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Picolinic acid spray stimulates the antioxidative metabolism and minimizes impairments on photosynthesis on wheat leaves infected by *Pyricularia oryzae*

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Fungal pathogens produce toxins that are important for their pathogenesis and/or aggressiveness towards their hosts. Picolinic acid (PA), a non-host selective toxin, causes lesions on rice leaves resembling those originated from *Pyricularia oryzae* infection. Considering that non-host selective toxins can be useful for plant diseases control, this study investigated whether the foliar spray with PA on wheat (*Triticum aestivum* L.) plants, in a non-phytotoxic concentration, could increase their resistance to blast, stimulate the anti-oxidative metabolism, and minimize alterations in photosynthesis. The PA spray at concentrations greater than 0.1 mg ml^{-1} caused foliar lesions, compromised the photosynthesis and was linked with greater accumulation of hydrogen peroxide (H_2O_2) and superoxide anion radical ($O_2^{\bullet-}$). Fungal mycelial growth, conidia production and germination decreased by PA at 0.3 mg ml^{-1}. Blast severity was significantly reduced by 59 and 23%, respectively, at 72 and 96 h after inoculation for plants sprayed with PA (0.1 mg ml^{-1}) at 24 h before fungal inoculation compared to non-sprayed plants. Reduction on blast symptoms was linked with increases on ascorbate peroxidase (EC 1.11.1.11), catalase (EC 1.11.1.6), glutathione peroxidase (EC 1.11.1.9), glutathione reductase (EC 1.8.1.7), glutathione-*S*-transferase (EC 2.5.1.18), peroxidase (EC 1.11.1.7), and superoxide dismutase (EC 1.15.1.1) activities, lower H_2O_2 and $O_2^{\bullet-}$ accumulation, reduced malondialdehyde production as well as less impairments to the photosynthetic apparatus. A more efficient antioxidative metabolism that rapidly scavenges the reactive oxygen species generated during *P. oryzae* infection, without dramatically decreasing the photosynthetic performance, was a remarkable effect obtained with PA spray.

Abbreviations – *A*, net CO_2 assimilation rate; APX, ascorbate peroxidase; BS, blast severity; CAT, catalase; C_i, internal CO_2 concentration; *E*, transpiration rate; F_m, maximum fluorescence image; F_v/F_m, variable-to-maximum chlorophyll fluorescence ratio; GPX, glutathione peroxidase; g_s, stomatal conductance to water vapor; GST, glutathione-*S*-transferase; hai, hours after inoculation; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; $O_2^{\bullet-}$, superoxide anion radical; PA, picolinic acid; POX, peroxidase; q_P, coefficient for photochemical quenching; SOD, superoxide dismutase; Y(II), effective photosystem II quantum yield; Y(NO), quantum yield of non-regulated energy dissipation; Y(NPQ), quantum yield of regulated energy dissipation.

Introduction

Blast, caused by the fungus *Pyricularia oryzae* Cavara (teleomorph *Magnaporthe grisea* [T. T. Hebert] M. E. Barr), was first reported on wheat (*Triticum aestivum* L.) in 1985 in Paraná State, Brazil (Igarashi et al. 1986). Since then, blast has caused significant yield losses (up to 60%) on commercial wheat fields in Argentina, Bolivia, Brazil and Paraguay when weather conditions are favorable such as rainy periods, high relative humidity and temperatures ranging from 21 to 27 °C (Goulart et al. 2007, Kohli et al. 2011). In 2016, wheat blast was reported in eight districts in Bangladesh, southwestern Asia, affecting about 16% of the cultivated area in this country (Malaker et al. 2016). It is evident that conidia dispersion of *P. oryzae* to other continents is a real problem that may put wheat production at risk.

On wheat leaves, the first blast symptoms appear at 48 hours after inoculation (hai) as small water-soaked lesions which quickly expand and exhibit dark green borders that become light tan in color with necrotic borders (Igarashi et al. 1986, Goulart et al. 2007, Cruz et al. 2016). Gray-brown lesions are noticed on both spikes and spikelets and bright black spots on the rachis (Goulart et al. 2007). The foliar application of systemic fungicides and the use of resistant cultivars have contributed to some extent to blast control on wheat (Cruz et al. 2010, Castroagudín et al. 2015, Rios et al. 2016).

The infection process of pathogens is favored by the intensive production of both hydrolytic enzymes and toxins at the infection sites (Willis et al. 1991, Howlett 2006). Toxins, named as host- and non-host selective, are molecules of low molecular weight with a high structural diversity originated from the primary metabolism of necrotrophic and hemibiotrophic fungal pathogens (Howlett 2006, Berestetski 2008). Perturbations caused by toxins on plant physiology include the suppression of cell division, inhibition of photosynthesis photochemistry, changes on the primary and secondary metabolism, increased production of reactive oxygen species (ROS), loss of cellular homeostasis and damage of the cell membranes that will ultimately cause cell death (Berestetski 2008, Dong et al. 2012, Chen et al. 2014). Pathogens producing host-selective toxins cause diseases only on the hosts unable to detoxify them while the non-host selective toxins maximize, together with the hydrolytic enzymes, the intensity of the diseases (Wheeler and Luke 1963, Wolpert et al. 2002, Tsuge et al. 2013). The non-host selective toxins pyricularin, pyriculol-related compounds (e.g. epipyriculol, epidihydropyriculol, pyriculariol and pyricuol), tenuazonic acid and picolinic acid (PA) were produced by *P. oryzae* on rice leaves (Kim et al. 1998, Tsurushima et al. 2010). The tenuazonic acid, PA and pyriculol are of greater importance for blast symptoms development on rice (Umetsu et al. 1972, Yoder 1980, Iwahashi et al. 1999, Jacob et al. 2016).

The use of non-host selective toxins can be of some value at aiming disease control. The non-host selective is used in plant breeding programs to avoid the uneven exposure to the pathogen and improving the selection processes (Daub 1986). The spray of PA to rice callus culture of different cultivars allowed the selection of genotypes with resistance to blast (Prabhu and Rush 1997). The spray of rice leaves with tenuazonic acid and picolinic acid inhibited hyphae growth of *P. oryzae* on leaf tissues due to a high production of ROS (Pasechnik et al. 1993, Zhang et al. 2004, Aver'yanov et al. 2007). Rice resistance to blast mediated by PA spray was associated with an increase on NADPH oxidase activity, a rapid increased of H_2O_2 pools and the accumulation of transcripts of defense-related genes (Zhang et al. 2004).

Considering the potential of using a non-host selective toxin to increase host resistance to disease, this study aimed to test the hypothesis that wheat plants sprayed with PA in a non-phytotoxic concentration could increase their resistance to blast through a better adjustment of the antioxidant metabolism accompanied by the preservation of the photosynthetic capacity of the infected plants.

