plants from the PA/*Po* treatment (Fig. 9).

Enzymes activities

Activities of APX, GR and GST at 48 hai and GR activity at both 48 and 96 hai were significantly higher for the PA/*Po* treatment compared to the other ones (Fig. 11A, F and G). APX and GST activities, respectively, at 72 and 96 hai were significantly higher for the PA/*Po* and *Po* treatments in comparison to the control treatment (Fig.

11A). Activities of APX, CAT and SOD at 96 hai and POX activity at 48, 72 and 96 hai were significantly higher for the PA/*Po* and *Po* treatments in comparison to the other treatments (Fig. 11A, B and D). SOD, GPX and GST activities were significantly higher at 48, 96 and 72 hai, respectively, in comparison to the control treatment (Fig. 11D, E and G). At 48 hai, CAT activity was significantly higher for the PA and PA/*Po* treatments in comparison to the other treatments. At 72 hai, CAT activity was significantly higher for the PA/*Po* and *Po* treatments in comparison to the PA treatment and also for the *Po* treatment in comparison to the control treatment (Fig. 11B). At 48 hai, SOD activity was significantly higher for the PA/*Po* and *Po* treatments in comparison to the PA treatment. At 72 hai, SOD activity was significantly higher for the *Po* treatment in comparison to the control and PA treatments (Fig. 11D). At 48 hai, GPX activity was significantly higher for the control and PA/*Po* treatments in comparison to the other treatments. At 72 hai, GPX activity was significantly lower for the *Po* treatment in comparison to the other treatments (Fig. 11E). At 48 hai, GR activity was significantly lower for the PA and *Po* treatments in comparison to the control treatment. At 72 hai, GR activity was significantly lower for the PA/*Po* treatment in comparison to the control and PA treatments (Fig. 11F). At 48 hai, GST activity was significantly lower for the PA treatment in comparison to the control treatment (Fig. 11G). The MDA concentration was significantly higher for the PA/*Po* treatment in comparison to the PA treatment at 48 hai and for the *Po* treatment in comparison to the other treatments at 96 hai (Fig. 11H).

Pearson correlations

For the *Po* treatment, the correlation of blast severity with the activities of APX, CAT, GR and with the parameters A, g_s, C_i, E, F_v/F_m and Y(II) was significantly negative, but significantly positive with the parameter Y(NO) and MDA concentration. For the

PA/*Po* treatment, the correlation of blast severity with the activities of APX, GR, GST, POX and SOD and with the parameters *A*, Y(II) and Y(NPQ) was significantly negative, but significantly positive with the parameter C_i and Y(NO) (Table 1).

Discussion

The present study provides, to the best of the authors' knowledge, novel physiological and biochemical evidences that the non-host selective toxin PA contributed positively to wheat resistance against *P. oryzae* infection based on the greater efficience of the antioxidative metabolism as well as reduced losses in the photosynthetic capacity of the infected plants.

The non-selective host toxins play pivotal role in the changes on the physiological and biochemical processes occurring in plants. For instance, pyricularina, tenuazonic acid as well as the filtrate of *P. oryzae* culture sprayed into rice leaves resulted in the typical blast symptoms besides affecting plant growth (Tamari and Kaji, 1959; Umetsu et al., 1972; Singburaudom et al., 1998). In the present study, necrotic lesions were formed on wheat leaves after 48 hours of spraying them with a solution of PA at the concentration of 0.3 mg mL^{-1}. In comparison to the typical blast symptoms, these necrotic lesions did not have their central region of a grayish color. The necrotic lesions in rice leaves and in suspension cells culture developed due to a high concentration of H_2O_2 (Zhang et al., 2004).

In the present study, the spray of PA solutions at concentrations above 0.3 mg mL^{-1} caused the necrosis of leaf tissue that resulted in drastic reductions in the values of A and E due to diffusive limitations that were associated with lower g_s values. Similar results demonstrated that high concentrations of toxins produced by *Helminthosporium maydis* caused a rapid reduction of the photosynthesis due to stomatal closure associated to limitations in the potassium uptake by the guard cells (Artzen et al., 1973). In the case of the fusaric acid at a concentration of 100 ppm, a non-host selective toxin produced by all *Fusarium* species caused drastic reductions in stomatal conductance and transpiration rate on the leaves of banana plants

compromising, therefore, the water balance of the infected plants (Gong et al., 2012). In the present study, the reductions in the values of F_v/F_m and q_P were accompanied to increases in the values of Y(NO), an indication of damage to the PSII photosynthetic machinery, for plants sprayed with PA at concentrations greater than 3 mg mL^{-1}. Based on these results, the direct or indirect effects of exogenous application of PA into the leaves of wheat and subsequent inoculation with *P. oryzae* were tested. Based on the *in vitro* scenario, fungal mycelial growth, sporulation and conidia germination were affected using 0.3 mg PA mL^{-1} in the culture medium suggesting, therefore, that the PA may interfere with fungal growth. In this regard, Pasechnik et al. (1993) demonstrated that concentrations of 1 to 3 mM of PA caused a drastic decrease in the conidia germination of *P. oryzae*. In the present study, the use of 0.3 mg PA mL^{-1} was not considered due to the occurrence of necrotic lesions coupled with drastic reductions in the photosynthesis of plants.

An efficient antioxidant system, especially based on the high activities of APX, POX, SOD and GST involved in the removal of ROS, limited the cell damage caused by *P. oryzae* infection on wheat leaves contributing, therefore, to a reduced blast severity (Debona et al., 2012). In the present study, the antioxidative metabolism of wheat plants in response to *P. oryzae* infection was due to a significant increase in the activities of all enzymes studied, except GPX, indicating alterations in cellular homeostasis due to the high production of ROS associated with host defense responses and the restoration of cellular oxidative balance (Torres et al., 2006; Torres, 2010). In addition, this response was greater for plants sprayed with a solution of PA at the concentration of 0.1 mg mL^{-1}, especially during the first hours after inoculation suggesting, therefore, the involvement of this non-selective toxin in favor a more efficient antioxidant metabolism that culminated in reduced blast severity. The better

antioxidative metabolism of inoculated plants that were sprayed with PA can be explained to a high activity of NADPH oxidase. It is known that NADPH oxidase was involved in the oxidative burst of rice plants sprayed with PA that exhibited increased resistance to blast (Zhang et al., 2004).

Different cellular compartments in the plant cells are responsible for the metabolism of ROS, a fact that is closely linked to the metabolic processes that are involved in their production (Mittler, 2002; Lima et al., 2002; Das and Roychoudhur, 2014). In the present study, wheat plants sprayed with PA and inoculated with *P. oryzae* showed increased activities of APX, POX, SOD, GR GPX and GST at 48 hai demonstrating, therefore, that different ROS detoxification pathways were operating in response to PA spray. In addition, these results confirmed the importance of SOD and APX as the major ROS-scavenging enzymes and that a balance on their activities is of detrimental importance to determine the state of metabolizing the superoxide radicals and H_2O_2 (Asada and Takahashi, 1987; Bowler et al., 1992; Willenkens et al., 1997). An increase in the resistance of plants against pathogens infection are strongly linked to their greater antioxidative metabolism (Resende et al., 2012; Cavalcanti et al., 2006). In the case of CAT, its activity was high for both non-inoculated and inoculated plants that were sprayed with PA indicating the contribution of this enzyme for ROS metabolism. The CAT activity increased on wheat plants sprayed with abscisic acid, salicylic acid, calcium and H_2O_2 (Agarwal et al., 2005). The POX is considered one of the antioxidant enzymes that are involved in the plant defense response to pathogens infection (Milavec et al., 2001). In the present study, POX activity increased from 48 to 96 hai on plants sprayed with PA and inoculated with *P. oryzae* in comparison to the non-inoculated plants. Similar results were reported for the *Cucurbita pepo*-zucchini yellow mosaic virus interaction using the salicylic acid

as an inducer (Radwan et al., 2007). The GPX and GST are important enzymes involved in the reduction of the oxidative stress in plant cells by using the GSH to reduce H_2O_2 and other hydroperoxides (Gill and Tuteja, 2010; Noctor et al., 2002). For wheat plants sprayed with PA and inoculated with *P. oryzae*, there was a consistent increase in the activities of GPX and GST during the infection process of *P. oryzae*. For the soybean-*Corynespora cassiicola* interaction, SOD, CAT, POX, APX, GPX, GR and GST activities were greater especially for a moderately resistant cultivar (Fortunato et al., 2015). In wheat cultivars partially resistant to blast in comparison to the most susceptible ones, GST activity was significantly higher and was considered to be the most important antioxidant enzyme among the other ones evaluated (Debona et al., 2012). Therefore, it seems reasonable to assume that GPX and GST played a key role in increasing wheat resistance to blast mediated by PA through the removal of H_2O_2 and, consequently, reducing lipid peroxidation. The GR is an enzyme located mostly in the chloroplasts in comparison to the mitochondria and cytosol. The GR is involved in the ASH-GSH cycle and plays an essential role in the plant defense system against ROS by sustaining the reduced status of GSH (Gill and Tuteja 2010). In the present study, GR activity was higher for inoculated plants in comparison to the non-inoculated ones. At 48 hai, plants sprayed with PA showed greater GR activity in comparison to plants non-sprayed with PA. The GR activity was also high in melon plants from a cultivar resistant to *Colletotrichum lagenarium* and wheat plants from a cultivar partially resistant to blast (Ge et al., 2013; Debona et al., 2012). Several studies reported the pivotal role of GR in promoting the resistance of plants to different types of stress considering the role of this enzyme in regenerating glutathione (GSH) which is important to protect the cells against the oxidative stress by maintaining the ascorbic acid (ASH) pool (Noctor et al., 2012; Gill and Tuteja 2010; Ding et al., 2009).

The MDA is considered to be one of the best lipid peroxidation products that indicates the level of cell damage caused by the oxidative stress generated during pathogens infection and an indirect indication of the ability of plants to counter act their infection (Weber et al., 2004; Debona et al., 2012; Resende et al., 2012; Bispo et al., 2014; Fortunato et al., 2015). In the present study, the MDA concentration was higher in the leaves of plants infected with *P. oryzae* at 96 hai in comparison to the non-inoculated plants. Similarly, the inoculated plants previously sprayed with PA showed a reduction in the MDA concentration in comparison to plants infected with *P. oryzae*.

In the present study, the gas exchange and Chl *a* fluorescence imaging parameters were virtually non-affected on wheat plants sprayed with PA and further inoculated with *P. oryzae* in contrast to the drastic alterationson the values of these parameters on the leaves of plants non-sprayed with PA. Indeed, the values for *A*, g_s, C_i and *E* were reduced in the leaves of plants non-sprayed with PA due to the greater blast severity in comparison to plants sprayed with PA. Debona et al. (2014) reported that the efficient activities of the enzymes involved in the antioxidant metabolism of wheat plants from a cultivar partially resistant to blast was strongly associated with their better photosynthetic performance during *P. oryzae* infection.

The infection of leaves by pathogens increase the resistance to CO_2 diffusion that culminates in lower g_s values (Erickson et al., 2003; Resende et al., 2012; Ge et al., 2013; Aucique-Pérez et al., 2014, Bispo et al., 2014). In the present study, the values of g_s and *E* were drastically reduced for inoculated plants in comparison to the non-inoculated ones. However, the greatest impact of *P. oryzae* occurred in the values of g_s and *E* on plants non-sprayed with PA. In fact, reductions in the values of g_s and *E* caused significant increases in the C_i values on the leaves of wheat plants infected

with *P.oryzae* and corroborates with the results from the corn-*Stenocarpella macrospora*, wheat-*P.oryzae* and grapevine-*Plasmopara viticola* interactions (Letousey et al., 2010; Debona et al., 2012; Bermudez-Cardona et al., 2015). In the leaves of wheat plants infected with *P. oryzae*, reduction in the values of *A* was associated with dysfunctions at the level of the biochemical reactions involving CO_2 fixation (Debona et al., 2014).

The imaging of the Chl *a* fluorescence parameters showed heterogeneity in the metabolic perturbations caused by *P. oryzae* infection on the leaves of plants non-sprayed or sprayed with PA being of less impact on the leaves of the former. The values of F_v/F_m, Y(II), Y(NPQ) and Y(NO) can increase or decreaseas reported for *Arabidopsis thaliana*, corn, rice and coffee infected by *Pseudomonas syringae*, *Stenocarpella macrospora*, *Monographella albescens* and *Hemileia vastatrix*, respectively (Bonfig et al., 2006; Tatagiba et al., 2014; Bermudez-Cardona et al., 2015;Honorato et al., 2015). Decreases in the values of F_v/F_m in plants infected by pathogens suggest that reactions at the PSII were damaged (Baker, 2008). Additionally, reductions in the values of Y(II) and Y(NPQ) coupled with an increase in the values of Y(NO) at 96 hai for plants non-sprayed with PA and inoculated with *P. oryzae* in contrast to plants sprayed with PA and inoculated with *P. oryzae* indicated that the necrotrophic phase of *P. oryzae* significantly compromised the ability of leaves on the photochemical energy conversion. Decreases in the values of Y(NPQ) in contrast to an increase in the values of Y(NO) suggest an inhibition in the electron flux necessary for the primary electron transport reactions of photosynthesis that resulted in irreversible damage (Bonfig et al., 2006). Therefore, the results from the present study indicated that *P. oryzae* infection caused drastic alterations in the photosynthetic machine of the wheat plants compromising, therefore, the dissipation mechanisms for

the photosynthetic process and photo-protection mechanisms and leading to oxidative stress coupled to increases in heat dissipation. However, these effects were relieved by anefficient antioxidant metabolism on plants sprayed with PA at the early stages of fungal infection.

In conclusion, lesions formed on the leaves of wheat plants sprayed with PA compromised their photosynthetic performance, which became more pronounced as the PA concentration increased. However, a low PA concentration was able to increase the efficiency of the antioxidative metabolism of the infected plantsleading, therefore, to a rapid removal of the ROS generated during fungal infection and a reduction of the oxidative damage in the infected cells which culminated to a greater wheat resistance to blast. Additionally, the reduction of the oxidative stress in plants imposed by fungal due to PA spray reflected in a better photosynthetic performance of the plants. Future research should focus on the biochemical and molecular analyzes to elucidate the mechanism that allows a greater antioxidative response of plants sprayed with PA in connections with possible changes in both primary and secondary metabolism to obtain an increase on wheat resistance to blast.

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List of Tableand Figures

Table 1. Pearson correlation coefficients among ascorbate peroxidases (APX), catalases (CAT), glutathione peroxidases (GPX), glutathione reductases (GR), glutathione-*S*-transferases (GST), peroxidases (POX), superoxide dismutases (SOD), net CO_2 assimilation rate (A), stomatal conductance to water vapor (g_s), internal CO_2 concentration (C_i), transpiration rate (E), maximum PSII quantum efficiency (F_v/F_m), effective PSII quantum yield Y(II), quantum yield of regulated energy dissipation Y(NPQ), quantum yield of non-regulated energy dissipation Y(NO), malondialdehyde (MDA) concentration and blast severity (SEV) determined on the leaves of wheat plants that were either non-sprayed (0 mg PA mL^{-1}) (-PA) or sprayed (0.1 mg PA mL^{-1}) (+PA) with picolinic acid (PA).

Fig. 1. Effect *in vitro* of different concentrations of picolinic acid (PA) on micelial growth (A and B), conidia production of *Pyricularia oryzae* (C) and their germination (D). Means of the treatments followed by an asterisk (*) are significantly different ($P \leq 0.05$) according to Dunnet´s test. Bars represent the standard desviation of the means from two pooled experiments. $n = 10$.

Fig. 2. Leaf gas exchange [net carbon assimilation rate (A) (A), stomatal conductance to water vapor (g_s) (C) and transpiration rate (E)] and chlorophyll *a* fluorescence [maximum PSII quantum efficiency (F_v/F_m) (B), yield of non-regulated dissipation energy (Y(NO) (D) and photochemical quenching coeficient (q_p) (F)] parameters determinedat 48 hours after spray of wheat leaves with different concentrations of picolinic acid (PA). Means of the treatements followed by an asterisk (*) are significantly different ($P \leq 0.05$) according to Dunnet´s test. Bars represent the standard desviation of the means from two pooled experiments. $n = 10$.

Fig. 3. Lesions and images of the chlorophyll *a* fluorescence parameters maximum PSII quantum efficiency (F_v/F_m), yield of non-regulated dissipation energy [Y(NO)] and photochemical quenching coeficient (q_p) determined at 48 hours after spray of wheat leaves with different concentrations of picolinic acid.

Fig. 4. Histochemical detection of hydrogen peroxideon the leaves of wheat plants at 24 hours after spray with different concentrations of picolinic acid.

Fig. 5. Histochemical detection of superoxide anion radical on the leaves of wheat plants at 24 hours after spray with different concentrations of picolinic acid.

Fig. 6. Histochemical detection of hydrogen peroxideonthe leaves of wheat plants that were either non-sprayed with picolinic acid and inoculated with *Pyricularia oryzae* (*Po*) and sprayed with picolinic acid and inoculated with *P. oryzae* (PA/*Po*).

Fig. 7. Histochemical detection of superoxide anion radicalonthe leaves of wheat plants that were either non-sprayed with picolinic acid and inoculated with *Pyricularia oryzae* (*Po*) and sprayed with picolinic acid and inoculated with *P. oryzae* (PA/*Po*).

Fig. 8. Blast development (A) and symptoms (B) onthe leaves of wheat plants that were non-sprayed (0 mg PA mL^{-1}) (-PA) or sprayed (0.1 mg PA mL^{-1}) (+PA) with picolinic acid (PA) and further inoculated with *Pyricularia oryzae*. Means for the -PA and +PA treatments followed by an asterisk (*), ateach evaluation time, are significantly different according to *F* test ($P \leq 0.05$). Bars represent the standard error of the means from two pooled experiments. $n = 10$.

Fig. 9. Net CO_2 assimilation rate (A) (A), stomatal conductance to water vapor (g_s) (B), internal CO_2 concentration (C_i) (C), transpiration rate (E) (D), maximum PSII quantum efficiency (F_v / F_m) (E), effective PSII quantum yield Y(II) (F), quantum

yield of regulated energy dissipation Y(NPQ) (G) and quantum yield of non-regulated energy dissipation Y(NO) (H) determined in the leaves of wheat plants that were sprayed with water (control), sprayed with 0.1 mg of picolinic acid (PA) mL^{-1} (PA), only inoculated with *Pyricularia oryzae* (*Po*) and sprayed with 0.1 mg PA mL^{-1} and inoculated with *P. oryzae* (PA/*Po*). Means from the treatments followed by different letters are significantly different according to Tukey's test ($P \leq 0.05$). Bars represent the standard error of the means from two pooled experiments. $n = 12$.

Fig. 10. Images of the parameters of chlorophyll *a* fluorescence: maximum PSII quantum efficiency (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) determined in the leaves of wheat plants that were sprayed with water (control), sprayed with 0.1 mg of picolinic acid (PA) mL^{-1} (PA), only inoculated with *Pyricularia oryzae* (*Po*) and sprayed with 0.1 mg PA mL^{-1} and inoculated with *P. oryzae* (PA/*Po*). Two experiments were conducted with consistent results; results from one representative experiment are shown.

Fig. 11. Activities of ascorbate peroxidases (APX) (A), catalases (CAT) (B), peroxidases (POX) (C), superoxide dismutases (SOD) (D), glutathione peroxidases (GPX) (E), glutathione reductases (GR) (F) and glutathione-*S*-transferases (GST) (G) as well as concentration of malondialdehyde (MDA) determined in the leaves of wheat plants that were sprayed with water (control), sprayed with 0.1 mg of picolinic acid (PA) mL^{-1}(PA), only inoculated with *Pyricularia oryzae* (*Po*) and sprayed with 0.1 mg PA mL^{-1} and inoculated with *P. oryzae* (PA/*Po*). Means from the treatments followed by different letters are significantly different according to Tukey's test ($P \leq 0.05$).Bars

represent the standard error of the means from two pooled experiments. n = 5. FW = fresh weight.

Table 1

Variables/Parameters	-PA	+PA
APX	-0.81*	-0.57*
CAT	-0.62*	-0.10
GPX	0.05	-0.44
GR	-0.53*	-0.63*
GST	0.11	-0.73*
POX	0.43	-0.60*
SOD	0.11	-0.63*
A	-0.95*	-0.66*
g_s	-0.65*	0.29
C_i	0.65*	0.84*
E	-0.77*	0.07
F_v/F_m	-0.67*	-0.34
Y(II)	-0.91*	-0.67*
Y(NPQ)	0.11	-0.54
Y(NO)	0.80*	0.83*
MDA	0.86*	0.32

* = significantat 0.05.

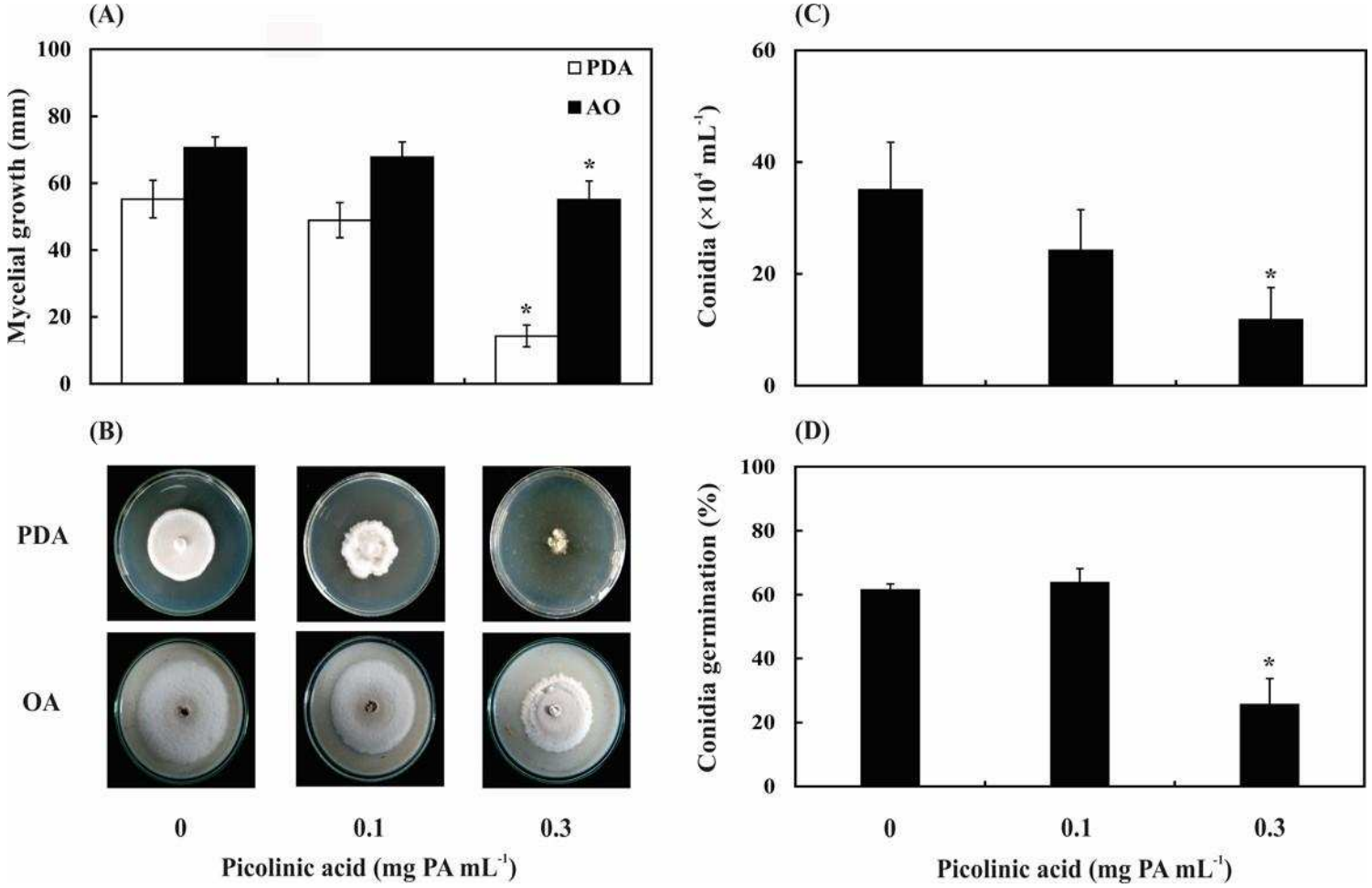
(A)
Mycelial growth (mm)
100
80
60
40
20
0
PDA
AO
*
*
*
(B)
PDA
OA
0
0.1
0.3
Picolinic acid (mg PA mL-1)
(C)
Conidia (×10⁴ mL⁻¹)
60
40
20
0
*
(D)
Conidia germination (%)
100
80
60
40
20
0
*
0
0.1
0.3
Picolinic acid (mg PA mL-1)

Figure 1

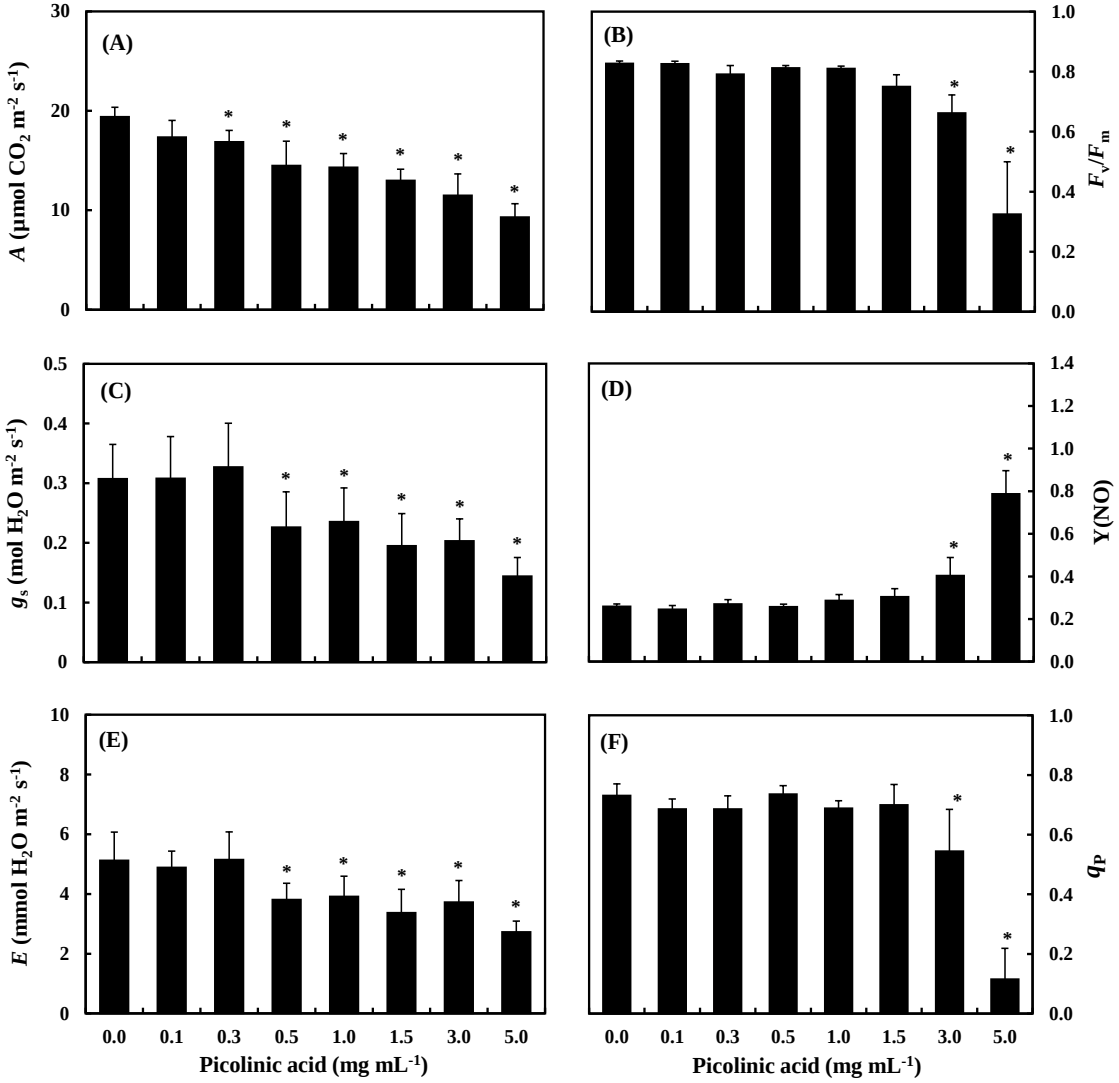
(A)
(B)
(C)
(D)
(E)
(F)
A (μmol CO2 m-2 s-1)
Fv/Fm
gs (mol H2O m-2 s-1)
Y(NO)
E (mmol H2O m-2 s-1)
qP
0.0 0.1 0.3 0.5 1.0 1.5 3.0 5.0
Picolinic acid (mg mL-1)

Figure 2

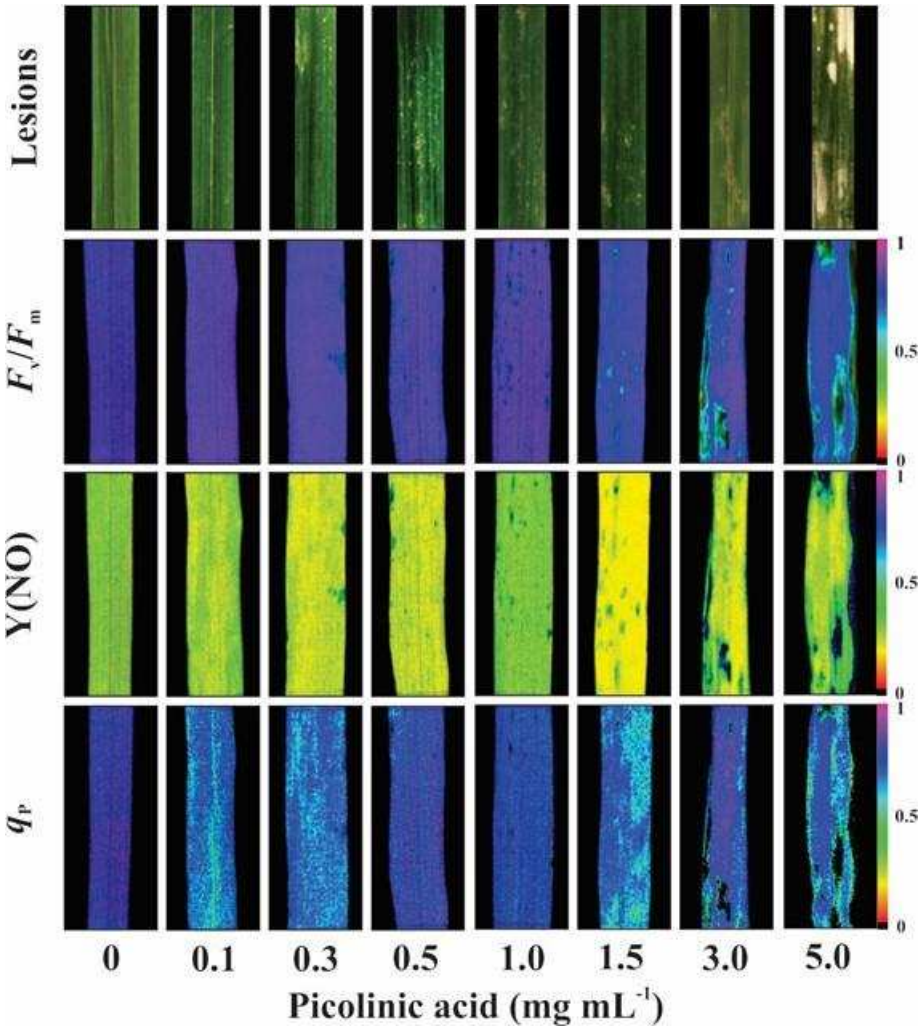
Lesions
F_v/F_m
Y(NO)
q_P
1
0.5
0
0
0.1
0.3
0.5
1.0
1.5
3.0
5.0
Picolinic acid (mg mL^{-1})

Figure 3

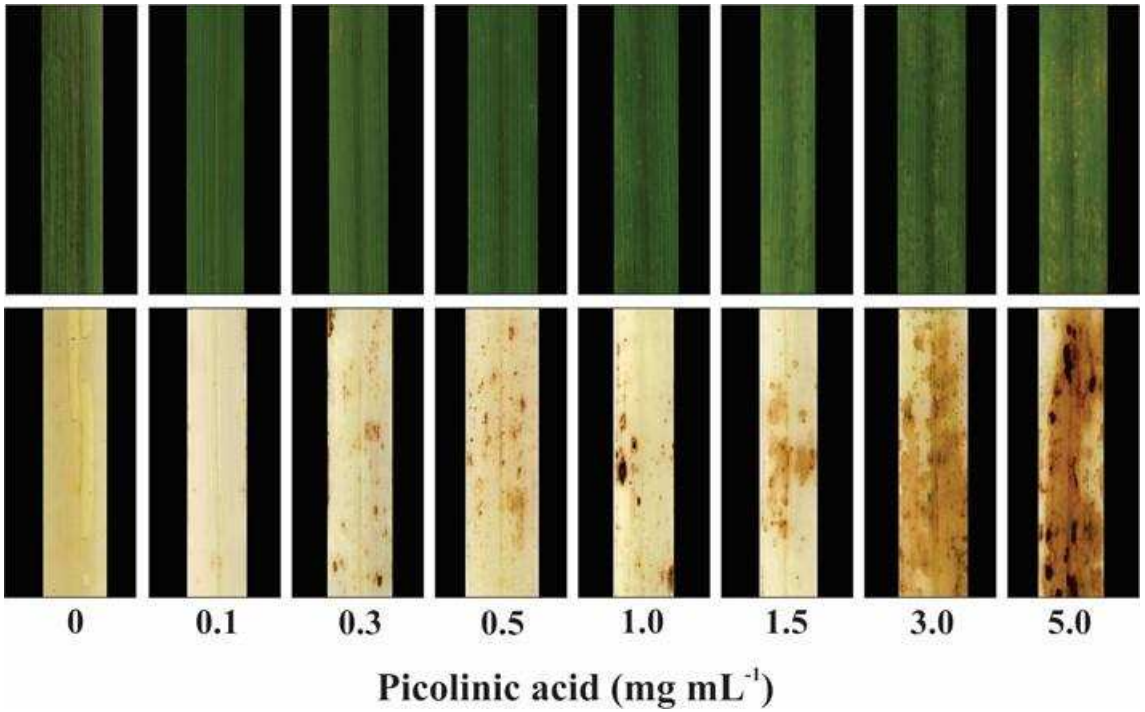

Figure 4

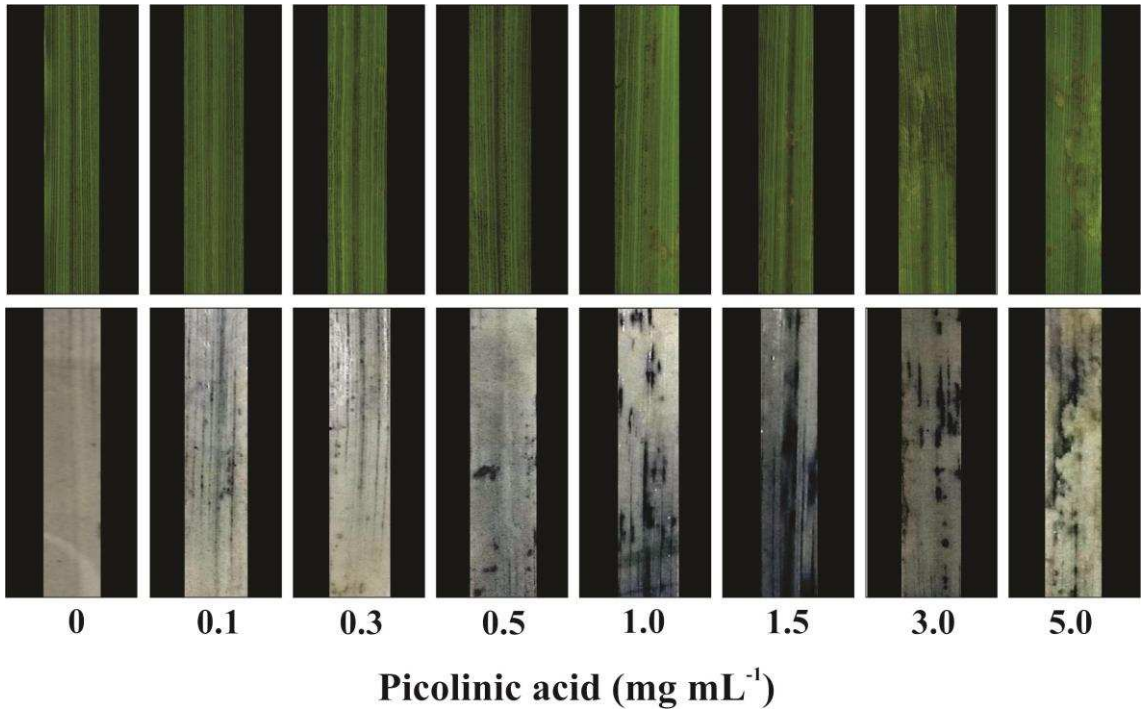

Figure 5

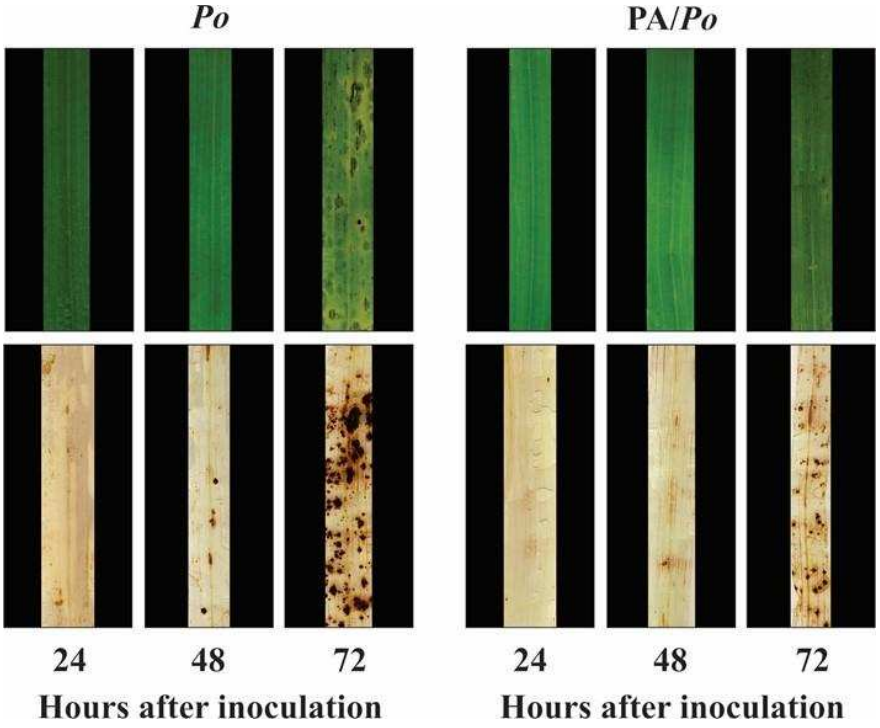
Po
PA/Po
24
48
72
24
48
72
Hours after inoculation
Hours after inoculation

Figure 6

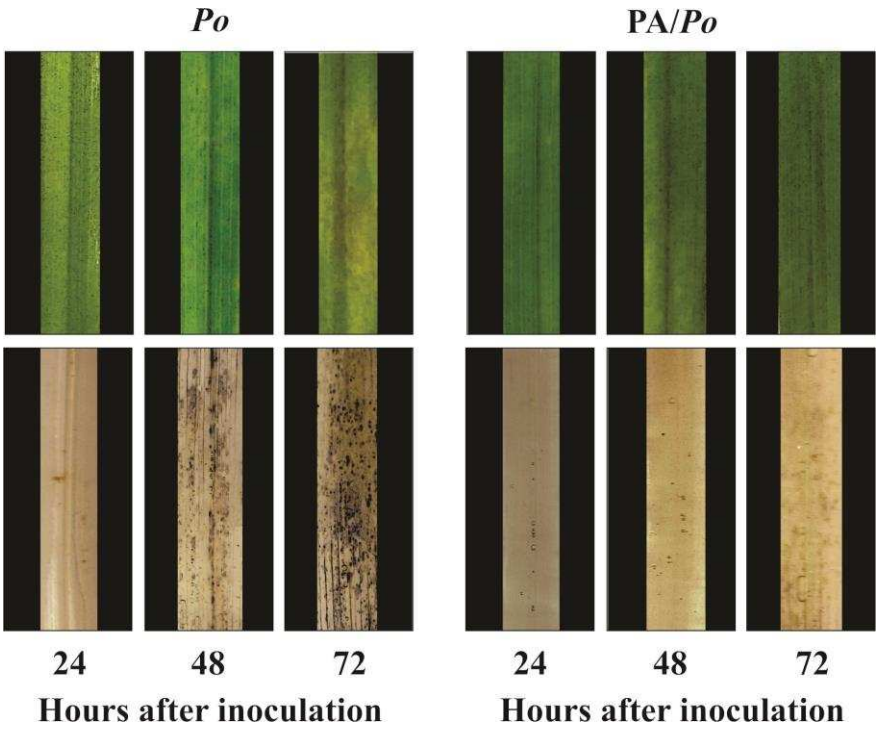
Po
PA/*Po*
24
48
72
24
48
72
Hours after inoculation
Hours after inoculation