Materials and methods

Plant growth

Wheat seeds (cultivar BRS-Guamirim; susceptible to blast) (Cruz et al. 2010) were surface-sterilized in 10% (v/v) 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 8 days after seedlings emergence, each pot was thinned to six seedlings. Substrate in each pot was fertilized with a nutrient solution containing, in gram per liter, 6.4 KCl, 3.48 K_2SO_4, 5.01 $MgSO_4 \bullet 7H_2O$, 2.03 $(NH_2)_2CO$, 0.009 $NH_4MO_7O_{24} \bullet 4H_2O$, 0.054 H_3BO_3, 0.222 $ZnSO_4 \bullet 7H_2O$, 0.058 $CuSO_4 \bullet 5H_2O$ and 0.137 $MnCl_2 \bullet 4H_2O$ (Xavier-Filha et al. 2011). A volume of 15 ml of nutrient solution containing 0.27 g of $FeSO_4 \bullet 7H_2O$ and 0.37 g of EDTA bisodic l^{-1} was

also applied after seedlings emergence. The nutrient solutions were prepared using deionized water and applied weekly. Plants were watered with deionized water as needed.

Effect of PA spray on the photosynthetic performance of plants

Wheat 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 using a portable open-flow gas exchange system (LI-6400XT; Li-Cor Inc.). Measurements, performed at 24 and 48 h after PA spray, were made at the attached fourth leaf, from the top to the base, of each plant per replication of each treatment from 09:00 to 11:00 h (solar time) when A was at its maximum. The experimental conditions during measurements were: artificial photosynthetically active radiation (PAR) of 1000 µmol photons m^{-2} s^{-1} at the leaf level; 400 µmol atmospheric CO_2 mol^{-1} air; 25 °C; vapor pressure deficit at approximately 1.0 kPa; and 10% of blue light (relative to total PAR) to optimize the stomatal aperture.

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). 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 plant 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} (1 Hz) at 24 and 48 h 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 maximum photosystem (PS) II photochemical efficiency ($F_v/F_m = (F_m - F_0)/F_m$), which denotes the maximum PS II quantum efficiency (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 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 [$Y(NO) = F_s/F_m$]; additionally, the coefficient of photochemical quenching, as $q_P = (F_m' - F)/(F_m' - F_0')$, was also calculated. 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 h after spray the PA solutions.

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

Fifteen fragments (~10 cm of length) of the fourth and fifth leaves, from top to base, of each plant per replication of each treatment, were collected at 24 h after PA spray were used. For detection of H_2O_2, fragments were placed in glass vials containing a solution of 3,3′-diaminobenzidine tetrahydrochloride (1 mg ml^{-1}) (Sigma-Aldrich), kept in the dark at 25 °C for 12 h, cleared in boiling aqueous 80% ethanol for 60 min thereafter and stored in glycerol solution (70%). For detection of $O_2^{\bullet-}$, leaf fragments placed in glass vials were infiltrated with a solution of 0.1% nitro blue tetrazolium (Sigma-Aldrich) in 10 mM potassium phosphate buffer (pH 6.8) for 40 min, and then cleared and stored as described above.

The *in vitro* effect of PA on fungal mycelial growth, conidia production and germination

The sensitivity of *P. oryzae* to PA was evaluated in vitro using PA at eight concentrations of 0, 0.1, 0.3, 0.5, 1.0, 1.5, 3.0 and 5.0 mg ml^{-1}. The PA solutions were incorporated into the potato-dextrose-agar (PDA) 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 10-day-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 7 days, fungal colony in each Petri dish was measured in two orthogonal directions using

a digital paquimeter to obtain its diameter. At this time, conidia production in each Petri dish containing only oat-agar (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 inside a laminar flow chamber. Conidia concentration was quantified in 500 µl of suspension per each treatment using a hemacytometer.

Conidia obtained from a 10-day-old colonies of *P. oryzae* in Petri dishes containing OA medium were removed using a camel-hair brush to obtain a conidial suspension of 1×10^5 conidia ml^{-1}. A total of 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 to determine the PA effect on conidia germination. 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 magnification. A conidium was considered germinated when the germ tube was longer than its diameter and the percentage of germination was calculated.

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

Plants were inoculated with the isolate UFV/DFP *Po*-01 of *P. oryzae* (Debona et al. 2012). Disks of filter paper containing fungal mycelia were transferred to Petri dishes containing OA medium. After growing the disks containing mycelia, the media with the fungus were transferred to new Petri dishes with 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}. At 24 h before inoculation, plants were sprayed with a solution of 0.1 mg PA ml^{-1} (35 ml per plant), which was selected based on a preliminary experiment that determined the effect of PA at different concentrations on the plant photosynthetic performance. The conidial suspension was sprayed with an atomizer (Paasche Airbrush Co.) on the adaxial surface of the leaves of plants at growth stage 39. After inoculation, plants were kept in a mist chamber under darkness at 25 °C during 24 h and transferred to a greenhouse (relative humidity of 80 ± 5%, temperature of 25 ± 3°C and natural PAR of 900 µmol photons m^{-2} s^{-1} as measured at midday) thereafter.

Blast severity assessment

Blast severity (BS) was evaluated on the fourth and fifth leaves, from top to base, of each plant per replication of each treatment, at 48, 72 and 96 hours after inoculation (hai) using a diagrammatic scale (Rios et al. 2013).

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

Leaf gas exchange and Chl *a* fluorescence parameters were measured on the attached fourth leaf, from top to 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 enzyme activities

The fourth and fifth leaves, from base to 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 −80 °C until further analysis. For determination of the activities of ascorbate peroxidases (APX, EC 1.11.1.11), catalases (CAT, EC 1.11.1.6), peroxidases (POX, EC 1.11.1.7), superoxide dismutases (SOD, EC 1.15.1.1), glutathione peroxidases (GPX, EC 1.11.1.9) and glutathione-*S*-transferases (GST, EC 2.5.1.18), 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 12 000 *g* for 15 min at 4°C and the supernatant was used as a crude enzyme extract. In order 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 15 W lamp light. After 10 min of light exposure, the light was turned off and the production of formazan blue, which resulted from the photoreduction of NTB, was monitored by the increase in absorbance at 560 nm in a spectrophotometer (Giannopolitis and Ries 1977). The reaction mixture for the control samples was kept in darkness. 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 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 CAT activity was determined by the rate of H_2O_2 decomposition at 240 nm during 1 min at 25 °C. The extinction coefficient of 36 M^{-1} cm^{-1} (Anderson et al. 1995) was used to calculate CAT activity, which was expressed as micromole per minute per milligram 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 during 1 min at 25 °C. The extinction coefficient of 2.47 mM^{-1} cm^{-1} (Chance and Maehley 1955) was used to calculate POX activity, which was expressed as millimole of purpurogallin produced per minute per milligram 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. The extinction coefficient of 9.6 mM^{-1} cm^{-1} (Habig et al. 1974) was used to determine GST activity, which was expressed as micromole per minute per milligram of protein.