Figure 7

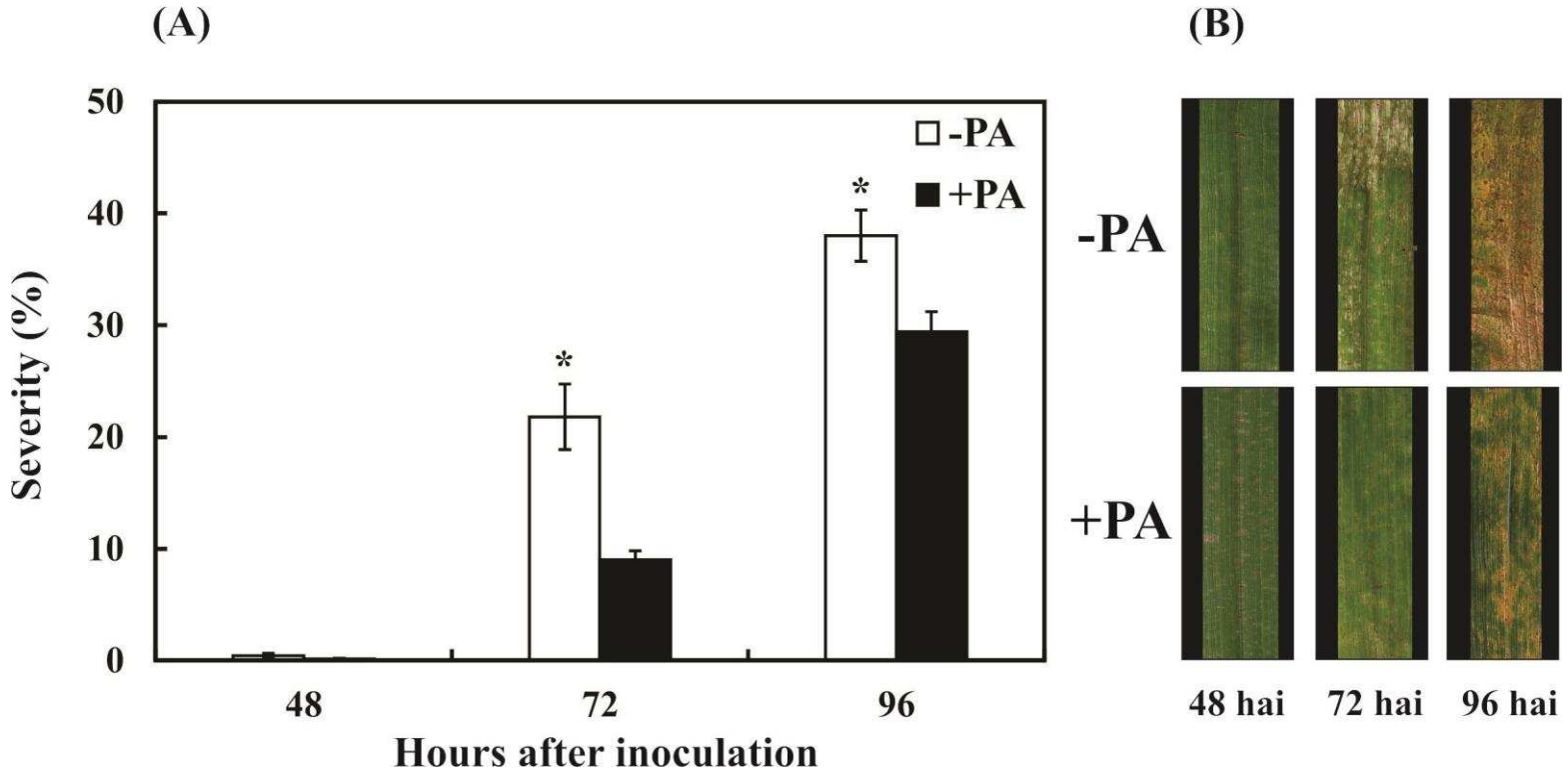
(A)
(B)
50
40
30
20
10
0
Severity (%)
-PA
+PA
*
*
48
72
96
Hours after inoculation
-PA
+PA
48 hai
72 hai
96 hai

Figure 8

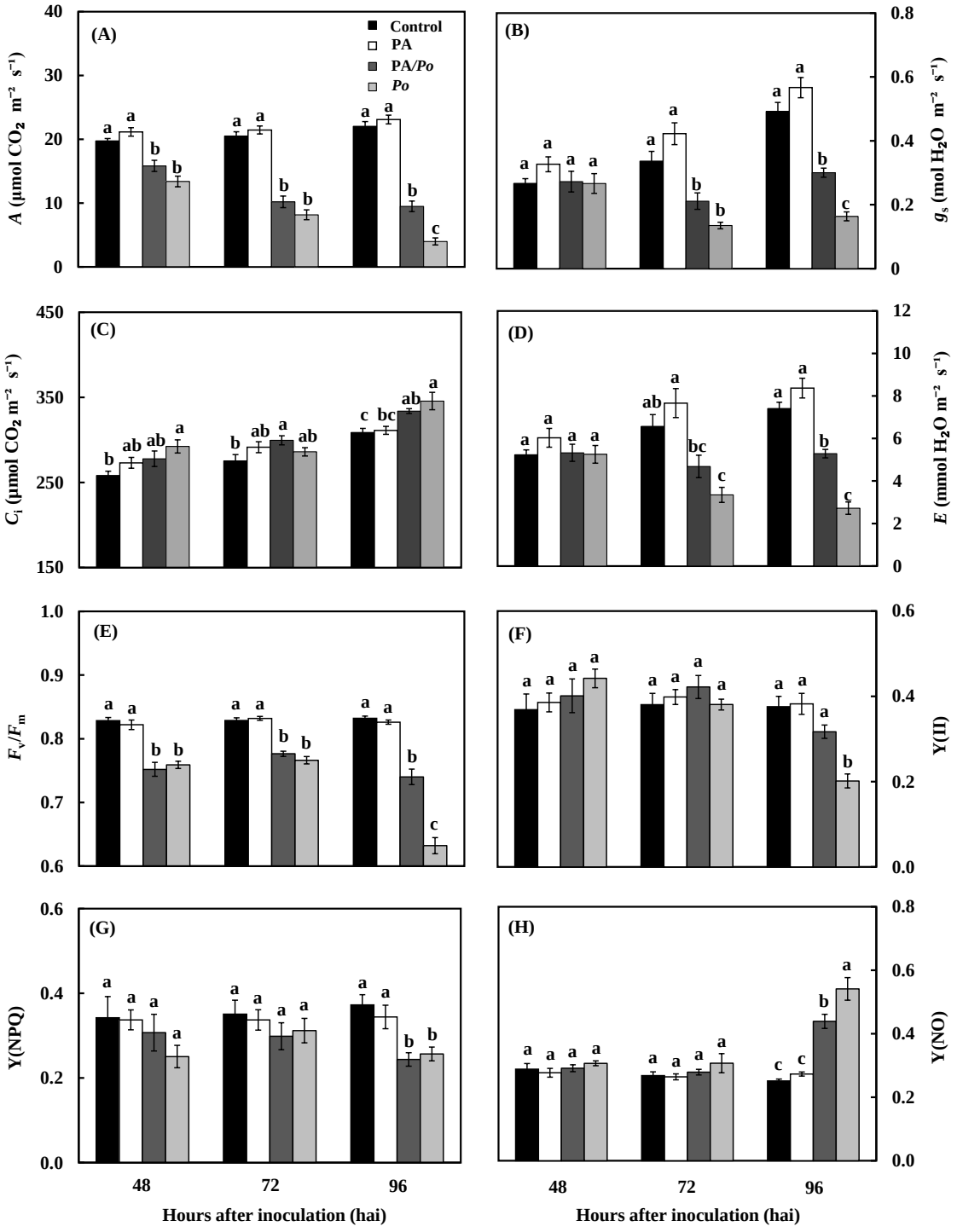
(A)
Control
PA
PA/Po
Po
A (µmol CO2 m−2 s−1)
(B)
gs (mol H2O m−2 s−1)
(C)
Ci (µmol CO2 m−2 s−1)
(D)
E (mmol H2O m−2 s−1)
(E)
Fv/Fm
(F)
Y(II)
(G)
Y(NPQ)
(H)
Y(NO)
48
72
96
Hours after inoculation (hai)
Hours after inoculation (hai)

Figure 9

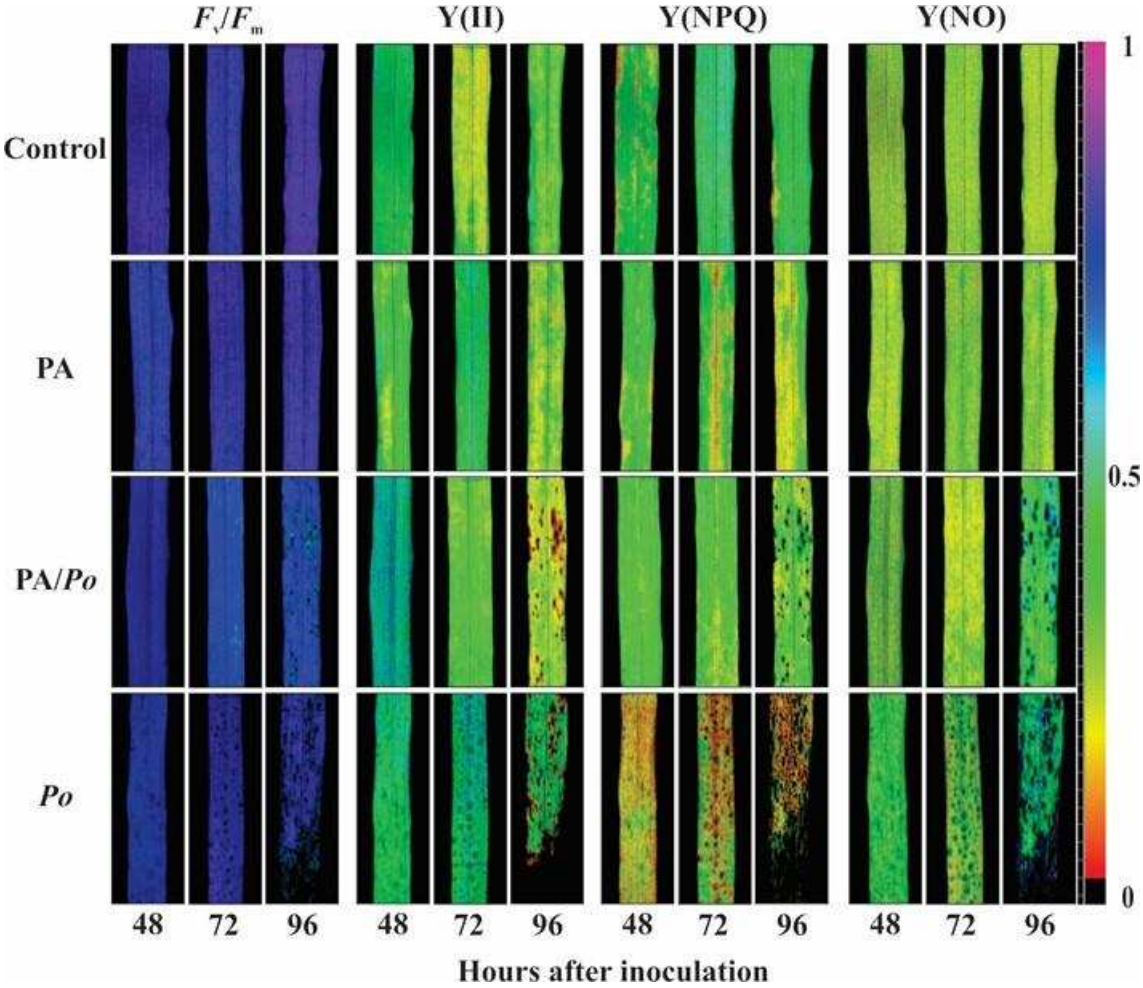
F_v/F_m
Y(II)
Y(NPQ)
Y(NO)
Control
PA
PA/*Po*
Po
1
0.5
0
48
72
96
Hours after inoculation

Figure 10

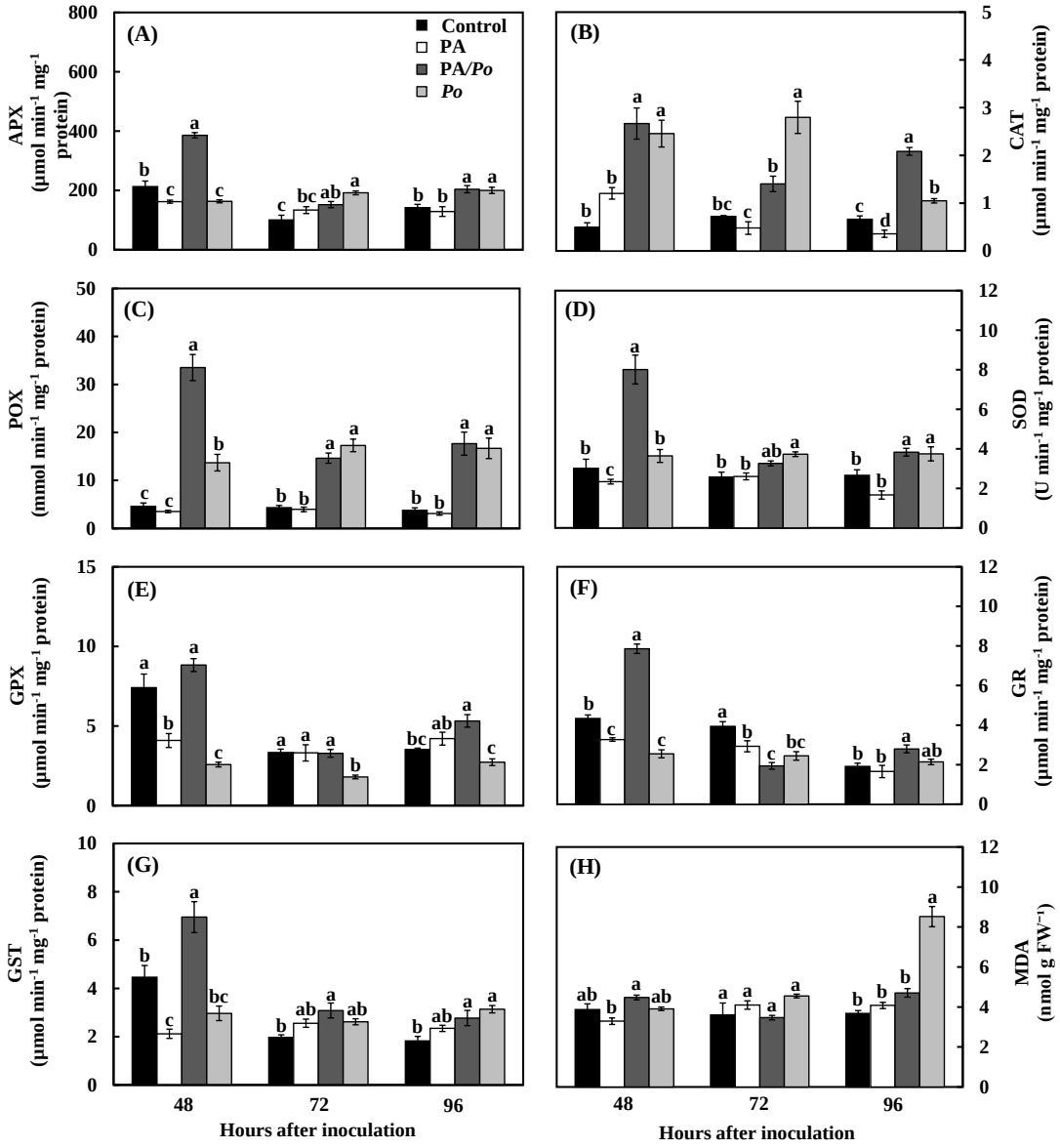
(A)
Control
PA
PA/Po
Po
APX (µmol min-1 mg-1 protein)
(B)
CAT (µmol min-1 mg-1 protein)
(C)
POX (mmol min-1 mg-1 protein)
(D)
SOD (U min-1 mg-1 protein)
(E)
GPX (µmol min-1 mg-1 protein)
(F)
GR (µmol min-1 mg-1 protein)
(G)
GST (µmol min-1 mg-1 protein)
(H)
MDA (nmol g FW-1)
48
72
96
Hours after inoculation
Hours after inoculation

Figure 11

Chapter 2

Metabolic Manipulation on the Leaves of Different Wheat Cultivars caused by *Pyricularia oryzae* Infection Reduce their Basal Level of Resistance to Blast

Abstract

Blast, caused by *Pyricularia oryzae*, can negatively affect wheat production. This study assessed the contrasting behavior of three wheat cultivars (BR-18, EMBRAPA-16 and BRS-Guamirim) differing in their basal level of resistance to blast in terms of changes in the carbohydrates and amino acids pools as well as on their photosynthetic performance and antioxidative metabolism. At 120 hours after inoculation, blast severity was significantly lower by 45 and 33% for cultivars EMBRAPA-16 and BR-18 in comparison to cultivar BRS-Guamirim. The photosynthetic performance of infected plants was altered due to diffusional and biochemical limitations for CO_2 fixation. During the asymptomatic phase of *P. oryzae* infection, drastic changes in the carbohydrates metabolism and on the levels of amino acids, intermediates compounds of the TCA cycle and polyamines occurred on plants from the three cultivars suggesting a metabolic manipulation exerted by the pathogen. However, a more efficient antioxidant metabolism was able to help the wheat plants to counteract against the deleterious effects of *P. oryzae* infection in association with great phenylalanine ammonia lyases and polyphenoloxidases activities and high concentrations of phenolics and lignin.

Keywords: GC-MS, host resistance, mechanisms of defense, metabolic profile, primary metabolism.

Introduction

Blast, which is caused by the hemibiotrophic fungus *Pyricularia oryzae* Cavara (teleomorph *Magnaporthe grisea* (T. T. Hebert) M. E. Barr) has negatively influenced wheat (*Triticum aestivum* L.) production in Brazil and in other South American countries such as Bolivia and Paraguay (Igarashi et al., 1986; Goulart and Paiva, 1992). In agricultural regions of Bolivia, Brazil and Paraguay where the climatic conditions (intense rainy periods, temperatures ranging from 21 to 27°C, cloudy days and high relative humidity) are favorable for the occurrence of blast epidemics, yield losses have been greater than 60% (Goulart et al., 2007; Kohli et al., 2011). In Bangladesh, blast occurred in about 15% of the total area used to grow wheat and yield losses reached around 90% due to the use of susceptible cultivars (Malaker et al., 2016). Since blast was reported in Brazil, breeding programs have been carried out in an effort to identify sources of resistance to be used in obtaining cultivars with a desirable level of resistance to blast (Cruz et al., 2010). Since then, most of the released cultivars showed susceptibility to blast with a few exceptions such as BRS 229, BRS 179, CNT 8, BRS 120, BRS Buriti, BR-18, MGS 3, Brilhante and EMBRAPA-16, which exhibited a high level of partial resistance to the disease under field conditions even though with some variations occurring according to the different regions of Brazil (Macielet al., 2008; Cruz et al., 2010).

The primary metabolism of plants infected by pathogens is greatly alterated in order to provide the necessary metabolites to beused in the pathways related to host defense mechanisms (Berger et al., 2007; Torres et al., 2015). Alterations in the levels of the hormones ethylene, jasmonic acid and salicylic acid, changes in the antioxidative metabolism and an increase in the production of phenylpropanoids and

phytoalexins are the remarkable alterations occurring in the secondary metabolism of plants when infected by pathogens of different life styles (Dixon and Paiva, 1995).

The infection of wheat leaves by *P. oryzae* results in dramatic reductions in the values of the leaf gas exchange parameters net CO_2 assimilation rate, stomatal conductance to water vapor and transpiration rate (*E*) in parallel with an increase in the internal CO_2. These changes are more pronounced for cultivars susceptible to blast than for the most resistant ones suggesting, therefore, that biochemical limitations likely related to a reduced Rubisco activity, rather than diffusive limitations, had better explain the alterations on these parameters (Debona et al., 2014). Indeed, a range of dysfunctions at the photochemical level occurred in the leaves of wheat plants infected with *P. oryzae*. The fungal infection caused a strong decrease in the maximum photochemical efficiency of photosystem II (F_v/F_m), in the efficiency of the excitation energy captured by the open PSII reaction centers and on the photochemical quenching coefficient indicating a chronic photoinhibition as well as the failure of the infected leaves for fully capture and exploit the absorbed energy to be used in photosynthesis (Krause and Weis 1991; Aucique-Pérez et al., 2014). A more efficient antioxidative metabolism in the removal of the excess of hydrogen peroxide and superoxide anion radical on the leaves of wheat plants infected by *P. oryzae* played a pivotal role in their resistance to blast (Debona et al., 2012). Wheat cultivars inoculated with non-adapted and adapted isolates of *P. oryzae* showed a differential expression of genes related to cell rescue, host defense pathways, plant metabolism, cellular transport and regulation of transcription (Tufan et al., 2009). Metabolomic analysis allows the qualitative and/or quantitative characterization of metabolites differentially produced in a certain host-pathogen interaction and the metabolite profiling obtained determines a group of related compounds involved in several metabolic pathways being the most ones related

to host defense mechanisms (Fukusaki and Kobayashi, 2005; Balmeret al., 2013; Kushalappa and Gunnaiah, 2013). Metabolomic platforms have been used to obtain information for the rice-*P. oryzae*, sunflower-*Sclerotinia sclerotiorum*, wheat-*Fusarium graminearum* and -*Zymoseptoria tritici* interactions (Paker et al., 2009; Peluffo et al., 2010; Gunnaiah et al., 2012).

Considering the importance of blast to decrease wheat yield, it is suitable to determine whether wheat resistance to this disease could be possibly associated with alterations in the host metabolism during the infection process of *P. oryzae*. Therefore, this study assessed the contrasting behavior of three cultivars differing on their basal level of resistance to blast in terms of changes in the carbohydrates and amino acids pools as well as on the photosynthetic performance and antioxidative metabolism.

Material and Methods

Plant growth

Wheat seeds from cultivars BR-18, EMBRAPA-16 and BRS-Guamirim were surface-sterilized in 10% (v v^{-1}) NaOCl for 2 min, rinsed in sterilized water for 3 min and germinated on plastic pots (20-cm diameter) filled with 1 kg of substrate made from a 1:1:1 mixture of pine bark, peat and expanded vermiculite (Tropstrato, Vida Verde, Mogi Mirim, SP, Brazil). The cultivars BR-18 and EMBRAPA-16 are moderately resistant to leaf blast while the cultivar BRS-Guamirim is susceptible (Urashima et al., 2004; Reis et al., 2006; Cruz et al., 2010). A total of 1.63 g of calcium phosphate monobasic was added to each plastic pot. A total of nine seeds were sown per pot and at eight days after seedlings emergence, each pot was thinned to six seedlings. Substrate in each pot was fertilized with a nutrient solution containing, in g L^{-1}, 6.4 KCl, 3.48 K_2SO_4, 5.01 $MgSO_4 \cdot 7H_2O$, 2.03 $(NH_2)_2CO$, 0.009 $NH_4MO_7O_{24} \cdot 4H_2O$, 0.054 H_3BO_3, 0.222 $ZnSO_4 \cdot 7H_2O$, 0.058 $CuSO_4 \cdot 5H_2O$ and 0.137 $MnCl_2 \cdot 4H_2O$ (Xavier-Filha et al., 2011). A volume of 15 mL of nutrient solution containing 0.27 g of $FeSO_4 \cdot 7H_2O$ and 0.37 g of EDTA bisodic L^{-1} was also applied after seedlings emergence. The nutrient solution was prepared using deionized water and applied weekly. Plants were watered with deionized water as needed. Plants were grown in a greenhouse (relative humidity of 75 ± 5%, temperature of 25 ± 3°C during day and 22 ± 2°C during night and natural photosynthetically active radiation of 900 ± 15 µmol photons m^{-2} s^{-1} measured at midday) until they were inoculated with *P. oryzae*.

Inoculum production and plant inoculation with *P. oryzae*

A pathogenic isolate of *P. oryzae* (UFV/DFP *Po*-01), obtained from the spikes of wheat plants (cultivar BR-18), was used to inoculate the plants (Debona et al., 2012). Disks of filter paper containing fungal mycelia were transferred to Petri dishes

containing oat-agar medium. After growing the disks containing mycelia, the media with the fungus were transferred to new Petri dishes containing the same medium. The dishes were incubated in a growth chamber at 25°C with a 24 h photoperiod for 10 days. After this period, conidia were carefully removed from the Petri dishes with a soft bristle brush using water containing gelatin (1% w/v). The conidial suspension was calibrated with a hemacytometer to obtain a concentration of 1×10^5 conidia mL^{-1}. The conidial suspension was sprayed with an atomizer (Paasche Airbrush Co., Chicago, IL, USA) on the adaxial surface of the leaves of wheat plants at growth stage 39 (35 days after emergence) (Lancashire et al., 1991). After inoculation, plants were kept in a mist chamber under darkness at 25°C during 24 h and thereafter to a greenhouse (relative humidity of 80 ± 5%, temperature of 25 ± 2°C during day and 22 ± 1°C during night and natural photosynthetically active radiation of 915 ± 10 µmol photons m^{-2} s^{-1} measured at midday).

Assessment of blast severity

The fourth and fifth leaves, from the top to the base, of each plant per replication of each treatment were marked and used to evaluate blast severity at 48, 72 and 96 hours after inoculation (hai) using the diagrammatic scale proposed by Rios et al. (2013).

Leaf gas exchange measurements

The leaf gas exchange parameters net CO_2 assimilation rate (A), stomatal conductance to water vapor (g_s), internal CO_2 concentration (C_i) and transpiration rate (E) were measured by using a portable open-flow gas exchange system (LI-6400XT; Li-Cor Inc., Lincoln, NE) at the attached fourth leaf, from the top to the base, of non-inoculated and at 72, 96 and 120 hours after inoculation of plants (growth stage 39) from 0900 to 1100 h (solar time). At this time, A was at its maximum under artificial photosynthetically active radiation (1000 µmol photons m^{-2} s^{-1} at the leaf level and 390

± 10 µmol atmospheric CO_2 mol^{-1}). All measurements were performed at 25°C and the vapor pressure deficit was maintained at approximately 1.0 kPa and the amount of blue light was set to 10% of the photosynthetic photon flux density to optimize the stomatal aperture.

Biochemical assays

The fourth and fifth leaves, from the base to the top, of each plant per replication of each treatment were collected between 1200 to 1400 (solar time) at 72, 96 and 120 hai, immediately frozen in liquid nitogen during samplings and then stored at -80°C until further analysis. Leaf samples were also collected from non-inoculated plants at the same sampling times.

Determination of the concentrations of sugars, starch, malate, fumarate and amino acids

A total of 10 mg of leaf tissue was lyophilized and homogenized with 500 µL of 100% ethanol, 500 µL of 80% ethanol and 500 µL of 50% ethanol and the mixture was incubated at 80°C for 30 min. The mixture was centrifuged at 13000 × *g* during 5 min and the supernatant was used to determine the concentrations of sucrose (Suc), fructose (Fru) and glucose (Glu) through NAD^+ reduction (Stittet al., 1989; Tretheweyet al., 1998). The pellet was washed with 1 mL of 80% ethanol, re-suspended with 400 µL of 1M KOH, incubated at 95°C for 60 min and neutralized with 70µL of 1M acetic acid to determine starch concentration (Tretheweyet al., 1998; Fernie et al., 2001). The concentrations of malate and fumarate were determined as described by Nunes-Nesiet al. (2007) and of proteins and amino acids according to Gibon et al. (2004).

Determination of the concentrations of total soluble phenolics (TSP), lignin-thioglycolic acid (LTGA) derivatives, malondialdedyde (MDA) and hydrogen peroxide (H_2O_2)

A total of 15 mg of leaf tissue was lyophilized and used to determine the concentrations of total soluble phenolics and LTGA derivatives according to Dallagnol et al. (2011). A total of 200 mg of fresh leaf tissue per sample was ground into a fine powder in a mortar and pestle with liquid N_2 and used to determine the concentrations of H_2O_2 and MDA as described by Cakmak & Horst (1991) and Bispo et al. (2015), respectively.

Determination of enzyme activities involved in the carbon and antioxidative metabolisms

A total of 300 mg of fresh leaf tissue per sample was ground into a fine powder in a mortar and pestle with liquid nitrogen to determine the activities ofascorbate peroxidases (APX, EC 1.11.1.11), catalases (CAT, EC 1.11.1.6), glutathione peroxidases (GPX, EC 1.11.1.9), glutathione reductases (GR, EC 1.8.1.7), glutathione-*S*-transferases (GST, EC 2.5.1.18), peroxidases (POX, EC 1.11.1.7), phenylalanine ammonia-lyases (PAL, EC 4.3.1.5), polyphenoloxidases (PPO, EC 1.10.3.1) and superoxide dismutases (SOD, EC 1.15.1.1) according to Bispo et al. (2015). The activities of sucrose-phosphate synthase (SPS, EC 2.4.1.14), fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11), ADP-glucose pyrophosphorylase (AGPase, EC 2.2.7.27), sucrose synthase (Susy, EC 2.4.1.13) as well as acid and alkaline invertases (EC 3.2.1.26) were determined according to Praxedes et al. (2006). The concentration of soluble proteins was determined following the method of Bradford (1976) using bovine serum albumin as a standard.

Metabolite profiling determined

The metabolite profiling was determined using an established gas chromatography-mass spectrometry (GC-MS) system. The extraction, derivatization, standard addition and samples injection were performed as described by Lisec et al. (2006). The metabolites were identified in comparison to database entries of authentic standards (Kopka et al., 2005). The metabolite levels were determined in a targeted fashion using the TargetSearch software package (Cuadros-Inostroza et al., 2009). In order to determine alterations of selected metabolites and visualize the metabolite profile heat maps, the package g plots in R and the web-based pipeline MetaboAnalyst version 3.0 was used (Xia et al., 2015).

Experimental design and data analysis

A 3 × 2 factorial experiment (Experiment 1), consisting of three wheat cultivars (BR-18, EMBRAPA-16 and BRS-Guamirim) and non-inoculated and inoculated plants, was arranged in a completely randomized design with six replications to evaluate blast severity and the parameters of leaf gas exchange. Another 3 × 2 factorial experiment (Experiment 2), consisting of three wheat cultivars and non-inoculated and inoculated plants, was arranged in a completely randomized design with six replications to obtain the leaf samples for the biochemical assays. All experiments were repeated at least twice. Considering that there was no significant effect of the factors cultivar (C) and sample time (ST), as well as the interaction C × ST for most of the variables and parameters evaluated for the non-inoculated plants (Table S1), their values were averaged per each evaluation and further used as the unique control treatment for the comparisons with the inoculated plants. Each experimental unit corresponded to a plastic pot containing six plants. Data from the variables and parameters evaluated were analyzed by ANOVA and means from the treatments were compared using the

test of *F*, Student´s *t* and Tukey ($P \leq 0.05$) (SAS version 6.12; SAS Institute, Inc., Cary, NC).

Results

Blast severity

Blast severity was significantly influenced by the factors cultivars (C), sampling times (ST) and the interaction C × ST (Table 1). Blast severity on plants from the three cultivars were similar on the two experiments (r = 0.97, P< 0.01) (Fig. 1A). Blast severity on plants from cultivar BRS-Guamirim was significantly higher by 40% in comparison to plants from cultivar EMBRAPA-16 at 96 hai. On plants from cultivars EMBRAPA-16 and BR-18, blast severity was significantly lower by 45 and 33%, respectively, in comparison to plants from cultivar BRS-Guamirim at 120 hai (Fig. 1B).

Leaf gas exchange parameters

The parameters A, g_s, C_i and E were significantly influenced by at least one of the factors studied and their interaction (Table 1). For the non-inoculated plants, there was no significant difference among cultivars for none of the leaf gas exchange parameters evaluated regardless of the evaluation time (Fig. S1). A significantly increased by 23 and 29% for cultivars EMBRAPA-16 and BR-18, respectively, in comparison to cultivar BRS-Guamirim at 96 hai. At 120 hai, A significantly increased by 32 and 23% for cultivars EMBRAPA-16 and BR-18, respectively, in comparison to cultivar BRS-Guamirim (Fig. 2A). There were significant decreases of 8 and 14% on g_s for cultivars EMBRAPA-16 and BRS-Guamirim, respectively, in comparison to cultivar BR-18 (Fig. 2B). For C_i, there were significant increases of 11 and 19% for cultivars EMBRAPA-16 and BRS-Guamirim, respectively, in comparison to cultivar BR-18.

A, g_s and E significantly decreased at 72, 96 and 120 hai in comparison to 0 hai regardless of the cultivar (Fig. 2C). C_i significantly decreased at 72 hai in comparison to 0 hai regardless of the cultivar (Fig. 2C).

Concentrations of carbohydrates, malate, fumarate and amino acids

The concentrations of carbohydrates (Glu, Fru, Suc and starch), malate, fumarate and amino acids were significantly influenced by at least one of the factors studied and their interaction (Table 1). For the non-inoculated plants, there was no significant difference among cultivars for the concentrations of carbohydrates, malate, fumarate and amino acids regardless of the evaluation time (Fig. S2). The Glu concentration significantly increased by 38 and 44%, respectively, for cultivars EMBRAPA-16 and BRS-Guamirim in comparison to cultivar BR-18 at 0 hai. At 96, Glu concentration significantly decreased by 75 and 201%, respectively, for cultivars BR-18 and EMBRAPA-16 in comparison to cultivar BRS-Guamirim. At 120 hai, Glu concentration significantly decreased by 67 and 40%, respectively, for cultivars BR-18 and BRS-Guamirim in comparison to cultivar EMBRAPA-16. The Fru concentration significantly decreased by 188 and 179% at 0 hai, by 59 and 152% at 96 hai and by 45 and 205% at 120 hai for cultivars BR-18 and EMBRAPA-16, respectively, in comparison to cultivar BRS-Guamirim. At 120 hai, Fru concentration significantly increased by 53 and 67%, respectively, for cultivars BR-18 and BRS-Guamirim in comparison to cultivar EMBRAPA-16. The Suc concentration significantly increased by 38 and 39% at 96 hai and by 47 and 38% at 120 hai for cultivars BR-18 and BRS-Guamirim, respectively, in comparison to cultivar EMBRAPA-16. For cultivar BR-18, the starch concentration significantly decreased by 102% in comparison to cultivar BRS-Guamirim at 72 hai. At 0 hai, fumarate concentration significantly increased by 20 and 26%, respectively, for cultivars BR-18 and EMBRAPA-16 in comparison to cultivar BRS-Guamirim. At 72 hai, fumarate concentration significantly increased by 37 and 31%, respectively, for cultivars BR-18 and BRS-Guamirim in comparison to cultivar EMBRAPA-16. At 120 hai, fumarate

concentration significantly increased by 57 and 49% for cultivars EMBRAPA-16 and BRS-Guamirim, respectively, in comparison to cultivar BR-18. The amino acids concentration significantly decreased by 50% for cultivar BR-18 in comparison to cultivar EMBRAPA-16 at 120 hai.

The Glu, Fru and malate concentrations significantly increased at 72, 96 and 120hai in comparison to 0 hai regardless of the cultivar. The Suc concentration significantly increased at 72 hai, but decreased at 96 and 120 hai in comparison to 0 hai regardless of the cultivar. At 72 hai, starch concentration significantly increased in comparison to 0 hai regardless of the cultivar. In comparison to 0 hai, fumarate concentration significantly increased at 72 hai only for cultivars BR-18 and BRS-Guamirim and at 96 hai for all cultivars, but significantly decreased only for cultivar BR-18 at 120 hai. In comparison to 0 hai, the amino acids concentration significantly increased at 96 hai regardless of the cultivar and at 120 hai only for cultivars EMBRAPA-16 and BRS-Guamirim.

Activities of enzymes involved in the carbon metabolism

The SPS, acid invertase, alkalin invertase, SuSy, AGPase and FBPase activities were significantly influenced by at least one of the factors studied and their interaction (Table 1). For the non-inoculated plants, there was no significant difference among cultivars for none of the enzymes involved in the carbon metabolism regardless of the evaluation time (Fig. S3). At 0 hai, SPS, alkaline invertase, SuSy, AGPase and FBPase activities were significantly reduced by 37, 35, 93, 72 and 41% for cultivar EMBRAPA-16 and by 27, 33, 43, 51 and 106% for cultivar BRS-Guamirim, respectively, in comparison to cultivar BR-18. At 72 hai, alkaline invertase and AGPase activities were significantly reduced by 53 and 128% for cultivar EMBRAPA-16 and by 27 and 39% for cultivar BRS-Guamirim, respectively, in comparison to

cultivar BR-18. At 96 hai, FBPase activity was significantly reduced by 68 and 46%, respectively, for cultivars EMBRAPA-16 and BRS-Guamirim in comparison to cultivar BR-18. The SPS activity significantly decreased by 75 and 32% for cultivars BR-18 and BRS-Guamirim, respectively, in comparison to cultivar EMBRAPA-16 at 72 hai. The SPS activity significantly decreased by 33% for cultivar BR-18 in comparison to cultivar BRS-Guamirim at 72 hai. The acid invertase activity significantly decreased by 33 and 30% at 96 hai, respectively, for cultivars BR-18 and EMBRAPA-16 in comparison to cultivar BRS-Guamirim. The alkaline invertase activity significantly decreased by 114 and 43%, respectively, for cultivars BR-18 and BRS-Guamirim in comparison to cultivar EMBRAPA-16 at 96 hai. The SuSy activity significantly decreased by 35% for cultivar EMBRAPA-16 in comparison to cultivar BRS-Guamirim at 0 hai. The SuSy activity was significantly increased by 69 and 71%, respectively, for cultivars BR-18 and BRS-Guamirim in comparison to cultivar EMBRAPA-16 at 72 hai and by 68 and 61% for cultivars EMBRAPA-16 and BRS-Guamirim, respectively, in comparison to cultivar BR-18 at 96 hai. The AGPase activity significantly decreased by 64% for cultivar EMBRAPA-16 in comparison to cultivar BRS-Guamirim at 72 hai. The FBPase activity significantly decreased by 46% for cultivar BRS-Guamirim in comparison to cultivar EMBRAPA-16 at 0 hai.

In comparison to 0 hai, SPS activity significantly increased only for cultivars EMBRAPA-16 and BRS-Guamirim at 72 and 96 hai and for all cultivars at 120 hai. At 72 and 120 hai, acid invertase, alkaline invertase and SuSy activities increased for all cultivars in comparison to 0 hai. At 96 hai, acid invertase activity increased for all cultivars in comparison to 0 hai. In comparison to 0 hai, AGPase activity significantly increased only for cultivars EMBRAPA-16 and BRS-Guamirim and the FBPase activity significantly increased only for cultivar BRS-Guamirim at 120 hai.

Activities of enzymes involved in the antioxidative metabolism

The APX, CAT, POX, SOD, GPX, GR and GST activities were significantly influenced by at least one of the factors studied and their interaction (Table 1). For the non-inoculated plants, there was no significant difference among cultivars for none of the enzymes involved in the antioxidative metabolism regardless of the evaluation time (Fig. S4). POX activity significantly increased by 39 and 36%, respectively, for cultivars EMBRAPA-16 and BRS-Guamirim in comparison to cultivar BR-18 at 72 hai, by 37% for cultivar BRS-Guamirim in comparison to cultivar BR-18 at 96 hai and by 88% for cultivar EMBRAPA-16 in comparison to cultivar BRS-Guamirim at 120 hai. GPX activity significantly increased by 57% for cultivar BR-18 in comparison to cultivar BRS-Guamirim at 0 hai and by 82 and 85%, respectively, for cultivars BR-18 and BRS-Guamirim in comparison to cultivar EMBRAPA-16 at 72 hai. There were significant decreases of 93 and 85% for GPX activity at 96 hai and of 436 and 353% at 120 hai, respectively, for cultivars BR-18 and BRS-Guamirim in comparison to cultivar EMBRAPA-16. GR activity significantly decreased by 120 and 217% at 0 hai and by 144 and 55% at 96 hai, respectively, for cultivars BR-18 and BRS-Guamirim in comparison to cultivar EMBRAPA-16. There were significant increases of 51 and 38% at 72 hai and of 26 and 39% at 120 hai, respectively, for cultivars EMBRAPA-16 and BRS-Guamirim in comparison to cultivar BR-18. GR activity significantly increased by 36% for cultivar BRS-Guamirim in comparison to cultivar BR-18 at 96 hai. GST activity significantly decreased by 120 and 73% at 0 hai and by 68 and 27% at 96 hai, respectively, for cultivars EMBRAPA-16 and BRS-Guamirim in comparison to cultivar BR-18. GST activity significantly increased by 24% for cultivar BRS-Guamirim in comparison to cultivar EMBRAPA-16 at 96 hai and by 35% for cultivar EMBRAPA-16 in comparison to cultivar BR-18 at 72 hai.

In comparison to 0 hai, APX and CAT activities did not significantly increase only for cultivars BR-18 and EMBRAPA-16 at 72 hai. At 72, 96 and 120 hai, POX activity significantly increased in comparison to 0 hai regardless of the cultivar. In comparison to 0 hai, GPX activity did not significantly increase only for cultivar BRS-Guamirim at 72 hai and for cultivars BR-18 and BRS-Guamirim at 120 hai. At 72 hai, GR and GST activities significantly increased only for cultivars BRS-Guamirim and EMBRAPA-16 in comparison to 0 hai. In comparison to 0 hai, GR activity significantly increased for all cultivars at 96 and 120 hai. At 120 hai, GST activity significantly increased only for cultivar EMBRAPA-16 in comparison to 0 hai.

Concentrations of H_2O_2 and MDA

The concentrations of H_2O_2 and MDA were significantly influenced by at least one of the factors studied and their interaction (Table 1). For the non-inoculated plants, there was no significant difference among cultivars for the concentrations of H_2O_2 and MDA regardless of the evaluation time (Fig. S4). The concentration of H_2O_2 significantly decreased by 66 and 83% for cultivars BR-18 and BRS-Guamirim, respectively, in comparison to cultivar EMBRAPA-16 at 96 hai. At 120 hai, the concentration of H_2O_2 significantly increased by 52 and 55% for cultivars EMBRAPA-16 and BRS-Guamirim, respectively, in comparison to cultivar BR-18. The MDA concentration significantly increased by 29 and 26% at 72 hai and by 40 and 30% at 96 hai for cultivars EMBRAPA-16 and BRS-Guamirim in comparison to cultivar BR-18.

In comparison to 0 hai, H_2O_2 concentration significantly increased only for cultivar EMBRAPA-16 at 96 hai and for cultivars EMBRAPA-16 and BRS-Guamirim at 120 hai. In comparison to 0 hai, MDA concentration significantly increased only for cultivar EMBRAPA-16 at 96 hai and for all cultivars at 120 hai.