The APX activity was determined according to the 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. The extinction coefficient of 2.8 mM^{-1} cm^{-1} (Nakano and Asada 1981) was used to calculate APX activity, which was expressed as micromole per minute per milligram of protein.

In order to determine 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. The extinction coefficient of 6.22 mM^{-1} cm^{-1} (Foyer and Halliwell 1976) was used to calculate GR activity, which was expressed as micromole per minute per milligram 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 2001). The decrease in absorbance was measured at 340 nm for 1 min at 30 °C. The extinction coefficient of 6.22 mM^{-1} cm^{-1} (Anderson and Davis 2004) was used to calculate GPX activity, which was expressed as micromole per minute per milligram 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 enzyme activity determinations. 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 (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 12 000 *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. The extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer 1968) was used to calculate MDA concentration, which was expressed as nanomole per gram of fresh weight (FW).

Experimental design and data analysis

Seven experiments were carried out with different goals. Experiments 1 to 4 were arranged in a completely randomized design with at least five replications to determine the effect of PA concentrations on leaf gas exchange and Chl *a* fluorescence parameters (Exp. 1); on H_2O_2 and $O_2^{\bullet-}$ production (Exp. 2); on fungal mycelial growth and conidia production (Exp. 3); and on conidia germination (Exp. 4). Experiments 5 to 7 were arranged in a completely randomized design with four treatments combinations [plants non-sprayed (−PA) or sprayed (+PA) with PA and non-incoulated (−*Po*) or inoculated (+*Po*) with *P. oryzae*] (referred as −PA/−*Po*, +PA/−*Po*, −PA/+*Po*, and +PA/+*Po* treatments thereafter) and five replications to determine the effect of a low concentration of PA (0.1 mg ml^{-1}) on leaf gas exchange and Chl *a* fluorescence parameters (Exp. 5), on the production of H_2O_2 and $O_2^{\bullet-}$ in the leaf tissue (Exp. 6), and on biochemical variables (MDA concentration and APX, CAT, GPX, GR, GST, POX and SOD activities). All of these experiments were repeated once. Each experimental unit corresponded to a plastic pot containing six plants. Data obtained from Exps 1 to 7 were analyzed using the MIXED procedure of the SAS software (Release 8.02 Level 02M0 for Windows, SAS Institute, Inc., 1989, Cary, NC) to determine if data from the repeated experiments could be combined (Moore and Dixon, 2015). Data from all variables and parameters were analyzed using ANOVA and treatments mean were compared either with F, Dunnet or Tukey tests ($P \leq 0.05$) using SAS (version 6.12; SAS Institute, Inc.). Principal components analysis (PCA) technique was used to determine the relationship among the variables evaluated on Exps 5 and 7. Data were analyzed using the Minitab software (version 15; Minitab Corporation).

Results

Effect of different PA concentrations on leaf gas exchange and Chl *a* fluorescence parameters

Above 0.1 mg PA ml^{-1}, the *A* values decreased significantly with increasing PA concentrations and were 52% lower at the highest concentration (5.0 mg PA ml^{-1}) than at 0 or 0.1 mg PA ml^{-1} (Fig. 1A). Similar decreases for g_s (Fig. 1B) and *E* (Fig. 1C), observed at concentrations above 0.3 mg PA ml^{-1}, were also found; at 5.0 mg PA ml^{-1} both g_s and *E* values were 53% lower than those of control plants. At the concentrations of 3.0 and 5.0 mg PA ml^{-1}, significant decreases in both F_v/F_m (9–11%) and q_P (10–18%) (Fig. 1D,E) were coupled with significant increases in Y(NO) (28–34%) in comparison to the other PA concentrations (Fig. 1F). Leaves of plants sprayed with PA solutions displayed visible necrotic lesions at 48 hai, but only at concentrations ≥0.3 mg PA ml^{-1} (Fig. S1). The imaging of the F_v/F_m, Y(NO) and q_P parameters became more pronounced (seen as larger necrotic lesions) with increasing PA concentrations (Fig. S1).

Histochemical localization of H_2O_2 and $O_2^{\bullet-}$ on leaves sprayed with different PA concentrations

The deposition of H_2O_2 and $O_2^{\bullet-}$ at 24 hai on the leaves was stronger as the concentrations of PA increased from 0.3 to 5.0 mg ml^{-1}. The brown and blue colors were less remarkable on the leaves of plants sprayed with 0.1 mg PA ml^{-1} resembling the leaves of plants from the control treatment (Fig. 2A,B).

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

Mycelial growth, conidia production and germination were not affected by the concentration of 0.1 mg PA ml^{-1} (Fig. 3A–D). Compared to the control treatment, mycelial growth was reduced by 22% on PDA media amended with 0.3 mg PA ml^{-1} (Fig. 3A), and reduced on OA media amended with 0.1 mg PA ml^{-1} (Fig. 3B). Mycelial growth was completely inhibited on PDA media with PA at concentrations above 0.5 mg ml^{-1} (data not shown). Conidia production and germination were significantly reduced by 58% on OA media amended with 0.3 mg PA ml^{-1} compared to the control treatment. Mycelia growth, conidia production and germination were not affected by the concentration of 0.1 mg PA ml^{-1} (Fig. 3A–D).

Blast severity

BS significantly decreased by 59 and 23% at 72 and 96 hai, respectively, for plants sprayed with PA compared to non-sprayed plants (Fig. 4A). Many necrotic lesions with intense chlorosis and coalescence were noticed on leaves of plants non-sprayed with PA. By contrast, the number and size of the necrotic lesions were reduced, the quantity of coalescing lesions was less, and chlorosis was reduced on leaves of plants sprayed with PA (Fig. 4B).

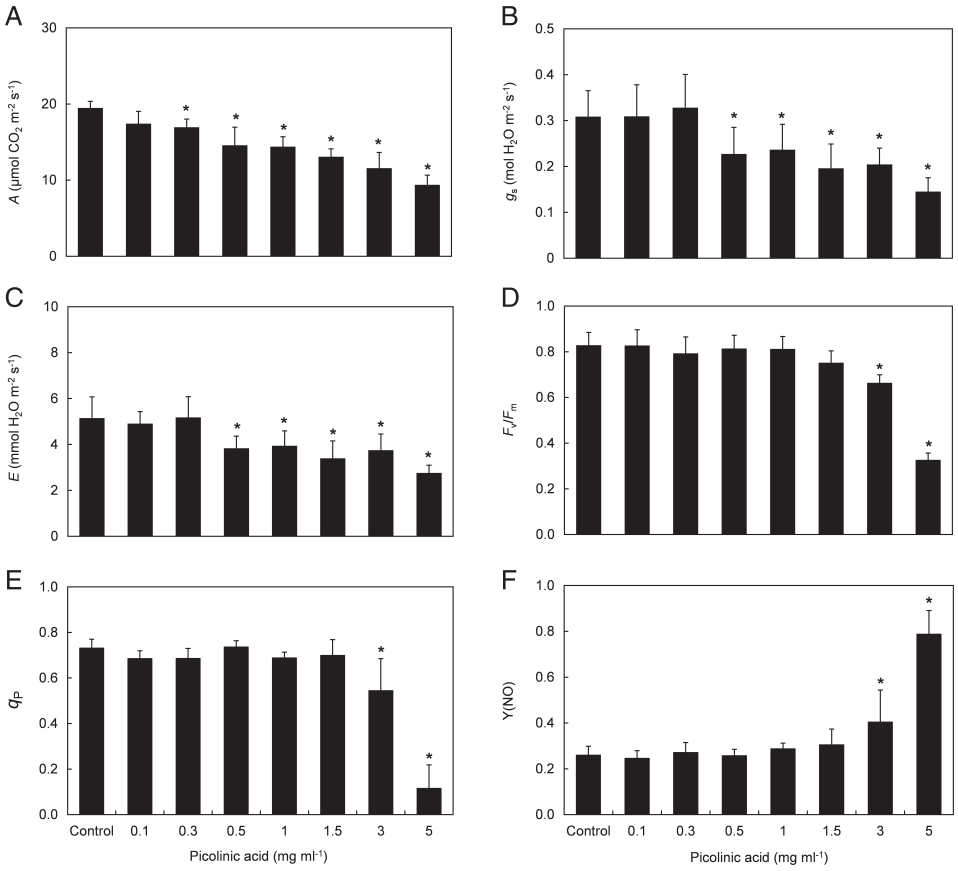

Fig. 1. Leaf gas exchange [net carbon assimilation rate (*A*), stomatal conductance to water vapor (g_s) (B), and transpiration rate (*E*) (C)] and chlorophyll *a* fluorescence [maximum PSII quantum efficiency (F_v/F_m) (D), photochemical quenching coefficient (q_p) (E), and yield of non-regulated dissipation energy (Y(NO)) (F)] parameters determined at 48 hours after the spray of wheat leaves with water (control) and with picolinic acid (PA) at different concentrations. Mean for each PA concentration with an asterisk (*) differ significantly ($P \leq 0.05$) from the control treatment according to Dunnet's test. Bars represent the standard deviation of the means from two pooled experiments. n = 10.

Histochemical localization of H_2O_2 and $O_2^{\bullet-}$ on infected leaves sprayed with a non-phytotoxic PA concentration

The production of H_2O_2 and $O_2^{\bullet-}$ was greater on leaves of plants from the −PA/+*Po* treatment than plants from the +PA/+*Po* treatment especially at 72 hai (Fig. 5).

Enzyme activities and MDA concentration

The activities of SOD and APX at 48 hai (22 and 24%, respectively), GR at 48 and 72 hai (24 and 26%, respectively), CAT at 96 hai (46%), and GPX and GST at 48 hai (45 and 52%, respectively) significantly decreased for the +PA/−*Po* treatment in comparison to the −PA/−*Po* treatment (Fig. 6A–D,F,G). There were significant increases for SOD (120%), GR (208%), APX (136%), POX (145%) and GST (134%) activities at 48 hai, for CAT activity at 96 hai (99%) and for GPX activity at 48, 72 and 96 hai (242, 45 and 95%, respectively), but significant decrease for CAT activity at 72 hai (50%) for the +PA/+*Po* treatment in comparison to the −PA/+*Po* treatment (Fig. 6A–G). For the −PA/+*Po* treatment, there were significant increases at 48, 72 and 96 hai for SOD (20, 45 and 41%, respectively), CAT (396, 289 and 58%, respectively) and POX (197, 300 and 342%, respectively) activities and for APX at 72 and 96 hai (48 and 29%, respectively) in comparison to the −PA/−*Po* treatment (Fig. 6A,C,D and E). For the +PA/+*Po* treatment, there were significant increases at 48 and 96 hai for SOD (201 and 130%, respectively), GR (310 and 69%, respectively) and APX (138 and 58%, respectively) activities, at 48, 72 and 96 hai for CAT (121, 193 and 477%, respectively) and POX (854, 270 and 469%, respectively) activities and for GPX (116%) and GST (228%) activities at 48 hai in comparison to the +PA/−*Po* treatment (Fig. 6A–G). GR activity significantly decreased at 72 hai (41%) for the +PA/+*Po* treatment in comparison to the treatment +PA/−*Po* while GPX significantly decreased at 48, 72 and 96 hai (65, 43 and 23%, respectively) for the −PA/+*Po* treatment in comparison to the −PA/−*Po* treatment (Fig. 6B and F). The MDA concentration was significantly lower for plants from the +PA/+*Po* in comparison to those from the −PA/+*Po* treatment, but significantly higher at 48 hai (36%) for plants from the treatment +PA/+*Po* in comparison to the treatment +PA/−*Po* and at 96 hai (132%) for the treatment −PA/+*Po* in comparison to the treatment −PA/−*Po* (Fig. 6H).