Concentrations of TSP and LTGA derivatives and the PAL and PPO activities

The concentrations of TSP and LTGA derivatives and the activities of PAL and PPO were significantly influenced by at least one of the factors studied and their interaction (Table 1). For the non-inoculated plants, there was no significant difference among cultivars for the concentrations of TSP and LTGA derivatives as well as for the activities of PAL and PPO regardless of the evaluation time (Fig. S5). The TSP concentration significantly increased by 22 and 28% for cultivars BR-18 and BRS-Guamirim, respectively, in comparison to cultivar EMBRAPA-16 and by 7% for cultivar BRS-Guamirim in comparison to cultivar BR-18 at 0 hai. At 120 hai, the TSP concentration significantly increased by 20% for cultivar EMBRAPA-16 in comparison to cultivar BRS-Guamirim. The LTGA derivatives concentration significantly increased by 23 and 37% at 0 hai and by 23 and 24% at 96 hai, respectively, for cultivars EMBRAPA-16 and BRS-Guamirim in comparison to cultivar BR-18. The LTGA concentration significantly increased by 19 and 9% at 0 and 120 hai, respectively, for cultivar BRS-Guamirim in comparison to cultivar EMBRAPA-16. The LTGA concentration significantly increased by 13 and 36%, respectively, for cultivars BR-18 and EMBRAPA-16 in comparison to cultivar BRS-Guamirim at 72 hai. The LTGA concentration significantly decreased by 59 and 44%, respectively, for cultivars EMBRAPA-16 and BRS-Guamirim in comparison to cultivar BR-18 at 120 hai. PAL activity significantly decreased by 52 and 72% at 0 hai and by 111 and 72% at 96 hai, respectively, for cultivars EMBRAPA-16 and BRS-Guamirim in comparison to cultivar BR-18. At 72 hai, PAL activity significantly decreased by 156 and 167%, respectively, for cultivars BR-18 and BRS-Guamirim in comparison to cultivar EMBRAPA-16. PPO activity significantly decreased by 29 and 61%, respectively, for cultivars BR-18 and BRS-Guamirim in comparison to

EMBRAPA-16 and by 25% for cultivar BRS-Guamirim in comparison to cultivar BR-18 at 0 hai. PPO activity significantly increased by 47 and 42% at 72 hai and by 19 and 32% at 96 hai, respectively, for cultivars EMBRAPA-16 and BRS-Guamirim in comparison to cultivar BR-18. PPO activity significantly increased by 27% for cultivar EMBRAPA-16 in comparison to cultivar BRS-Guamirim at 72 hai and significantly decreased by 47 and 33% for cultivars EMBRAPA-16 and BRS-Guamirim, respectively, in comparison to cultivar BR-18.

In comparison to 0 hai, the TSP and LTGA derivatives concentrations did not significantly increase only for cultivar BRS-Guamirim at 72 hai. The TSP concentration significantly increase only for cultivar BR-18 at 96 hai and for cultivars BR-18 and BRS-Guamirim at 120 hai in comparison to 0 hai.The LTGA derivatives concentration significantly increased only for cultivar EMBRAPA-16 at 96 hai and for cultivars BR-18 and EMBRAPA-16 at 120 hai in comparison to 0 hai. PAL activity did not significantly increase only for cultivar EMBRAPA-16 at 72 hai, but significantly increased for all cultivars at 120 hai in comparison to 0 hai. The PPO activity did not significantly increase only for cultivar BR-18 at 72 hai and for cultivars BR-18 and EMBRAPA-16 at 96 hai in comparison to 0 hai.

Metabolic profile

The levels of the metabolites classified in the groups of amino acids, organic acids, sugars, sugar-alcohols and polyamines were significantly influenced by at least one of the factors studied and their interaction (Table 2). The metabolite profile of theinoculated plants from the three cultivars resulted in the identification of 57annotated metabolites which included 18 amino acids, 19 organic acids, 11 sugars, 7 sugar alcohols and 2 polyamines. The significance in terms of abundance of the non-identified metabolites associated factors (cultivars and sampling times and their

interaction) are shown in Table 2 and Fig. 7A, C and E.Considerable differences in the levels of amino acids were found for the inoculated plants. The levels of alanine, arginine, cysteine, glutamine, histidine, homoserine, isoleucine, leucine, methionine, ornithine, phenylalanine, proline, tryptophan and valine were higher on the leaves of plants from the 3 cultivars at 96 and 120 hai in comparison to the non-inoculated plants (0 hai). There was a significant increase in the level of glycine only at 72 hai regardless of the cultivar. For plants from cultivars EMBRAPA-16 and BRS-Guamirim, the level of serine was higher at 72 and 96 hai and the level of thymine at 120 hai (Fig. 7B). In comparison to plants from cultivars EMBRAPA-16 and BRS-Guamirim, plants from cultivar BR-18 showed higher increases in the levels of butanoic acid, fumaric acid, gluconic acid, glutaric acid, guanosine, malonic acid, malic acid, ribonic acid and succinic acid at 96 and 120 hai. On the other hand, significant reductions in the levels of aconitic acid, glycolic acid and citric acid occurred at 72, 96 and 120 hai forplants from cultivars EMBRAPA-16 and BRS-Guamirim in comparison to the non-inoculated plants from these cultivars. For cultivar BR-18 at 120 hai, the levels of cinnamic acid and oxaloacetate were higher in contrast to the other two cultivars (Fig. 7D). For inoculated plants from the three cultivars, there were significant reductions in the levels of inositol, loganin, sorbitol, galactinol and raffinose at 120 hai in comparison to the non-inoculated plants. The levels of fructose, glucose, mannose, mannitol, arabinose, idose, erythritol and ribose were high for the inoculated plants from cultivars BR-18 and BRS-Guamirim. The levels of mannitol, idose, erythritol and ribose were higher only for plants from cultivar EMBRAPA-16 at 120 hai. Considerable increases in the levels of sophorose, glycerol and maltose occurred for inoculated plants from cultivar BR-18 (Fig. 7F).

The relative content of putrescine significantly decreased by 82 and 64% at 72 hai and by 67 and 93% at 96 hai for cultivars BR-18 and EMBRAPA-16 in comparison to cultivar BRS-Guamirim. At 120 hai, the relative content of putrescine significantly increased by 50 and 53%, respectively, for cultivars BR-18 and BRS-Guamirim in comparison to cultivar EMBRAPA-16. At 72 hai, the relative content of spermidine significantly increased by 32 and 35%, respectively, for cultivars BR-18 and BRS-Guamirim in comparison to cultivar EMBRAPA-16 (Fig. S6).

In comparison to 0 hai, therelative content of putrescine significantly increased for all cultivars at 72, 96 and 120 hai. In comparison to 0 hai, therelative content of spermidine significantly increase for all cultivars at 72 hai, only for cultivars BR-18 and EMBRAPA-16 at 96 hai and only for cultivar BR-18 at 120 hai (Fig. S6).

Discussion

The present study brings novel insights into the alterations on the leaves of wheat plants from three cultivars differing on their basal level of resistance to blast at biochemical, physiological and metabolic levels upon being infected by *P. oryzae*. Currently, wheat blast management is based on the use of cultivars with a desirable level of resistance in association with fungicides application (Cruz et al., 2010; Castroagudín et al., 2015; Rios et al., 2016). However, alterations in the genetic structure of populations of *P. oryzae*, in conjunction with the occurrence of environmental conditions favorable for blast epidemics in most of the wheat growing regions, put the release of cultivars with high level of partial resistance at increased risk for becoming more susceptible to the disease (Castroagudín et al., 2016; Cruz et al., 2016; Maciel et al., 2014). In the present study, the cultivars BR-18, EMBRAPA-16 and BRS-Guamirim showed differential response to blast being the cultivar BRS-Guamirim more susceptible to blast in comparison to cultivars BR-18 and EMBRAPA-16 confirming, therefore, their level of partial resistance to the disease as previously reported (Maciel et al., 2008; Cruz et al., 2010).

Since the beginning of fungal infection, reductions in the values of*A*coupled with the lower values for both g_s and E were more evident for cultivar BRS-Guamirim, considered to be the most susceptible to blast, in contrast to cultivars BR-18 and EMBRAPA-16. It has been reported that an increase on blast severity was negatively correlated with both A and g_s suggesting, therefore, the deleterious effect of *P. oryzae* infection to lower the photosynthetic performance of the plants (Debona et al., 2014; Aucique-Pérez et al., 2014). Additionally, the increase in the C_i values at advanced stages of fungal infection, especially for cultivar BRS-Guamirim, confirmed the biochemical limitations that were associated with the reduction of Rubisco activity to

restrict the CO_2 influx into the carboxylation sites in the chloroplasts (Debona et al., 2014). This finding corroborates with what was reported for the maize-*Stenocarpella macrospora*, rice-*P. oryzae* and oil palm-*Phytophthora palmivora* interactions (Moreno-Chacón et al., 2013, Bermúdez-Cardona et al., 2015; Domiciano et al., 2015). Moreover, the reduced photosynthetic performance of the infected plants was associated with both structural and functional damage to the photochemical machinery. For the wheat-*P. oryzae* interaction, there was a chronic photoinhibition of the photosynthesis (lower F_v/F_m values) coupled to decreases in the efficiency of the excitation energy captured by the open PSII reaction centers (lower F_v'/F_m' values) as well as perturbation in the electron transfer rate (lower ETR values) and higher non-photochemical quenching (high NPQ values) (Aucique-Pérez et al., 2014).

Perturbations in the CO_2 assimilation on leaves infected by pathogens are coupled with changes in the carbohydrate metabolism in a way of manipulate the host´s physiologyin benefit of the pathogen to acquirethe necessary nutrients to infect the tissues successfully (Major et al., 2010). In the present study, different events determined the alterations onthe carbohydrate metabolism of the infected plants. During the asymptomatic phase of *P. oryzae* infection (from 24 to 72 hai), high concentrations of sucrose and starch and greater SPS and AGPase activities (except FBPase) were the common features for the three cultivars. An increase in the concentrations of sucrose, glucose and fructose was noticed on the leaves of different grasses before the occurrence of blast symptoms (Parker et al., 2009). This finding corroborates with the decrease in the *A* values on leaves infected by many pathogens and the accumulation of carbohydrates disrupts, therefore, the translocation of assimilates to the sink sources (Berger et al., 2004; Moreno-Chacón et al., 2013). The susceptibility of mango plants from cultivar Palmer cultivar to infection by

Ceratocystis fimbriata was associated with spatial and temporal inability of the infected plants to mobilize the carbon skeletons necessary for the production of compounds related to host defense and their reduced photosynthetic performance (Bispo et al., 2016).

Here, higher activities of SuSy, acid invertase and alkaline invertase on infected leaves of plants from the three cultivars correspondedto an increase in the pools of glucose and fructose and in a decrease on the export of assimilates from the infected tissues. This findings confirms the importance for plants of maintaining a high invertase activity upon infection by pathogens (Biemelt and Sonnewald, 2006; Bolton, 2009; Bispo et al., 2016). The repression of invertase in the roots of tobacco plants impaired and delayed defence-related processes in response to *Phytophthora nicotianae* infection suggesting the importance of hexoses as a component of the signaling system during host defense (Essmann et al., 2008). However, pathogens are able to use sucrose from their hosts as a source of hexoses due to the action of sucrolytic enzymes, a fact that establishes a dichotomy of the origin of hexoses during the infection process of the pathogens (Bolton, 2009). In the leaves of sunflower infected with *Sclerotinias clerotiorum*, the decrease in the concentration of sucrose was linked with the expression of the genes coding for invertase and its corresponding enzyme (Jobic et al., 2007). The manipulation of the metabolism of plants by the effectors released during the infection process of pathogens may suppress the invertase activity in order to prevent hexose-mediated defense signaling (Biemelt and Sonnewald, 2006). The relationship between an increase on invertase activity and the success of the infection of a certain pathogen on its host need to be better investigated (Berger et al., 2007).

The inositol, galactinol, raffinose and sorbitol are involved in helping plants suffering from osmotic stress (Nishizawa et al., 2008). In the present study, reduction in the abundance of these metabolites were related to the metabolic failure of wheat plants to counteract against *P. oryzae* infection coupled with their lower antioxidant capacity that was not sufficient to minimize the metabolic problems.

Plants respond against pathogen infection through the generation of reactive oxygen species (ROS), especially the hydrogen peroxide (H_2O_2) (Torres et al., 2006; Torres, 2010). At the beginning of the infection process, the ROS are involved in the activation of host defense mechanisms, but can show antimicrobial properties at advanced stages of pathogen infection (Heller and Tudzynski, 2011). An imbalance on water status on the plant tissues and the limitation on the concentration of CO_2 in the chloroplasts coupled with an excess of light and a decrease in the consumption of electrons released by the oxidation ofwater during the photochemical process favor the formation and accumulation of ROS in the stressed plants (Das and Roychoudhury, 2014). In the present study, the increase in the H_2O_2 concentration on the leaves of infected plants from cultivars BRS-Guamirim and EMBRAPA-16 at advanced stages of fungal infection was associated with lower activities of SOD, APX, CAT and POX involved in the metabolism of the ROS. Therefore, the inability of the antioxidant metabolism of the infected plants to metabolize the excess of H_2O_2 greatly contributed to the increase in the MDA concentration. High blast severity on plants from a susceptible cultivar was associated with the lower antioxidant capacity of these plants (reduced activities of SOD, POX, APX, GST, GR, and CAT) in contrast to plants from a more resistant cultivar (Debona et al., 2012). In the present study, increases in APX, CAT, POX, SOD, GR and GST activities occurred on plants from the three cultivars at 72 hai explaining, therefore, the reduced concentrations of both H_2O_2 and MDA that

resembled the non-inoculated plants. This finding indicates an early participation of these enzymes to counteract the deleterious action of *P. oryzae* infection, which were of less importance as the disease developed. On plants from cultivar EMBRAPA-16, blast severity was reduced due to the greater antioxidant capacity of these plants in comparison to the other cultivars. This finding confirms that an efficient antioxidative system that relieve the oxidative stress generated *P. oryzae* infection is an important component of wheat resistance to blast (Debona et al., 2012). On plants suffering from biotic stress, the GPX plays a pivotal role in the biosynthesis of lignin as well as preventing the degradation of indole acetic acid by using H_2O_2 in the process. Meanwhile, the GR activity is of crucial importance on the ASC-GSH cycle for being involved in the formation of a disulfide bond in glutathione disulfide in order to maintain a high cellular GSH/GSSG ratio (Das and Roychoudhury, 2014). According to Ge et al. (2013), the GR activity in the leaves of melon plants from a resistant cultivar was higherin response to *Colletotrichum lagenarium* infection. In the present study, the GPX and GR activities were considerably higher on infected plants from cultivar EMBRAPA-16 confirming its high level of resistance to blast.

The production of defense compounds by plants to relieve the negative effects of both abiotic and biotic types of stress demand a great amount of carbon skeletons and nutrients from primary metabolism, therefore a balance between the allocation of photoassimilates and nutrients must exist between plant growth and their strategies for defense (Sumbele et al., 2012). The *L*-phenylalanine is converted to *trans*-cinnamic acid by PAL resulting, therefore, in the synthesis of various phenolics with the lignin as the final product (Campbell and Sederoff, 1996). PPO and POX are involved in the polymerization of phenolics that culminates in the production of lignin (Mohammadi and Kazemi, 2002). According to the present study, the increase in the concentrations

of TPS and LTGA derivatives for plants from cultivars EMBRAPA-16 and BR-18 was supported by the greater PAL and PPO activities, especially in the former cultivar. An increase of 9 folds in the expression of the gene *PAL*was noticed on the leaves of wheat plans infected with *P. oryzae* confirming, therefore, the importance of the PAL enzyme for wheat resistance to blast (Cruz et al. 2016). Cai et al. (2008) found that reduced blast severity on rice plants from an isogenic line was associated with higher PAL and PPO activities and great lignin concentration.

The analyses of the metabolic profile of plants is an interesting way to better understand the mechanisms involved in their resistance when exposed to conditions of abiotic or biotic stress. Comparative metabolomic analyses of rice and other grasses with blast symptoms highlights that the biochemical alterations were conserved in the different host species of *Pyricularia* sp. (Parker et al., 2009; Jones et al., 2011). In the present study, the levels of amino acids, organic acids and sugar were raised on the infected leaves indicating that the metabolism of plants was reprogrammed and confirms what was reported for the rice-*P. oryzae* and *Brachypodium distachyon-P. grisea* interactions (Parker et al., 2009; Jones et al., 2011). It is important to stress out that the differential response of the three wheat cultivars against *P. oryzae* infection allowed to confirm their resistance or susceptibility to blast based on the analyses of the metabolic profile.

The amino acid metabolism has been associated with the resistance of plants to pathogens (Zeier, 2013). In the present study, thelevels of amino acids derived from glycolytic intermediates (*e.g.* alanine, cysteine, phenylalanineand valine) were greater for infected plants from cultivars BR-18 and BRS-Guamirim in comparison to their counterparts. Likewise, the levels of methionine, proline, histidine and tryptophan also increased for infected plants from cultivars BR-18 and BRS-Guamirim in comparison

to their counterparts. Rice plants were more susceptible against *P. grisea* and *Rhizoctonia solani* due to an increase in the levels of glutamate, glutamine, aspartate, pyruvate, GABA, glycine, histidine, phenylalanine, serine, tryptophan and tyrosine (Parker et al., 2009; Suharti et al., 2016). According to Salomon et al. (2003), high levels of methionine, arginine, tryptophan, cysteine, proline and histidine were important for the nutrition of biotrophic pathogens on their hosts. It is plausible to hypothesize that at the hemibiotrophic phase of *P. oryzae*, there will be a manipulation of the wheat plants by the fungus to obtain the necessary amino acids to favor infection and, consequently, a greater disease development.

Polyamines are amines involved in the control of cells division, plant growth, differentiation of leaves and flowers as well as in the host defense responses against pathogens infection (Aribaud et al., 1999; Walters, 2003). In the present study, it was observed drastic alterations in the metabolism of polyamines on infected leaves, especially for plants from cultivar BRS-Guamirim which culminated with great levels of putrescine and spermidine and with their precursor ornithine. It can be suggested that both putrescine and spermidine were rapidly accumulated in the infected leaf tissues and reflected in a more susceptibility of plants to blast, especially from cultivar BRS-Guamirim. An increase in the level of putrescine occurred in the roots of oil palm at advanced stage of *Phytophthora palmivora* infection whereas the levels of spermidine and spermine were kept at high levels during the entire infection process of the pathogen (Moreno-Chacón et al., 2013). For many host-pathogen interactions, the accumulation of H_2O_2 on infected tissues results from the polyamines catabolism and nitric oxide due to the induction of spermine/spermidine signaling pathways (Walters, 2003; Yamasaki and Cohen, 2006; Hussain et al., 2011).

In comparison to the non-inoculated plants, the metabolism of organic acids was not a common pattern among cultivars even though some metabolites showed slight increase on their levels during the infection process of *P. oryzae*. For cultivar BR-18, some metabolic intermediates of the TCA cycle such asoxaloacetic acid, malic acid, fumaric acid and succinic acid increased their levels on infected leaves, but for cultivars EMBRAPA-16 and BRS-Guamirim the levels of these metabolites was kept stable or tended to decrease during the infection process of *P. oryzae*. Reductions in the levels of citric acid and aconitic acid on plants from cultivars EMBRAPA-16 and BRS-Guamirim suggested that the TCA cycle was compromised with consequent reduction in the levels of other metabolites necessary for the synthesis of amino acids that are possibly involved in host defense responses. Interestingly, the continuous accumulation of malate on infected leaves indicated the importance of this metabolite as a source of energy to keep the several metabolic processes active (Casati et al., 1999). From the malate catabolism, CO_2, pyruvate and NADPH are produced which become important for respiration and compounds related to host defense such as phenolics, lignin and flavonoids (Casati et al., 1999). Paker et al. (2009) reported that an increasein the number of TCA cycle intermediates occurred during the asymptomatic phase of *P. oryzae* on rice leaves and indicated the capacity of the fungus to reprogram the metabolism of its host as well as to promote the transport of metabolites to the non-colonized tissues.

The results from the present study allow to conclude that the infection of wheat leaves by *P. oryzae* induced remarkable disturbances in the primary metabolism and some of them were conserved among the cultivars regardless of their basal level of resistance to blast. Moreover, for the most resistant cultivar, low concentrations of sucrose and starch, less damage to the photosynthesis and an improved antioxidative

metabolism that lowered the production of ROS allowed the plants to better counteract against *P. oryzae* infection through an investment in mechanisms of defense such as greater PAL and PPO activities and the high concentrations of phenolics and lignin.

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different ($P \leq 0.05$) according to Tukey's test. Bars represent the standard deviation of means. n = 10 and 20, respectively, for graphics A and B.

Figure 2. Net CO_2 assimilation rate (A) (A), stomatal conductance to water vapor (g_s) (B), internal CO_2 concentration (C_i) (C) and transpiration rate (E) (D) determined in the leaves of wheat plants from cultivars BR-18, EMBRAPA-16 (EMB-16) and BRS-Guamirim (BRS-G) non-inoculated (0 hours after inoculation (hai) or at 72, 96 and 120 hai with *Pyricularia oryzae*. Means from the three cultivars, at each evaluation time, followed by different letters are significantly different ($P \leq 0.05$) according to Tukey's test.For each parameter, mean from each cultivar at 72, 96 and 120 hai with an asterisk (*) is significantly different from the respective cultivar at 0 hai according to Ftest ($P \leq 0.05$).Bars represent the standard deviation of means. n = 6.

Figure 3. Concentrations of glucose (A), fructose (B), sucrose (C), starch (D), malate (E), fumarato (F) and amino acids (G) in the leaves of wheat plants from cultivars BR-18, EMBRAPA-16 (EMB-16) and BRS-Guamirim (BRS-G) non-inoculated (0 hours after inoculation (hai) or at 72, 96 and 120 hai with *Pyricularia oryzae*. Means from the three cultivars, at each evaluation time, followed by different letters are significantly different ($P \leq 0.05$) according to Tukey's test. For each parameter, mean from each cultivar at 72, 96 and 120 hai with an asterisk (*) is significantly different from the respective cultivar at 0 hai according to F test ($P \leq 0.05$).Bars represent the standard deviation of means. n = 6. DW = dry weight.