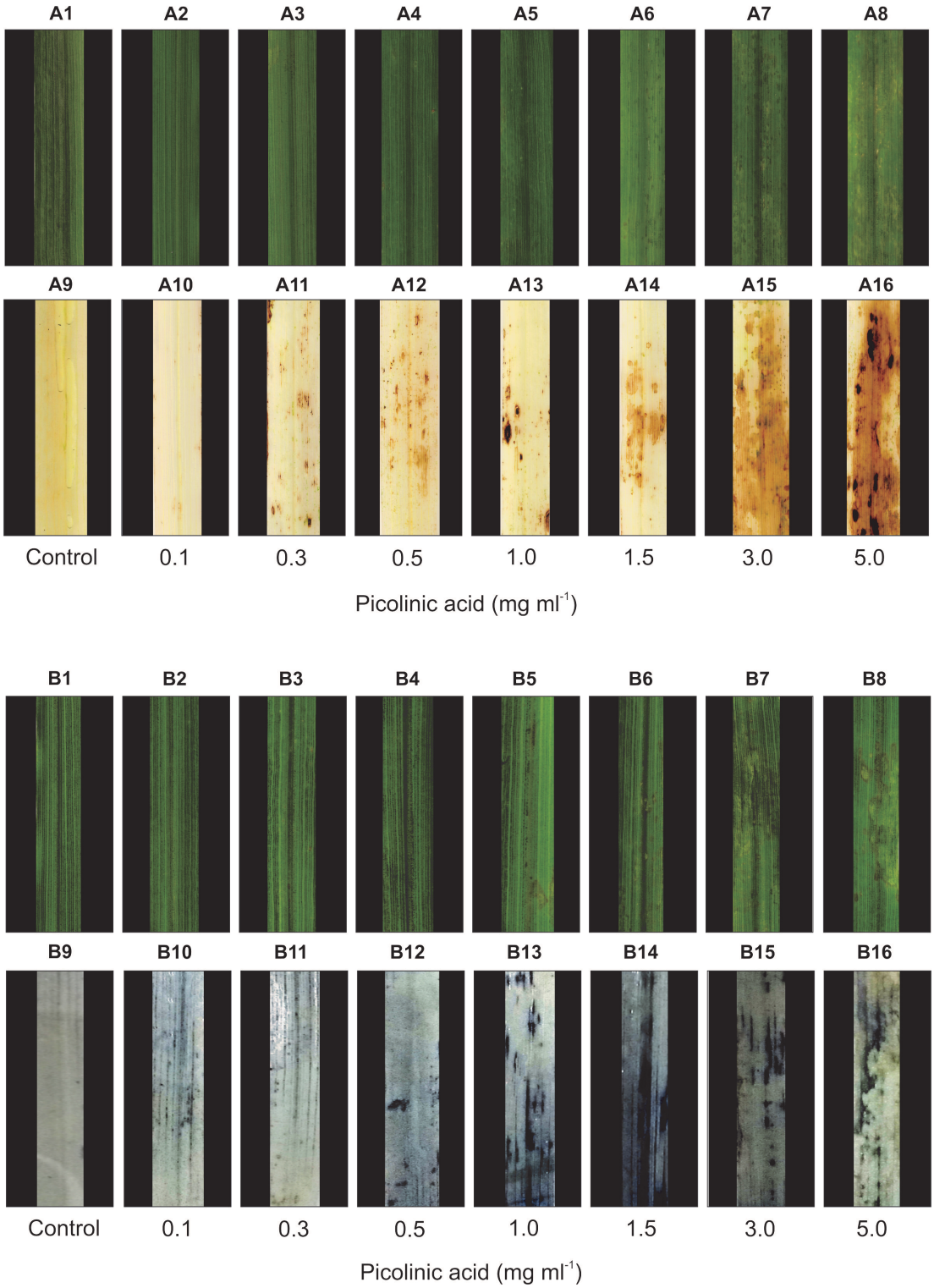

Fig. 2. Lesion development on the leaves of plants at 24 hours after the spray of picolinic acid at different concentrations (A2–A8 and B2–B8). Leaves of plants at 24 hours after spray with water (A1 and B1) served as the control treatment. These leaves were used for the histochemical detection of hydrogen peroxide [H_2O_2] (A9–A16) and superoxide anion radical [$O_2^{\bullet-}$] (B9–B16) as indicated by the brown and blue colors, respectively. n = 15.

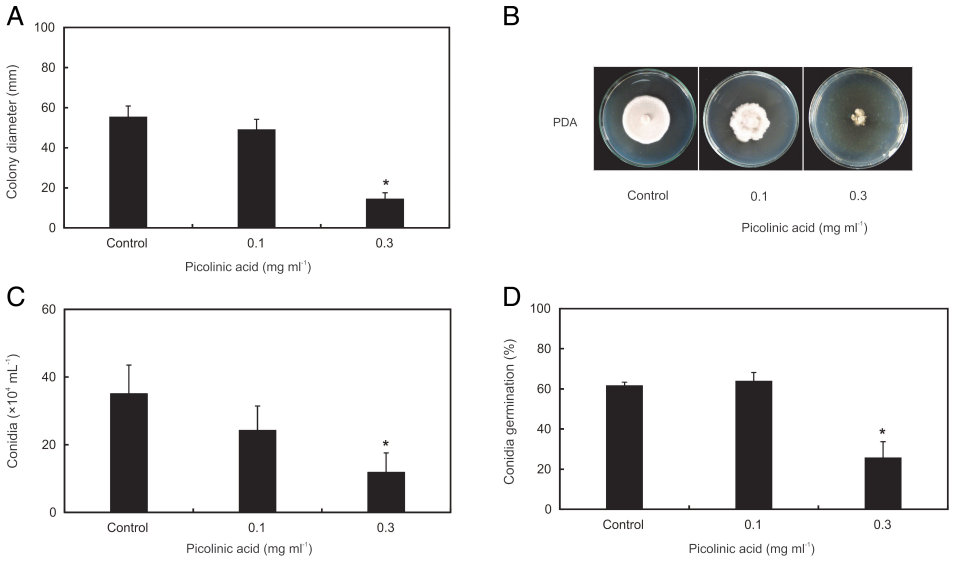

Fig. 3. In vitro effect of picolinic acid (PA) at different concentrations on mycelial growth (A and B), conidia production (C) and germination (D). Treatments means followed by an asterisk (*) are significantly different ($P \leq 0.05$) according to Dunnet's test. Bars represent the standard desviation of means from two pooled experiments. n = 8.

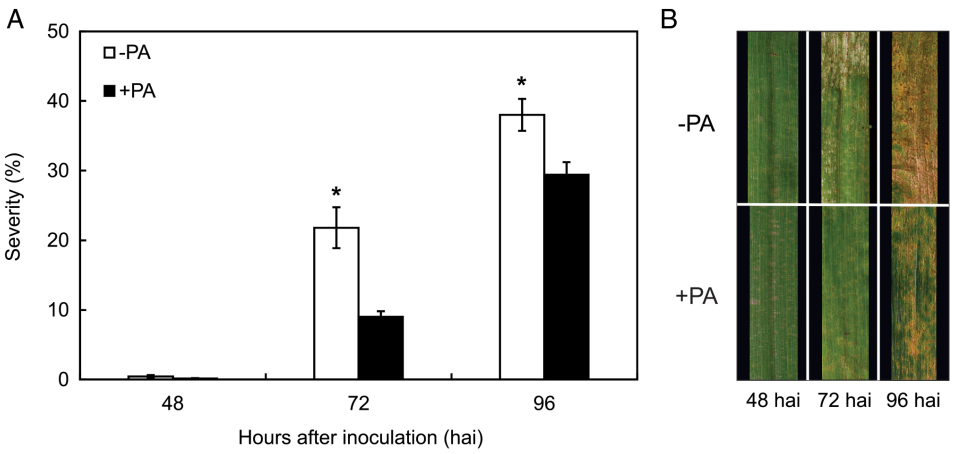

Fig. 4. Severity (A) and blast symptoms development (B) on the leaves of plants sprayed with water (−PA) or sprayed with picolinic acid (PA) (+PA). Means for the −PA and + PA treatments followed by an asterisk (*), at each evaluation time, are significantly different according to *F* test ($P \leq 0.05$). Bars represent the standard error of the means from two pooled experiments. n = 10.

Effect of a non-phytotoxic PA concentration and infection by *P. oryzae* on leaf gas exchange and Chl *a* fluorescence parameters

At 96 hai, the values of A, g_s, E, F_v/F_m and Y(II) were significantly higher (50, 54, 49, 17 and 57%, respectively) while those for Y(NO) (19%) were significantly lower for the +PA/+*Po* treatment in comparison to the −PA/+*Po* treatment (Fig. 7A,B,D,E,F and H). There were significantly decreases at 48, 72, 96 hai for A (21, 41 and 31%, respectively) and F_v/F_m (9, 7 and 12%, respectively), for g_s (49 and 51%) and E (41 and 37%) and at 96 hai for Y(NPQ) (41%) and significant increase for Y(NO) (38%) at 96 hai for the treatment +PA/+*Po* in comparison to the +PA/−*Po* treatment (Fig. 7A,B,D,E,G and H). For the −PA/+*Po* treatment, there were significant decreases at 48, 72, and 96 hai for A (10, 21, and 63%, respectively) and F_v/F_m (9, 8 and 32%, respectively), at 72 and 96 hai for g_s (29 and 62%, respectively) and E (22 and 50%, respectively), and at 96 hai for Y(II) (46%) and Y(NPQ) (31%) while significant increase occurred for C_i at 48 (13%) and 96 hai (14%) and Y(NO) at 96 hai (115%) in comparison to the -PA/-*Po* treatment (Fig. 6A–H).

Alterations on the images for the F_v/F_m, Y(II), Y(NPQ) and Y(NO) parameters were first noticed at 72 hai mainly on the leaves from plants of the −PA/+*Po* treatment compared to +PA/+*Po* treatment. Decreases on the F_v/F_m, Y(II) and Y(NPQ) values and high Y(NO) values at 96 hai were closely related to reductions in the light energy dissipassion for the photosynthetic process (black areas in the images) especially on leaves from plants of the −PA/+*Po* treatment compared to +PA/+*Po* treatment (Fig. S2).

Based on the principal component analysis (PCA) for all treatments at the different evaluation times, the treatments −PA/−*Po* and +PA/−*Po* were kept grouped at 48, 72 and 96 hai (Fig. 8A,C, and E). At 48 hai, the first two PCs accounted for 65% of the total data variance of the data (PC 1 = 40% and PC 2 = 25%) (Fig. 8A). The PC

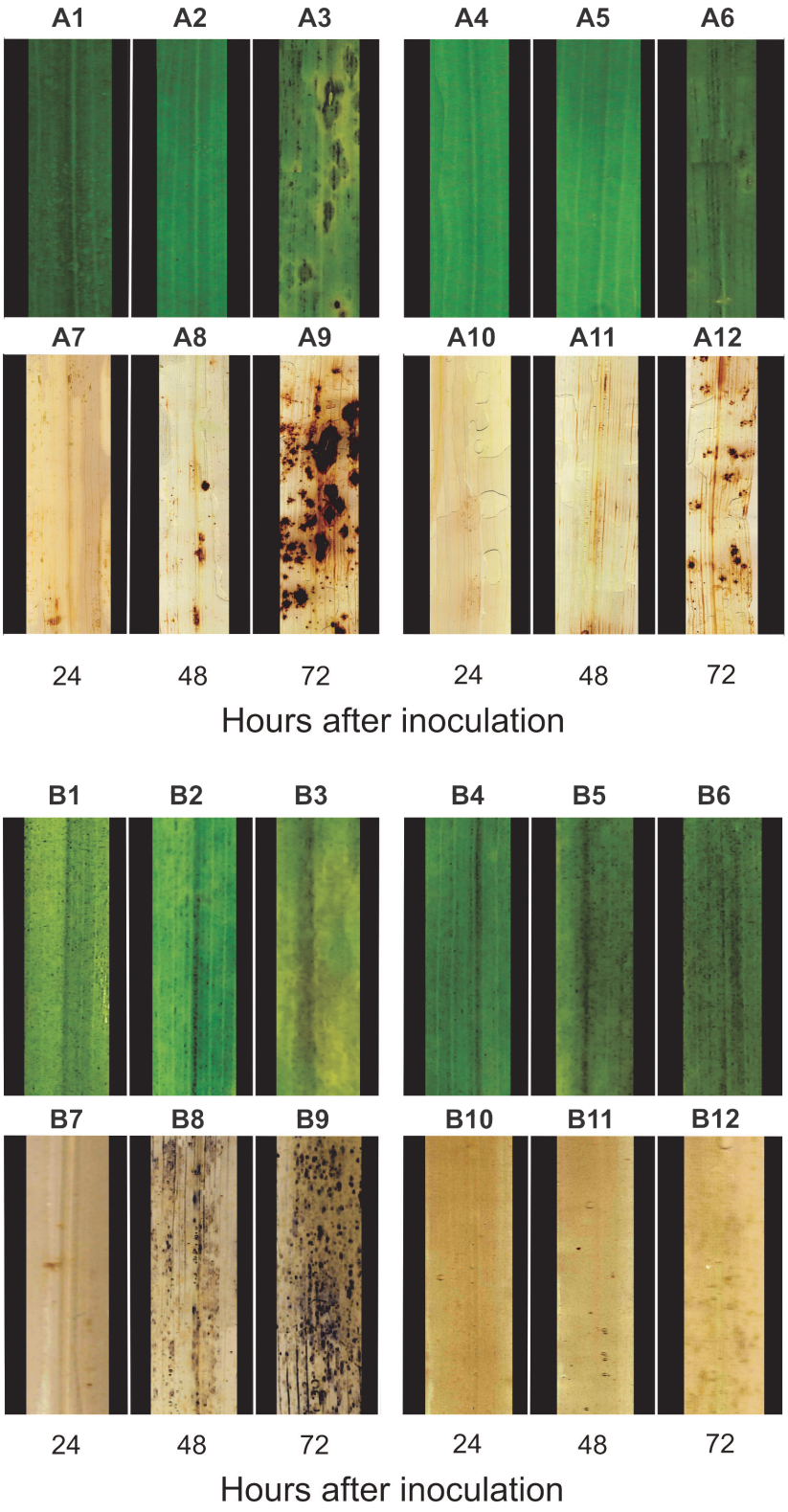

Fig. 5. Development of blast lesions on the leaves of plants at 24, 48, and 72 hours after inoculation with *Pyricularia oryzae* that were non-sprayed (A1–A3 and B1–B3) or sprayed with picolinic acid (A4–A6 and B4–B6). These leaves were used for the histochemical detection of hydrogen peroxide [H_2O_2] (A7–A12) and superoxide anion radical [$O_2^{\bullet-}$] (B7–B12) as indicated by the brown color and blue colors, respectively. n = 15.