Figure 4. Activities of sucrose-phosphate synthase (SPS) (A), acid invertase (B), alkaline invertase (C), sucrose synthase (Susy) (D), ADP-glucose pyrophosphorylase (AGPase) (E) and fructose 1,6-bisphosphatase (FBPase) (F) in the leaves of wheat plants from cultivars BR-18, EMBRAPA-16 (EMB-16) and BRS-Guamirim (BRS-G)

non-inoculated (0 hours after inoculation (hai) or at 72, 96 and 120 hai with *Pyricularia oryzae*. Means from the three cultivars, at each evaluation time, followed by different letters are significantly different ($P \leq 0.05$) according to Tukey's test. For each parameter, mean from each cultivar at 72, 96 and 120 hai with an asterisk (*) is significantly different from the respective cultivar at 0 hai according to *F* test ($P \leq 0.05$).Bars represent the standard deviation of means. $n = 6$. FW = fresh weight.

Figure 5. Activities of ascorbate peroxidase (APX) (A), catalase (CAT) (B), peroxidase (POX) (C), superoxide dismutase (SOD) (D), glutathione peroxidase (GPX) (E), glutathione reductase (GR) (F) and glutathione-*S*-transferase (GST) (G) as well as concentrations of hydrogen peroxide (H_2O_2)(H) and malondialdehyde (MDA) (I) in the leaves of wheat plants from cultivars BR-18, EMBRAPA-16 (EMB-16) and BRS-Guamirim (BRS-G) non-inoculated (0 hours after inoculation (hai) or at 72, 96 and 120 hai with *Pyricularia oryzae*. Means from the three cultivars, at each evaluation time, followed by different letters are significantly different ($P \leq 0.05$) according to Tukey's test. For each parameter, mean from each cultivar at 72, 96 and 120 hai with an asterisk (*) is significantly different from the respective cultivar at 0 hai according to *F* test ($P \leq 0.05$).Bars represent the standard deviation of means. $n = 6$. FW = fresh weight.

Figure 6. Concentrations of total soluble phenols (TSP) (A) andlignin-thioglycolic acid derivatives (LTGA; B) as well as activities of phenylalanine ammonia-lyases (PAL) (C) and polyphenoloxidases (PPO) (D) in the leaves of wheat plants from cultivars BR-18, EMBRAPA-16 (EMB-16) and BRS-Guamirim (BRS-G) non-inoculated (0 hours after inoculation (hai) or at 72, 96 and 120 hai with *Pyricularia oryzae*. Means from the three cultivars, at each evaluation time, followed by different

letters are significantly different ($P \leq 0.05$) according to Tukey's test. For each parameter, mean from each cultivar at 72, 96 and 120 hai with an asterisk (*) is significantly different from the respective cultivar at 0 hai according to *F* test ($P \leq 0.05$).Bars represent the standard deviation of means. $n = 6$. FW = fresh weight.

Figure 7. Metabolic profiles of amino acids, organic acids, sugars and sugar-alcohols in the leaves of wheat plants from cultivars BR-18, EMBRAPA-16 (EMB-16) and BRS-Guamirim (BRS-G)] non-inoculated (0 hours after inoculation (hai) or at 72, 96 and 120 hai with *Pyricularia oryzae*. Heat maps represent the significant changes in metabolites for each cultivar at 0, 72, 96 and 120 hai) (B, D and F). Normalized signal intensities (log transformed) can be visualized as a color spectrum and the scale from least abundant to highest ranges is from -3.0 or -2.0 to 2.0 or 3.0. The red and green colors of the title indicate, respectively, high and low abundance of the detected metabolites. Venn diagram shows the amount of metabolites that are significantly ($P \leq 0.05$) for the factors cultivar, sampling time and their interaction for amino acids (A), organic acids (C) and sugars and sugar-alcohols (E).

Supplementary Material

Table and Figures

Table S1. Analysis of variance of the effects of cultivars (C), sampling times (ST) and the interaction C × ST for non-inoculated plants on leaf gas exchange parameters (net CO_2 assimilation rate [*A*], stomatal conductance to water vapor [g_s], internal CO_2 concentration [C_i] and transpiration rate [*E*]); concentration of carbohydrates (glucose, fructose, sucrose and starch), malate, fumarate and amino acids; activities of enzymes involved in the carbon metabolism (acid invertase, alkaline invertase, sucrose-phosphate synthase [SPS], sucrose synthase [SuSy], fructose-1,6-bisphosphatase [FBPase] and ADP-glucose pyrophosphorylase [AGPase]) and in the antioxidative metabolism (ascorbate peroxidase [APX], catalase [CAT], peroxidase [POX], superoxide dismutase [SOD], glutathione peroxidase [GPX], glutathione reductase [GR] and glutathione-*S*-transferase [GST]); concentrations of hydrogen peroxide (H_2O_2), malondialdehyde (MDA), total soluble phenolics (TSP) and lignin-thioglycolic acid (LTGA) derivatives as well as activities of phenylalanine ammonia-lyases (PAL) and polyphenoloxidases (PPO).

Figure S1. Net CO_2 assimilation rate (*A*) (A), stomatal conductance to water vapor (g_s) (B), internal CO_2 concentration (C_i) (C) and transpiration rate (*E*) (D) determined in the leaves of wheat plants from cultivars BR-18, EMBRAPA-16 (EMB-16) and BRS-Guamirim (BRS-G) non-inoculated with *Pyricularia oryzae*. Plants from each cultivar were evaluated at the same time as for the inoculated plants (72, 96 and 120 hours after inoculation). Bars represent the standard deviation of means. $n = 6$.

Figure S2. Concentrations of glucose (A), fructose (B), sucrose (C), starch (D), malate (E), fumarato (F) and amino acids (G) determined in the leaves of wheat plants from

cultivars BR-18, EMBRAPA-16 (EMB-16) and BRS-Guamirim (BRS-G) non-inoculated with *Pyricularia oryzae*. Plants from each cultivar were evaluated at the same time as for the inoculated plants (72, 96 and 120 hours after inoculation). Bars represent the standard deviation of means. $n = 6$.

Figure S3. Activities of sucrose-phosphate synthase (SPS; A), acid (B), alkaline invertase (C), sucrose synthase (Susy; D), ADP-glucose pyrophosphorylase (AGPase; E) and fructose 1,6-bisphosphatase (FBPase; F) determined in the leaves of wheat plants from cultivars BR-18, EMBRAPA-16 (EMB-16) and BRS-Guamirim (BRS-G) non-inoculated with *Pyricularia oryzae*. Plants from each cultivar were evaluated at the same time as for the inoculated plants (72, 96 and 120 hours after inoculation). Bars represent the standard deviation of means. $n = 6$.

Figure S4. Activities of ascorbate peroxidase (APX) (A), catalase (CAT) (B), peroxidase (POX) (C), superoxide dismutase (SOD) (D), glutathione peroxidase (GPX) (E), glutathione reductase (GR) (F), glutathione-S-transferase (GST) (G) as well as concentrations of hydrogen peroxide (H_2O_2)(H) and malondialdehyde (MDA) (I)determined in the leaves of wheat plants from cultivars BR-18, EMBRAPA-16 (EMB-16) and BRS-Guamirim (BRS-G) non-inoculated with *Pyricularia oryzae*. Plants from each cultivar were evaluated at the same time as for the inoculated plants (72, 96 and 120 hours after inoculation). Bars represent the standard deviation of means. $n = 6$.

Figure S5. Concentrations total soluble phenols (TSP) (A) andlignin-thioglycolic acid derivatives (LTGA; B) as well as activities of phenylalanine ammonia-lyases (PAL) (C) and polyphenoloxidases (PPO) (D) determined in the leaves of wheat plants from cultivars BR-18, EMBRAPA-16 (EMB-16) and BRS-Guamirim (BRS-G) non-

inoculated with *Pyricularia oryzae*. Plants from each cultivar were evaluated at the same time as for the inoculated plants (72, 96 and 120 hours after inoculation). Bars represent the standard deviation of means. $n = 6$.

Figure S6. Relative concentrations of putrescine (A) and spermidine (B) on the leaves of wheat plants from cultivars BR-18, EMBRAPA-16 (EMB-16) and BRS-Guamirim (BRS-G) non-inoculated (0 hours after inoculation (hai) or at 72, 96 and 120 hai with *Pyricularia oryzae*. Means from the three cultivars, at each evaluation time, followed by different letters are significantly different ($P \leq 0.05$) according to Tukey's test. For each parameter, mean from each cultivar at 72, 96 and 120 hai with an asterisk (*) is significantly different from the respective cultivar at 0 hai according to Student´s *t* test ($P \leq 0.05$).Bars represent the standard deviation of means. $n = 6$.

Table 1

Categories	Variables/Parameters	*P* values		
		C	ST	C × ST
Disease	Blast	<0.001	<0.001	<0.001
Leaf gas exchange	*A*	<0.001	<0.001	<0.001
	g_s	0.534	< 0.001	0.042
	C_i	0.085	<0.001	<0.001
	E	0.228	<0.001	0.079
Carbohydrates	Glucose	<0.001	<0.001	<0.001
	Fructose	<0.001	<0.001	<0.001
	Sucrose	0.044	<0.001	0.006
	Starch	0.157	<0.001	0.002
Biochemical	Malate	0.807	<0.001	0.035
	Fumarate	0.006	<0.001	<0.001
	Aminoacids	0.039	<0.001	0.030
	Acid invertase	<0.001	<0.001	0.025
	Alkalin invertase	<0.001	<0.001	<0.001
	SPS	<0.001	<0.001	<0.001
	SuSy	<0.001	<0.001	<0.001
	FBPase	<0.001	<0.001	0.004
	AGPase	<0.001	<0.001	0.003
	APX	0.056	<0.001	0.581
	CAT	<0.001	<0.001	0.019
	POX	<0.001	<0.001	<0.001
	SOD	0.144	<0.001	0.086
	GPX	<0.001	<0.001	<0.001
	GR	<0.001	<0.001	<0.001
	GST	0.023	<0.001	<0.001
	H_2O_2	<0.001	<0.001	<0.001
	MDA	<0.001	<0.001	<0.001
	LAGT	0.120	<0.001	<0.001
	TSP	0.003	<0.001	<0.001
	PAL	<0.001	<0.001	<0.001
	PPO	<0.001	<0.001	<0.001

Table 2

Groups	Metabolites	*P* values		
		C	ST	C × ST
Amino acids	Ornithine	0.012	<0.001	0.012
	Asparagine	0.012	<0.001	0.365
	Serine	0.023	0.002	0.005
	Lysine	0.032	<0.001	0.264
	Tryptophan	0.042	<0.001	0.149
	Glutamicacid	0.042	0.051	0.641
	Histidine	0.052	<0.001	0.330
	Isoleucine	0.080	<0.001	0.762
	Thymine	0.089	0.001	0.013
	Homoserine	0.123	<0.001	0.024
	Leucine	0.195	0.006	0.886
	Glutamine	0.215	<0.001	0.176
	Phenilalanine	0.289	<0.001	0.589
	Glicine	0.347	0.031	0.024
	Valine	0.470	<0.001	0.792
	Proline	0.489	0.010	0.785
	Alanine	0.533	<0.001	0.909
	Methionine	0.680	<0.001	0.018
	Cysteine	0.939	<0.001	0.446
Organic acids	Ferulicacid	<0.001	<0.001	0.038
	Citricacid	<0.001	<0.001	0.004
	Cinnamicacid	<0.001	0.057	0.088
	Gluconicacid	<0.001	<0.001	0.189
	Nicotinicacid	0.002	0.060	0.337
	Ribonicacid	0.003	<0.001	0.485
	Aconitic acid	0.006	<0.001	0.004
	Malonicacid	0.007	<0.001	0.843
	Oxaloacetate	0.012	0.016	0.246
	Succinicacid	0.014	<0.001	0.080
	Glycolicacid	0.026	<0.001	0.076
	Glutaricacid	0.046	0.003	0.470
	Malicacid	0.047	<0.001	0.002
	Nicotinamide	0.052	<0.001	0.124
	Fumaricacid	0.052	<0.001	0.117
	Guanidine	0.312	0.233	0.047
	Butanoicacid	0.368	<0.001	0.808
	Guanosine	0.690	<0.001	0.457
sugars and sugar-alcohols	Maltitol	<0.001	0.006	0.960
	Sedoheptulose	<0.001	0.034	0.295
	Glycerol	<0.001	<0.001	<0.001
	Sophorose	<0.001	<0.001	0.018
	Inositol	<0.001	<0.001	0.320
	Arabinose	0.007	<0.001	0.015
	Mannose	0.021	<0.001	0.015
	Ribose	0.028	<0.001	0.318
	Loganin	0.044	<0.001	0.083
	Erythritol	0.053	<0.001	0.006
	Maltose	0.075	0.009	0.099
	Glucose	0.092	<0.001	0.036
	Idose	0.104	<0.001	0.332
	Galactinol	0.400	<0.001	0.665
	Fructose	0.576	<0.001	0.001
	Raffiinose	0.647	0.027	0.905
	Mannitol	0.820	0.009	0.979
	Sorbitol	0.988	0.027	0.694
Polyamines	Putrescine	<0.001	<0.001	<0.001
	Spermidine	0.066	<0.001	<0.001

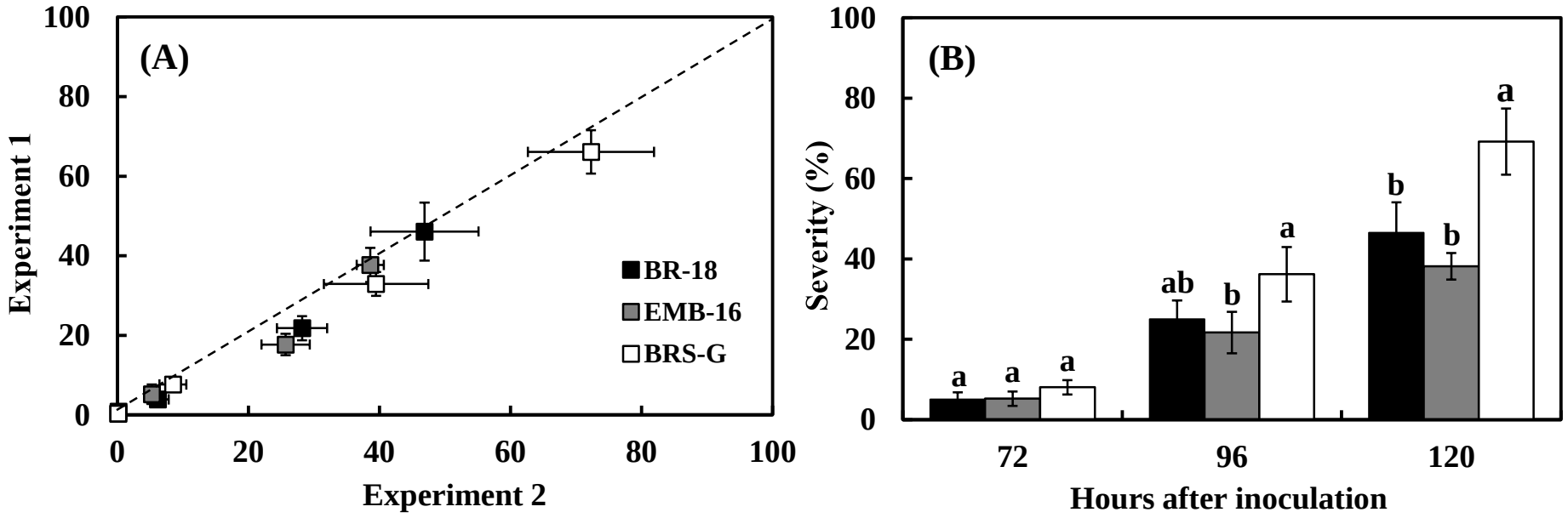
(A)
Experiment 1
Experiment 2
0
20
40
60
80
100
BR-18
EMB-16
BRS-G
(B)
Severity (%)
Hours after inoculation
72
96
120
a
a
a
ab
b
a
b
b
a

Figure 1

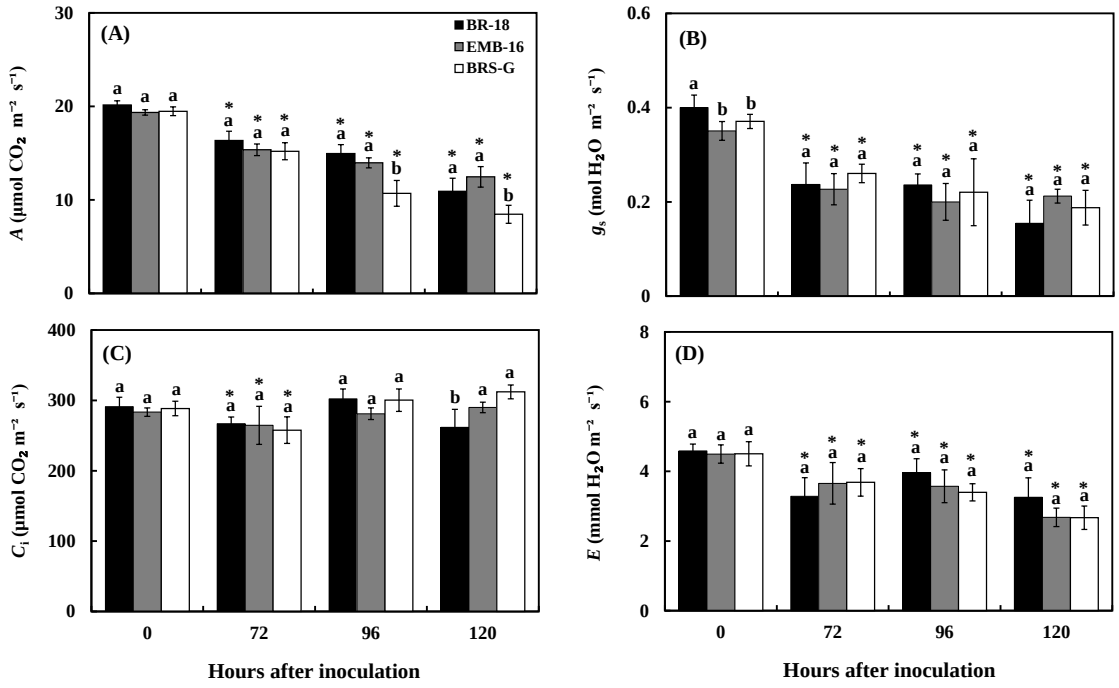
(A)
BR-18
EMB-16
BRS-G
A (μmol CO2 m−2 s−1)
(B)
gs (mol H2O m−2 s−1)
(C)
Ci (μmol CO2 m−2 s−1)
(D)
E (mmol H2O m−2 s−1)
0
72
96
120
Hours after inoculation
Hours after inoculation

Figure 2

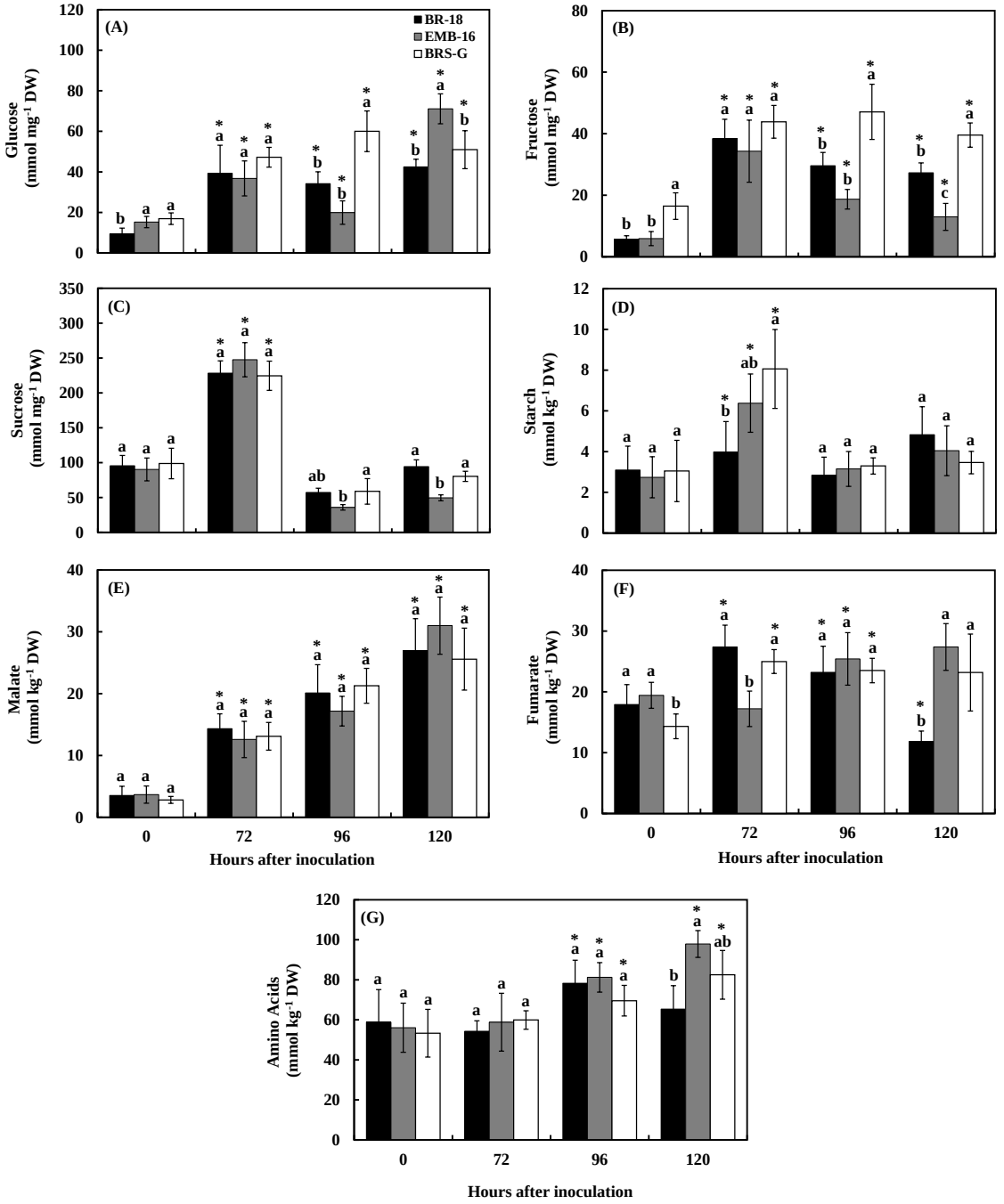
(A)
BR-18
EMB-16
BRS-G
Glucose (mmol mg-1 DW)
(B)
Fructose (mmol mg-1 DW)
(C)
Sucrose (mmol mg-1 DW)
(D)
Starch (mmol kg-1 DW)
(E)
Malate (mmol kg-1 DW)
(F)
Fumarate (mmol kg-1 DW)
(G)
Amino Acids (mmol kg-1 DW)
0
72
96
120
Hours after inoculation

Figure 3

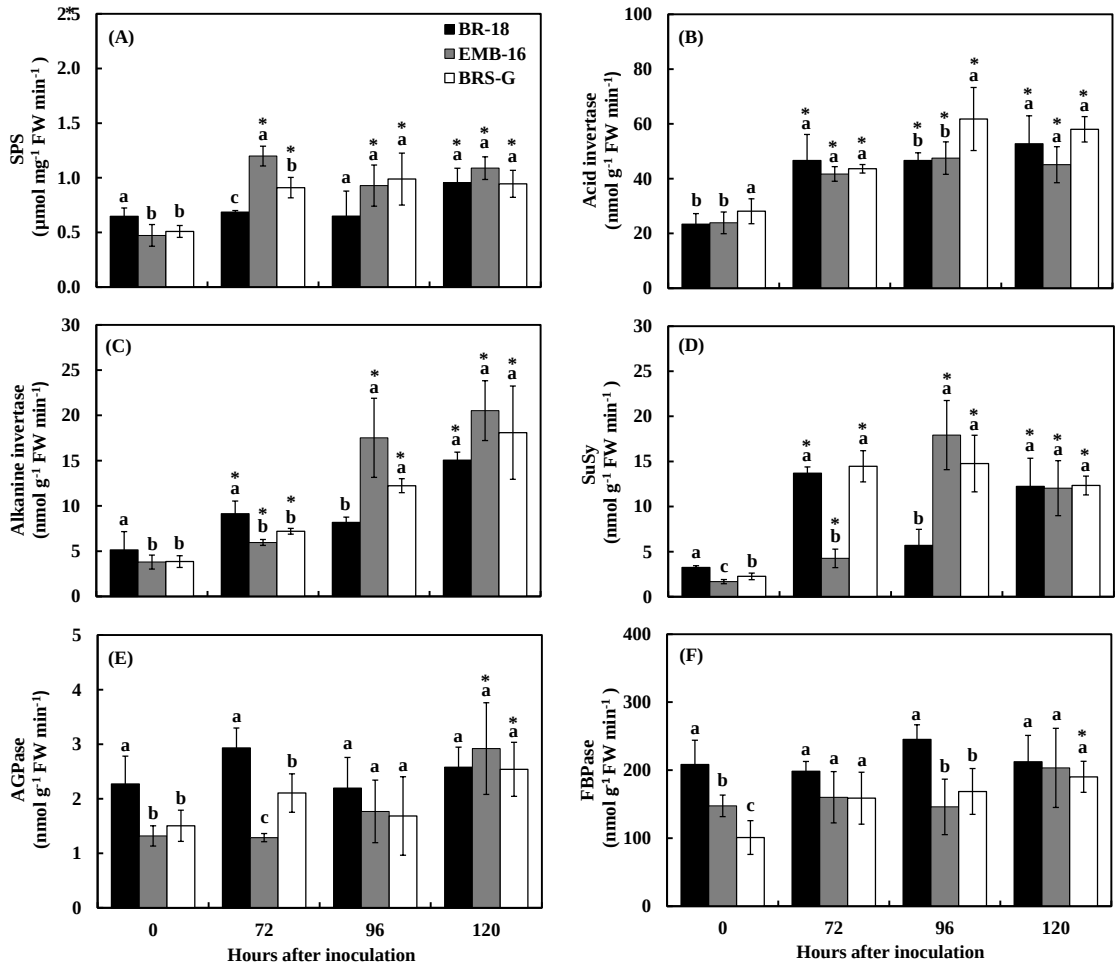
(A)
BR-18
EMB-16
BRS-G
SPS
(μmol mg-1 FW min-1)
(B)
Acid invertase
(nmol g-1 FW min-1)
(C)
Alkanine invertase
(nmol g-1 FW min-1)
(D)
SuSy
(nmol g-1 FW min-1)
(E)
AGPase
(nmol g-1 FW min-1)
(F)
FBPase
(nmol g-1 FW min-1)
0
72
96
120
Hours after inoculation

Figure 4

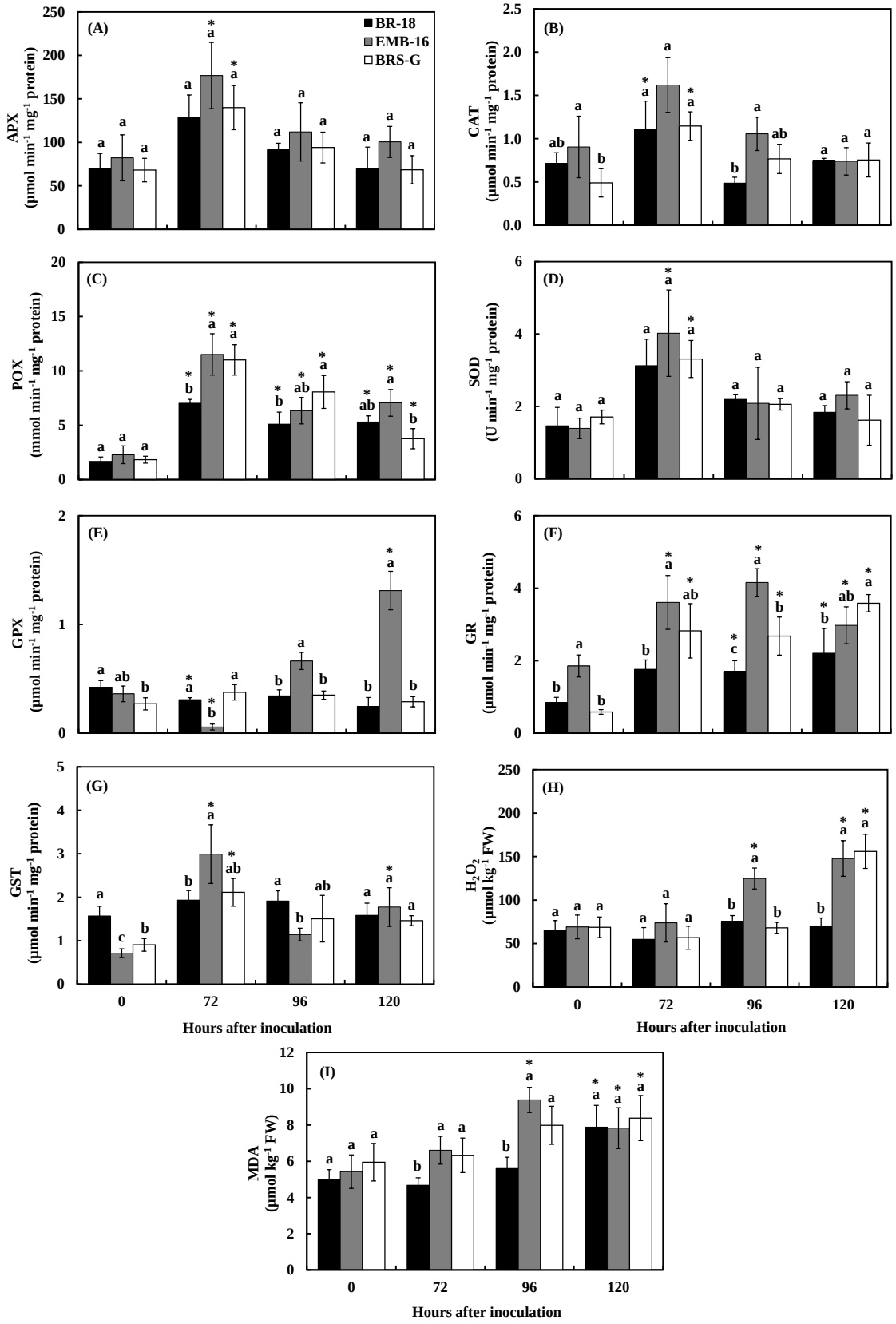
(A)
BR-18
EMB-16
BRS-G
APX
(µmol min-1 mg-1 protein)
(B)
CAT
(µmol min-1 mg-1 protein)
(C)
POX
(mmol min-1 mg-1 protein)
(D)
SOD
(U min-1 mg-1 protein)
(E)
GPX
(µmol min-1 mg-1 protein)
(F)
GR
(µmol min-1 mg-1 protein)
(G)
GST
(µmol min-1 mg-1 protein)
(H)
H2O2
(µmol kg-1 FW)
(I)
MDA
(µmol kg-1 FW)
0
72
96
120
Hours after inoculation

Figure 5

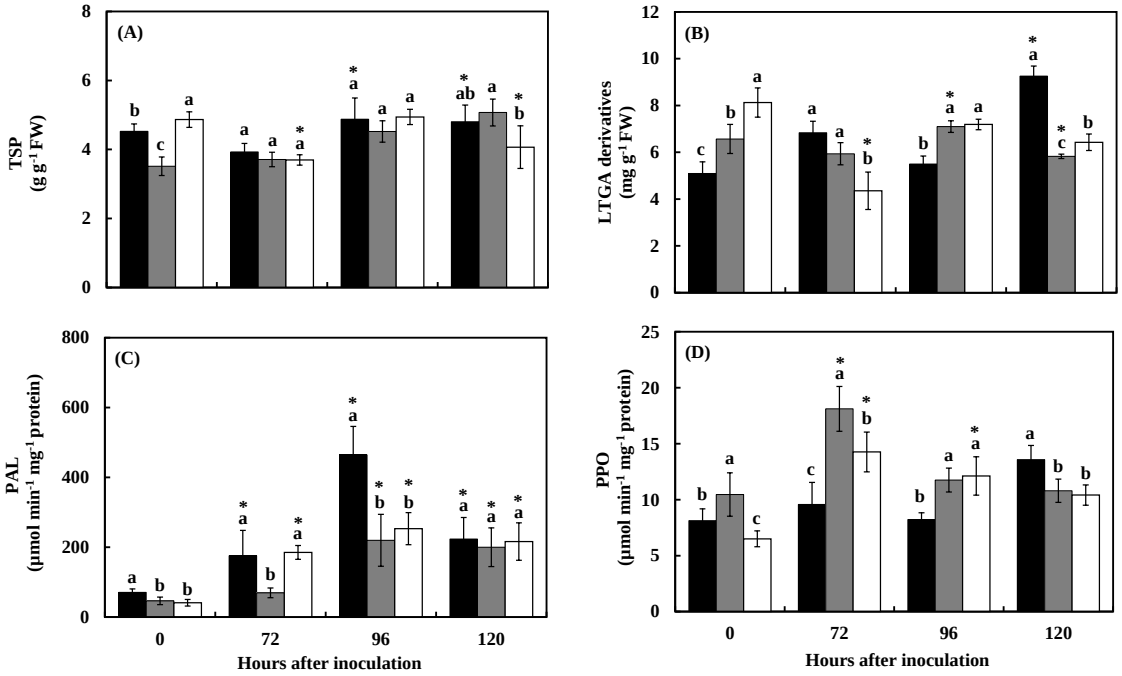
(A)
TSP
(g g-1 FW)
(B)
LTGA derivatives
(mg g-1 FW)
(C)
PAL
(µmol min-1 mg-1 protein)
(D)
PPO
(µmol min-1 mg-1 protein)
0
72
96
120
Hours after inoculation

Figure 6

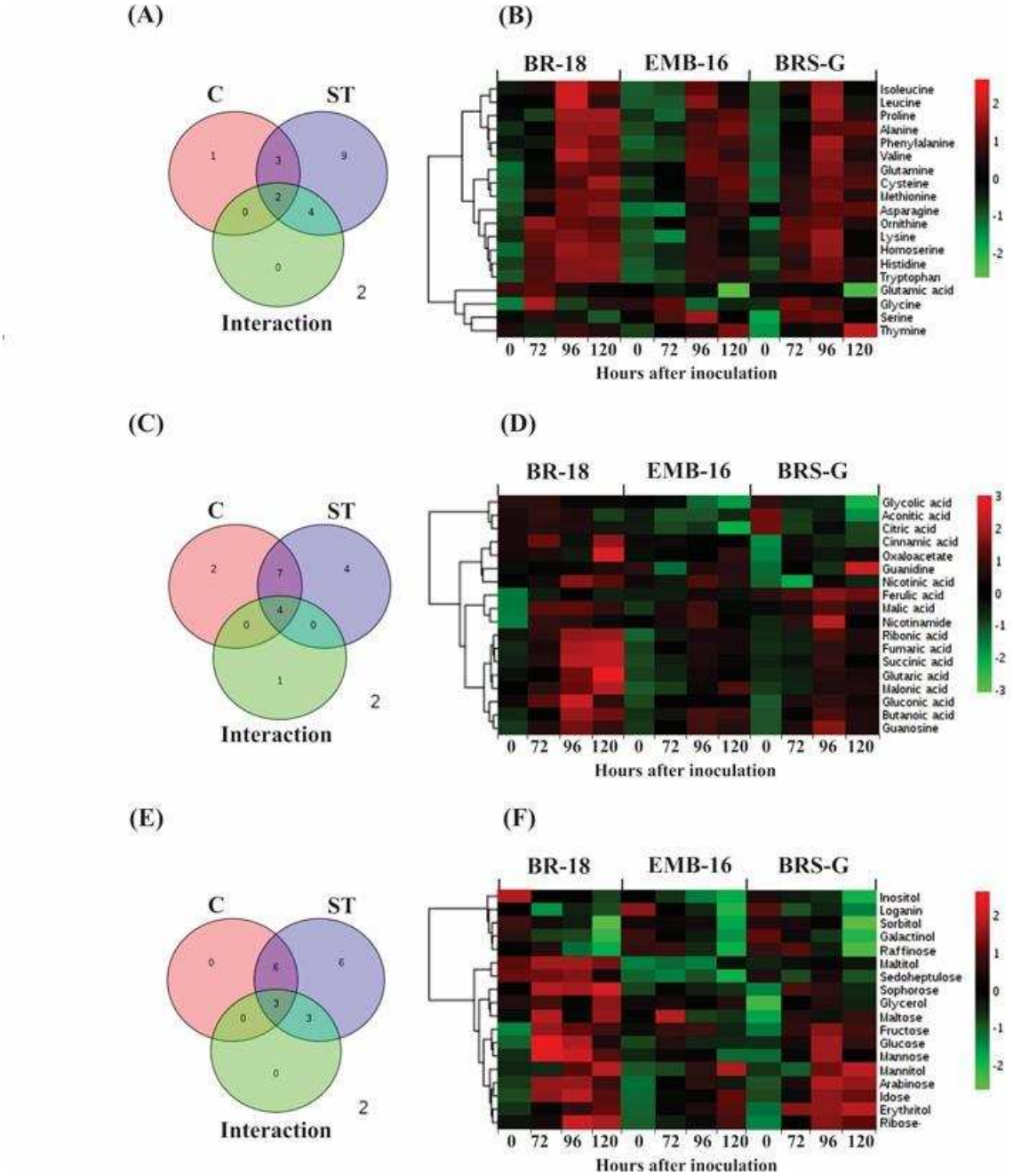
(A)
C
ST
1
3
9
2
0
4
0
2
Interaction
(B)
BR-18
EMB-16
BRS-G
Isoleucine
Leucine
Proline
Alanine
Phenylalanine
Valine
Glutamine
Cysteine
Methionine
Asparagine
Ornithine
Lysine
Homoserine
Histidine
Tryptophan
Glutamic acid
Glycine
Serine
Thymine
2
1
0
-1
-2
0 72 96 120 0 72 96 120 0 72 96 120
Hours after inoculation
(C)
C
ST
2
7
4
4
0
0
1
2
Interaction
(D)
BR-18
EMB-16
BRS-G
Glycolic acid
Aconitic acid
Citric acid
Cinnamic acid
Oxaloacetate
Guanidine
Nicotinic acid
Ferulic acid
Malic acid
Nicotinamide
Ribonic acid
Fumaric acid
Succinic acid
Glutaric acid
Malonic acid
Gluconic acid
Butanoic acid
Guanosine
3
2
1
0
-1
-2
-3
0 72 96 120 0 72 96 120 0 72 96 120
Hours after inoculation
(E)
C
ST
0
6
6
3
0
3
0
2
Interaction
(F)
BR-18
EMB-16
BRS-G
Inositol
Loganin
Sorbitol
Galactinol
Raffinose
Maltitol
Sedoheptulose
Sophorose
Glycerol
Maltose
Fructose
Glucose
Mannose
Mannitol
Arabinose
Idose
Erythritol
Ribose-
2
1
0
-1
-2
0 72 96 120 0 72 96 120 0 72 96 120
Hours after inoculation

Figure 7

Table S1

Categories	Variables/Parameters	*P* values		
		C	ST	C × ST
Physiological	*A*	0.044	0.105	0.919
	g_s	0.003	0.092	0.042
	C_i	0.393	0.119	0.735
	E	0.414	0.430	0.483
Carbohydrates	Glucose	<0.001	0.390	0.580
	Fructose	<0.001	0.051	0.138
	Sacarose	0.531	0.366	0.829
	Starch	0.771	0.893	0.650
Biochemical	Malate	0.243	0.901	0.727
	Fumarate	<0.001	0.108	0.003
	Amino Acids	0.630	0.441	0.591
	LAGT	<0.001	0.325	0.851
	TSP	<0.001	0.054	0.006
	Acid invertase	0.009	0.125	0.186
	Alkalin invertase	0.014	0.065	0.241
	SPS	<0.001	0.110	0.628
	SuSy	<0.001	0.069	0.545
	FBPase	<0.001	0.363	0.427
	AGPase	<0.001	0.279	0.562
	APX	0.103	0.171	0.015
	CAT	<0.001	0.212	0.104
	POX	0.002	0.082	0.024
	SOD	0.058	0.078	0.133
	GPX	<0.001	0.381	0.583
	GR	<0.001	0.139	0.426
	GST	<0.001	0.124	0.384
	H_2O_2	0.650	0.394	0.236
	MDA	0.023	0.818	0.767
	PAL	<0.001	0.449	0.719
	PPO	<0.001	0.850	0.532

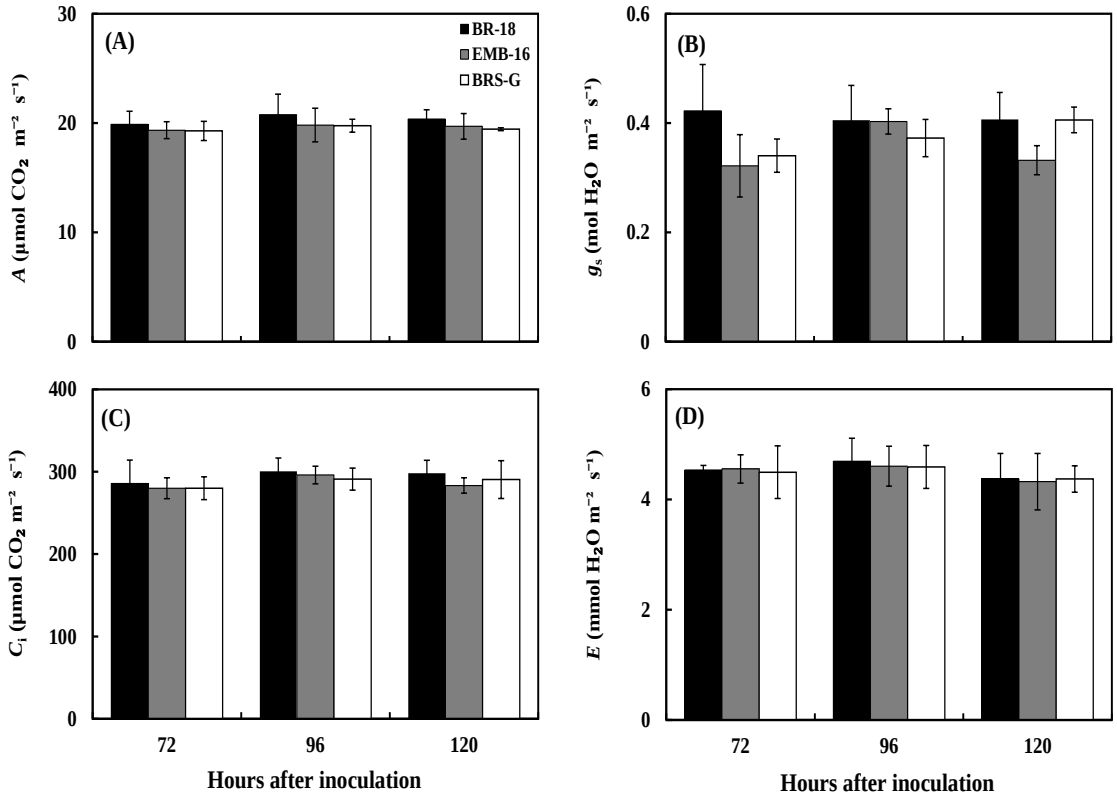
(A)
BR-18
EMB-16
BRS-G
A (µmol CO2 m−2 s−1)
(B)
gs (mol H2O m−2 s−1)
(C)
Ci (µmol CO2 m−2 s−1)
(D)
E (mmol H2O m−2 s−1)
72
96
120
Hours after inoculation