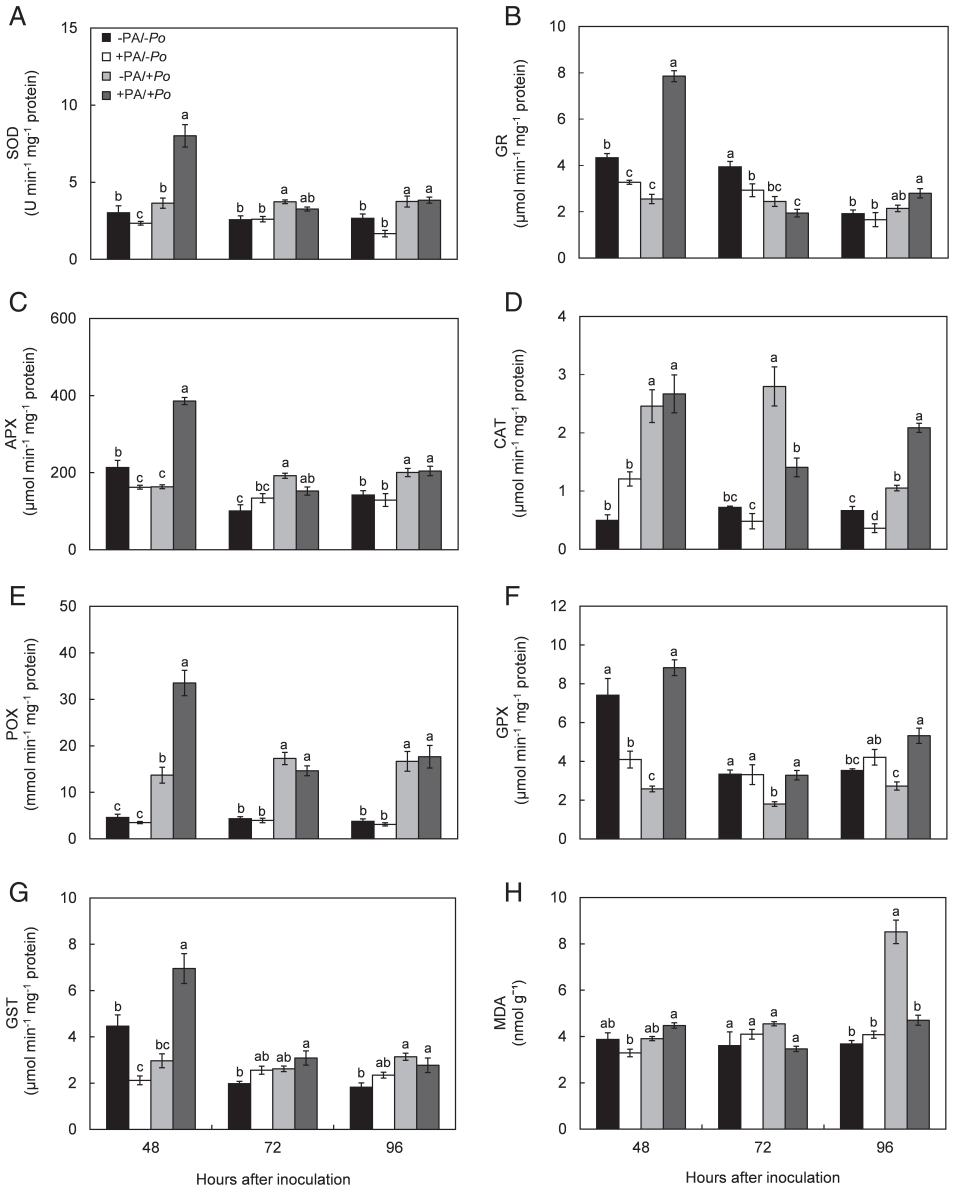

Fig. 6. Activities of superoxide dismutases (SOD) (A), glutathione reductases (GR) (B), ascorbate peroxidases (APX) (C), catalases (CAT) (D), peroxidases (POX) (E), glutathione peroxidases (GPX) (F) and glutathione-*S*-transferases (GST) (G) as well as concentration of malondialdehyde (MDA) (H) determined in the leaves of wheat plants that were sprayed with water (−PA/−*Po*), sprayed with 0.1 mg of picolinic acid (+PA/−*Po*) ml^{-1} (PA), only inoculated with *Pyricularia oryzae* (−PA/+*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.

1, characterized by positive scores for APX, GST, POX and SOD, showed negative correlations with F_v/F_m; the PC 2 showed positive scores for Y(NPQ), GPX and GR and negative correlation with Y(II) (Fig. 8B). At 72 hai, the PCA explained 67% of the observed variation (PC 1 = 52% and PC 2 = 15%) (Fig. 8C). Positive scores for BS, POX, and SOD and negative correlation between A and g_s parameters were noticed for the PC 1. For PC 2, positive score occurred for Y(II) and negative correlations with Y(NPQ), CAT and MDA (Fig. 8D). At 96 hai, 85% of the total data variance was explained for the two PCs (PC 1 = 69% and PC 2 = 16%) (Fig. 8E). For PC 1, there were positive scores for C_i, Y(NO), BS, APX, POX, and SOD and negative scores for A, g_s, E, Y(II) and F_v/F_m (Fig. 8F). For the +PA/+*Po* treatment, the PCA indicated positive correlation with APX, CAT, GR, GPX and GST at 48 and 96 hai. On the other hand, for the −PA/+*Po* treatment there were positive correlation for C_i, MDA and Y(NO) and negative correlations for A, g_s, E, Y(II) and F_v/F_m.