Figure S1

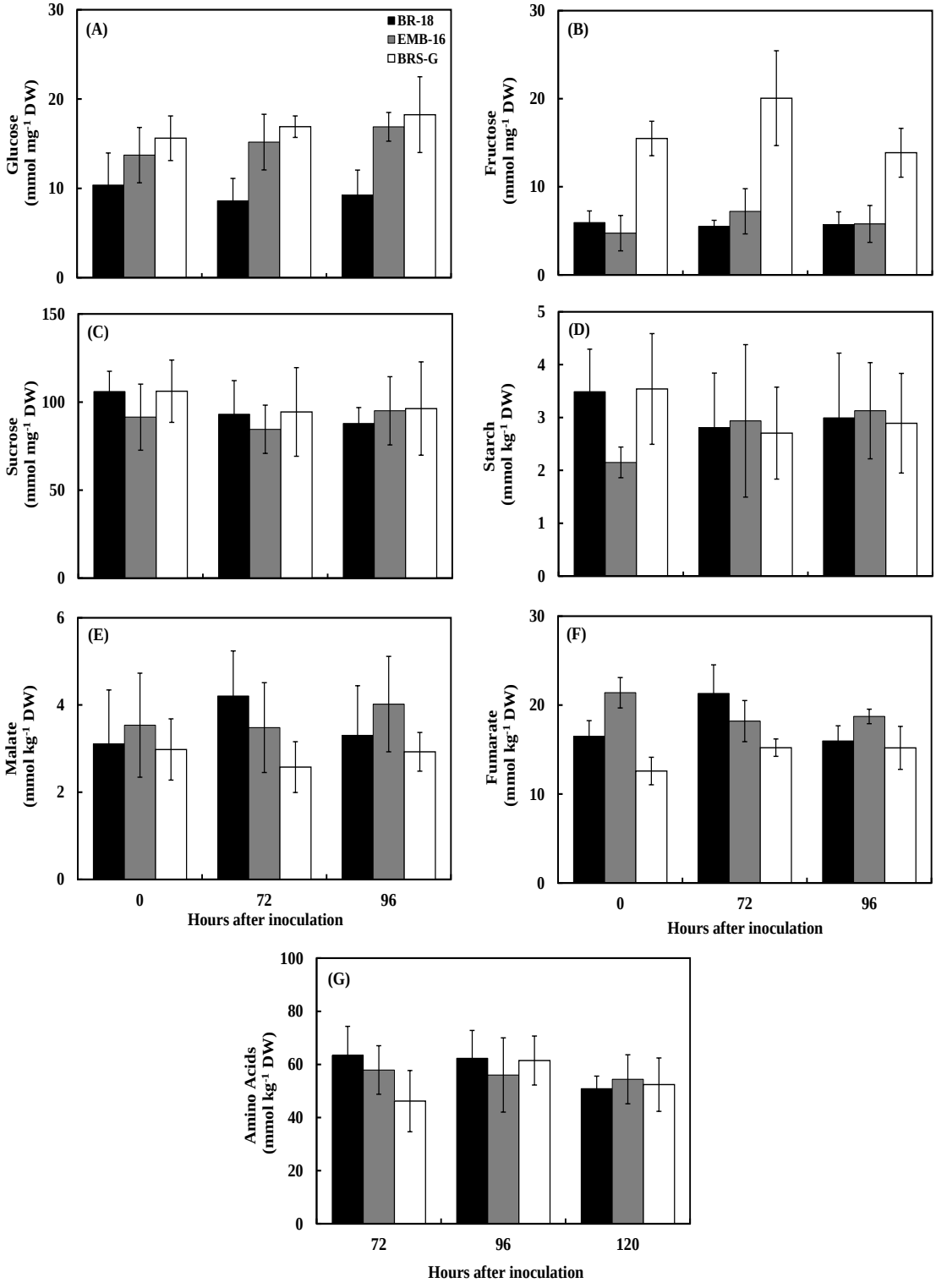
(A)
BR-18
EMB-16
BRS-G
Glucose (mmol mg-1 DW)
(B)
Fructose (mmol mg-1 DW)
(C)
Sucrose (mmol mg-1 DW)
(D)
Starch (mmol kg-1 DW)
(E)
Malate (mmol kg-1 DW)
(F)
Fumarate (mmol kg-1 DW)
Hours after inoculation
(G)
Amino Acids (mmol kg-1 DW)
Hours after inoculation

Figure S2

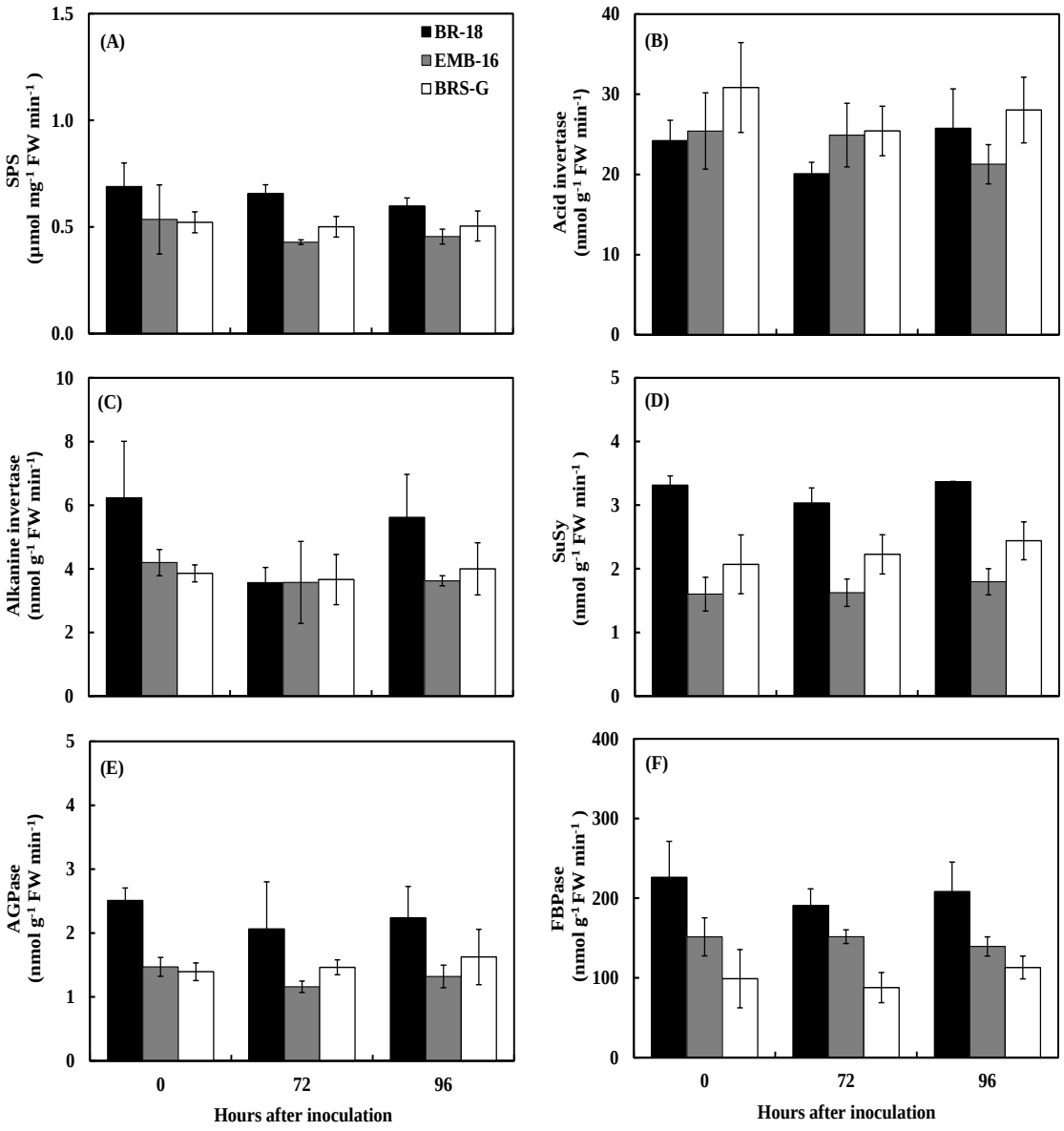
(A)
BR-18
EMB-16
BRS-G
SPS
(µmol mg-1 FW min-1)
(B)
Acid invertase
(nmol g-1 FW min-1)
(C)
Alkanine invertase
(nmol g-1 FW min-1)
(D)
SuSy
(nmol g-1 FW min-1)
(E)
AGPase
(nmol g-1 FW min-1)
(F)
FBPase
(nmol g-1 FW min-1)
0
72
96
Hours after inoculation

Figure S3

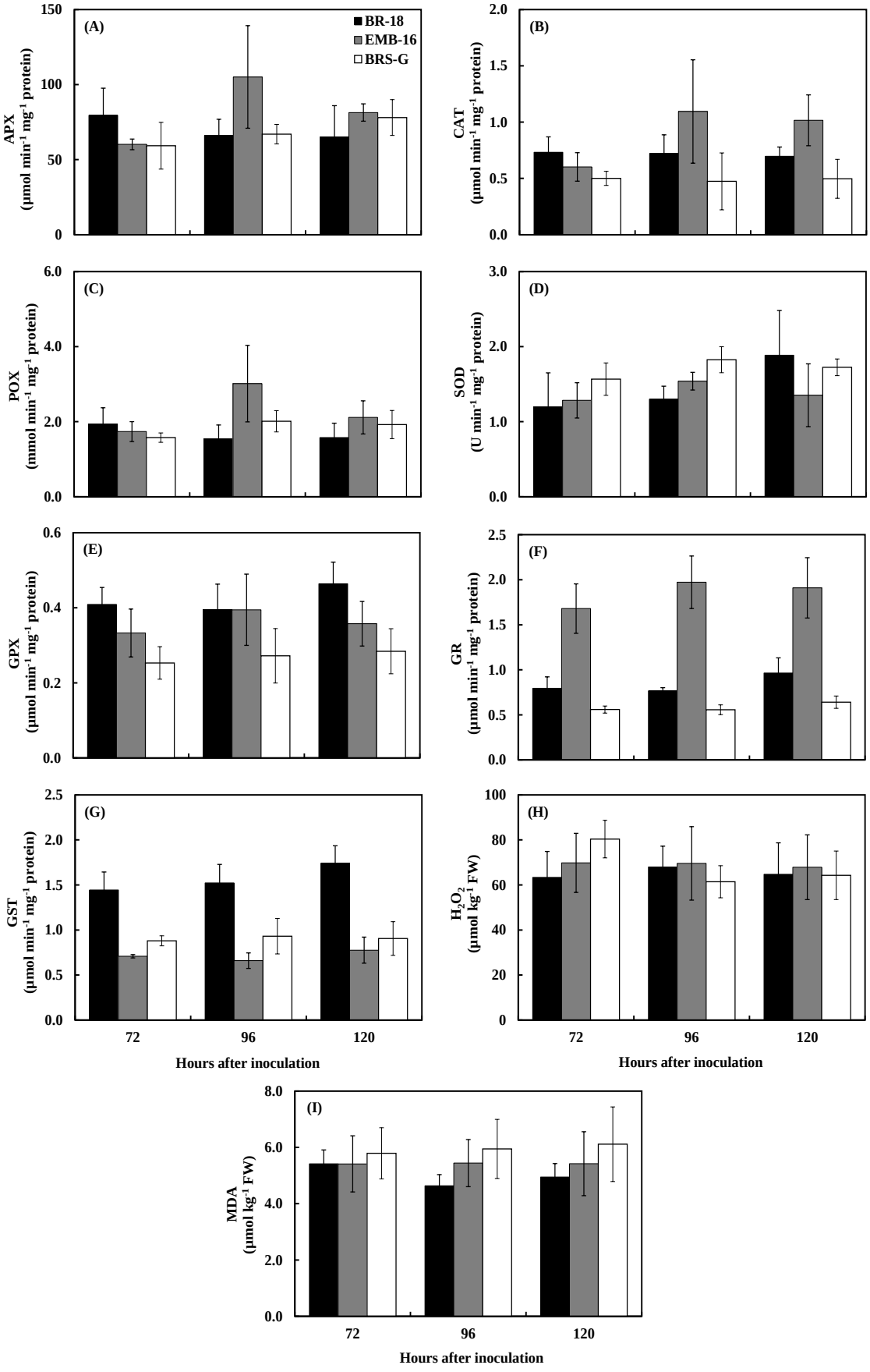
(A)
BR-18
EMB-16
BRS-G
APX
(µmol min-1 mg-1 protein)
(B)
CAT
(µmol min-1 mg-1 protein)
(C)
POX
(mmol min-1 mg-1 protein)
(D)
SOD
(U min-1 mg-1 protein)
(E)
GPX
(µmol min-1 mg-1 protein)
(F)
GR
(µmol min-1 mg-1 protein)
(G)
GST
(µmol min-1 mg-1 protein)
(H)
H2O2
(µmol kg-1 FW)
72
96
120
Hours after inoculation
(I)
MDA
(µmol kg-1 FW)

Figure S4

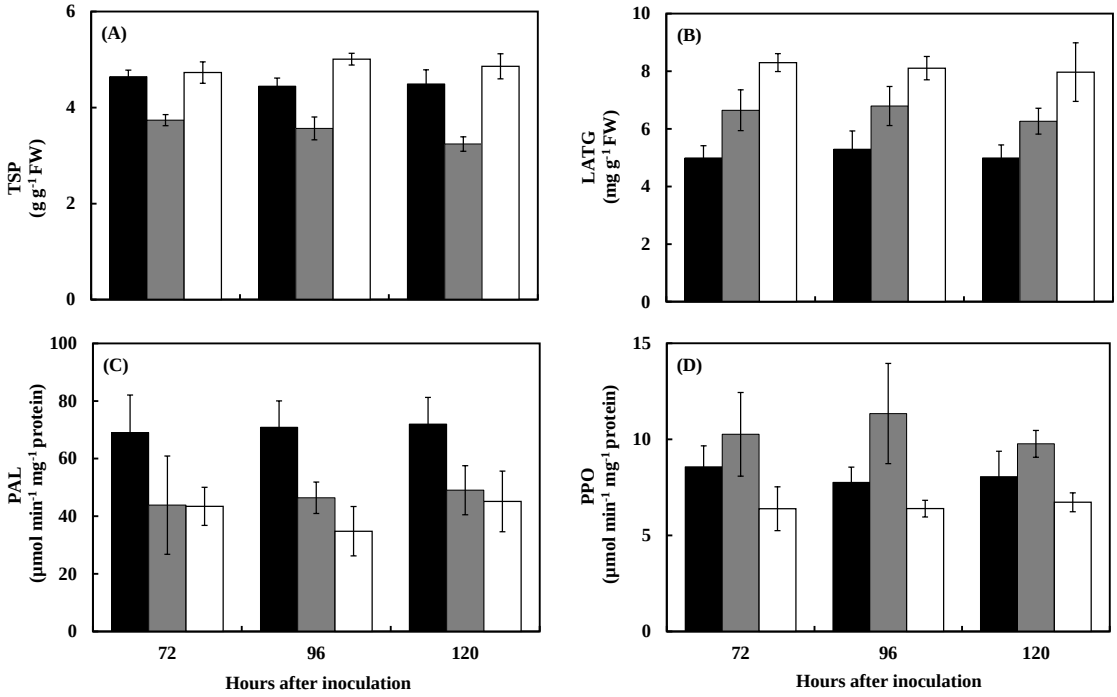
(A)
TSP
(g g-1 FW)
0
2
4
6
(B)
LATG
(mg g-1 FW)
0
2
4
6
8
10
(C)
PAL
(µmol min-1 mg-1 protein)
0
20
40
60
80
100
(D)
PPO
(µmol min-1 mg-1 protein)
0
5
10
15
72
96
120
Hours after inoculation
72
96
120
Hours after inoculation

Figure S5

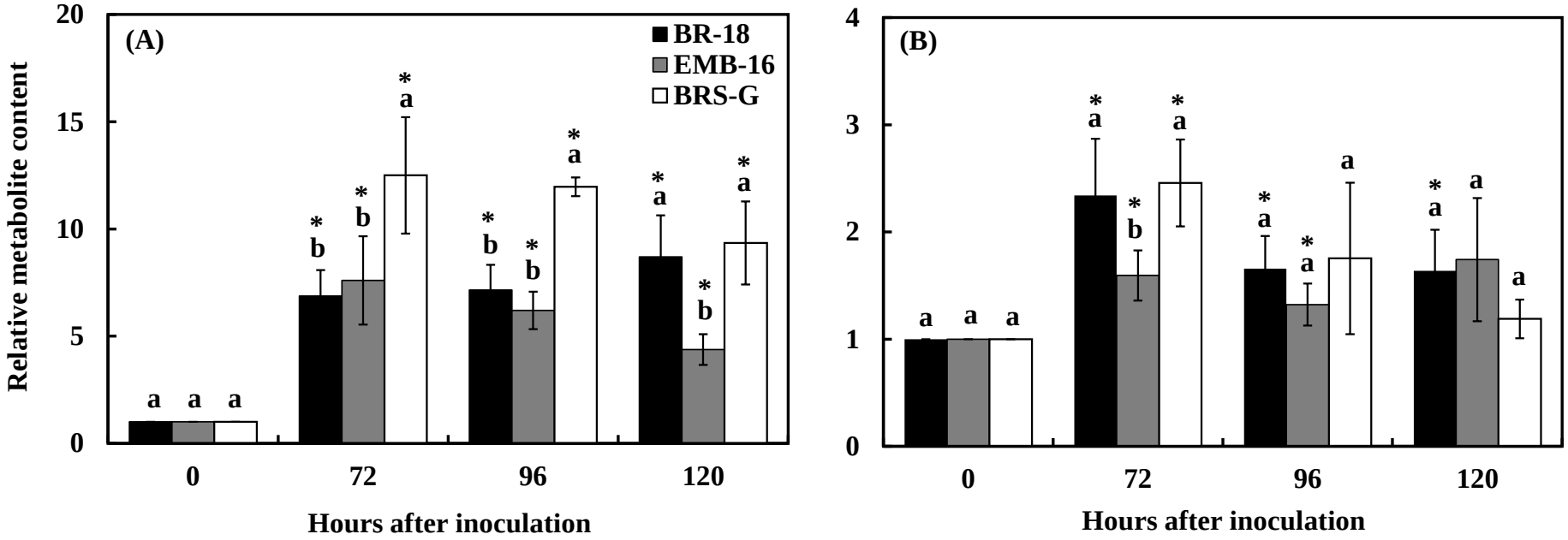
(A)
BR-18
EMB-16
BRS-G
Relative metabolite content
20
15
10
5
0
a a a
*b *b *a
*b *b *a
*a *b *a
0
72
96
120
Hours after inoculation
(B)
4
3
2
1
0
a a a
*a *b *a
*a *a a
*a a a
0
72
96
120
Hours after inoculation

Figure S6

GENERAL CONCLUSIONS

Wheat blast, caused by *Pyricularia oryzae* currently considered one of the major threat that limit the production of wheat grain. Since their first discovered in Paraná State, Brazil in 1986, wheat blast has been the cause of reductions of 10-100% in yields, similar situations have been reported in neighboring countries like Bolivia, Paraguay and Argentina. The application of foliar fungicides and the use of genetic resistance are the strategies most widely recommended for minimizing losses caused by wheat blast.

From the results obtained by this investigation, is concluded that picolinic acid (aggressive factor of *P. oryzae*) spray on wheat leaves with a non-phytotoxicconcentration of 0.1 mg mL^{-1} resulted in less blast symptoms in association with a better photosynthetic performance, an improvement on the antioxidant metabolism and reduced concentrations of H_2O_2, $O_2^{\bullet-}$ and malondialdehyde.

On the other hand, in three wheat cultivars (BR-18, EMBRAPA-16 and BRS-Guamirim) was verified differences in basal level of resistance to blast. In addition, the photosynthetic performance of infected plants was altered and during the asymptomatic phase of *P. oryzae* infection, drastic changes in the carbohydrates metabolism and on the levels of amino acids, intermediates compounds of the TCA cycle and polyamines occurred on plants from the three cultivars suggesting a metabolic reprogramming exerted by the pathogen. However, a more efficient antioxidant metabolism was able to help the wheat plants to counteract against the deleterious effects of *P. oryzae* infection in association with great phenylalanine ammonia lyases and polyphenoloxidases activities and high concentrations of phenolics and lignin.