Discussion

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

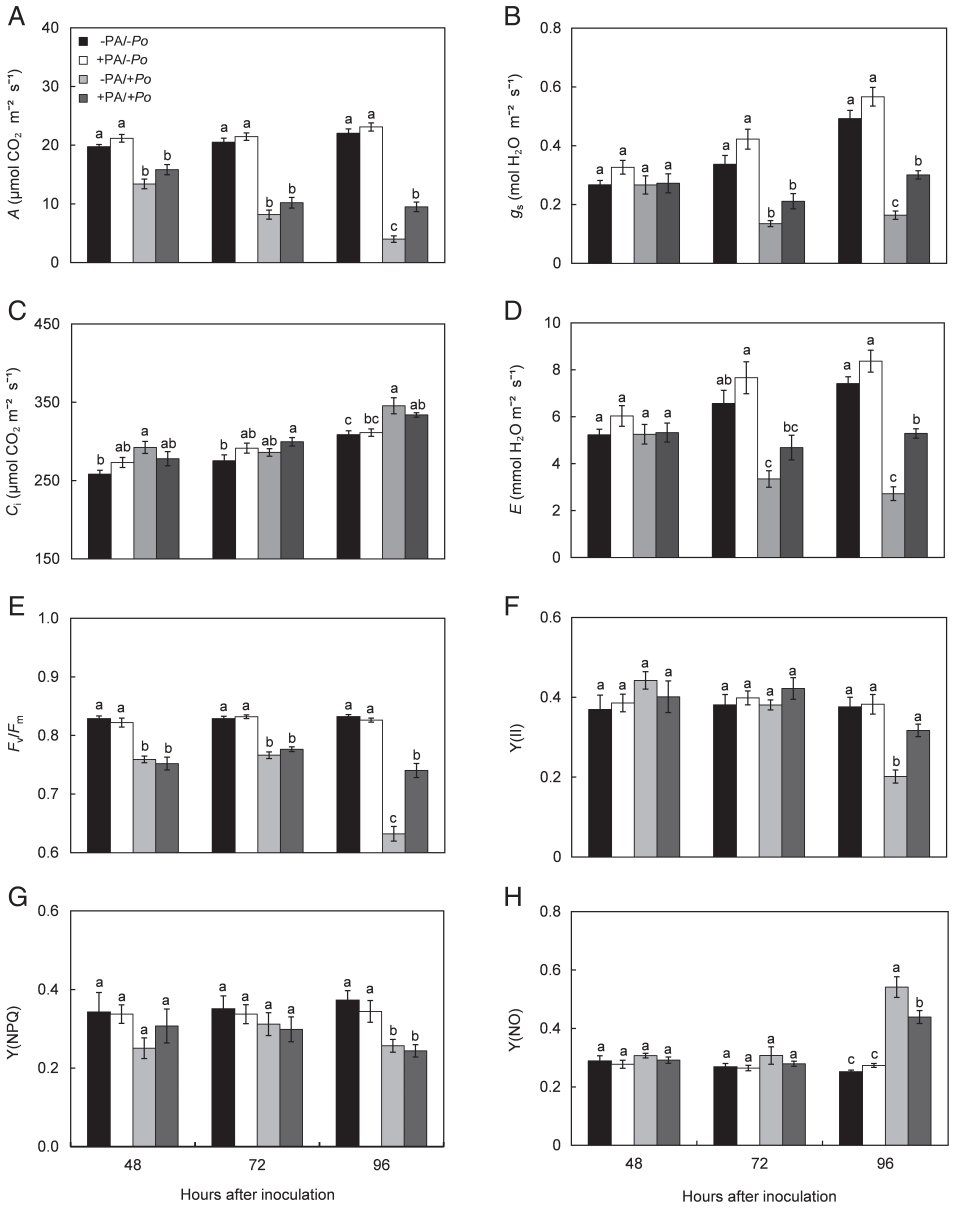

Fig. 7. Net CO_2 assimilation rate (*A*) (A), stomatal conductance to water vapor (g_s) (B), internal CO_2 concentration (C_i) (C), transpiration rate (*E*) (D), maximum PSII quantum efficiency (F_v/F_m) (E), effective PSII quantum yield Y(II) (F), quantum yield of regulated energy dissipation Y(NPQ) (G) and quantum yield of non-regulated energy dissipation Y(NO) (H) determined in the leaves of wheat plants that were sprayed with water (–PA/–*Po*), sprayed with 0.1 mg of picolinic acid (PA) ml^{-1} (+PA/–*Po*), only inoculated with *Pyricularia oryzae* (–PA/+*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 = 10.

The non-selective host toxins can markedly alter a range of physiological and biochemical processes of plants. For instance, pyricularina, tenuazonic acid, pyriculol 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, Jacob et al. 2016). In this study, necrotic lesions were noticed on wheat leaves at 48 h after spray with a PA solution. On wheat leaves, the lesions caused by PA were accompanied by the accumulation of H_2O_2 and $O_2^{\bullet-}$, being more evident as the concentrations of PA increased. Similar results with H_2O_2 were reported in rice leaves sprayed with PA at concentrations starting with 1 mg ml^{-1} (Zhang et al. 2004).

In this study, the spray of PA solutions caused necrosis of leaf tissues that were accompanied by dramatic reductions in *A* and *E* due to diffusive limitations that were linked to lower g_s values. Decreases in photosynthetic gas exchanges associated with toxins have been reported elsewhere, e.g. (1) rapid decreases in *A* (caused by high concentrations of toxins produced by *Helminthosporium maydis*) due to stomatal closure associated with limitations in potassium uptake by the guard cells on the corn leaves (Arntzen et al. 1973) and (2) drastic reductions in g_s and E on the banana leaves caused by fusaric

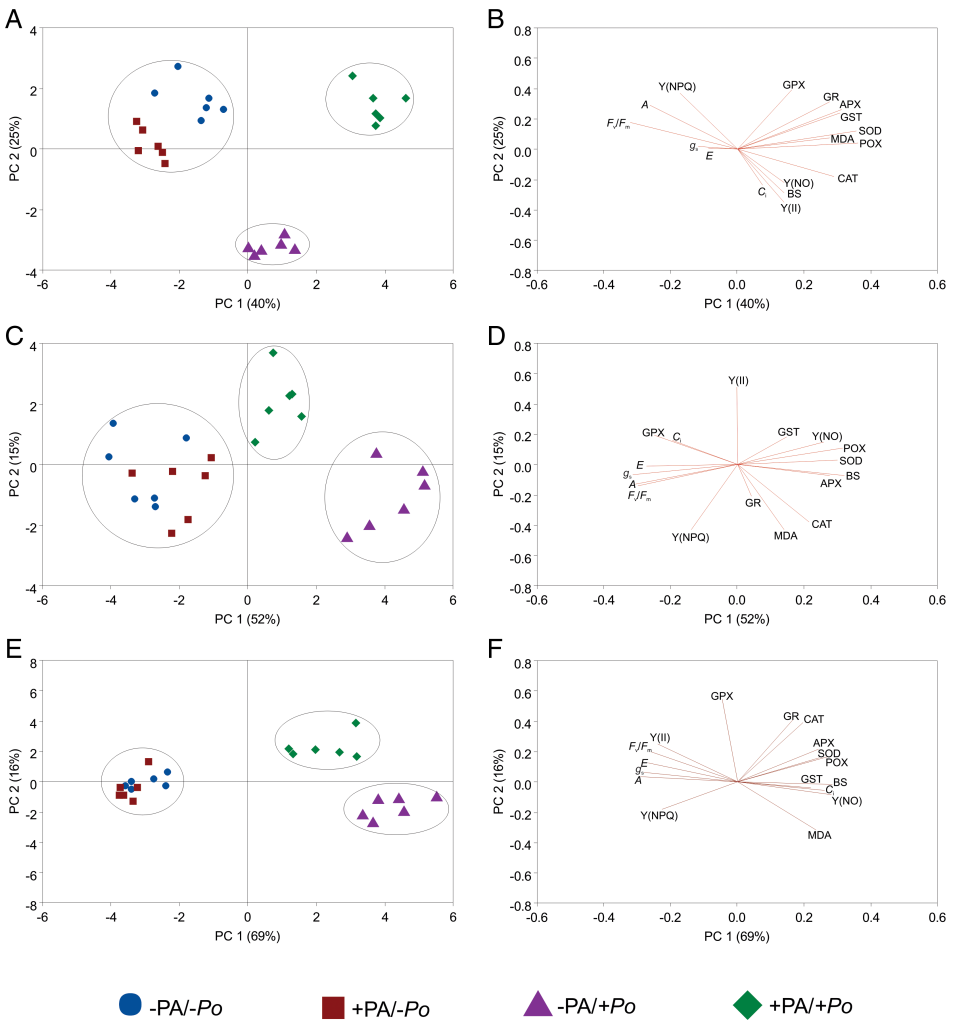

Fig. 8. Score plot (A, C and E) and loading values (B, D and F) of principal components analysis comparing blast severity (BS), the leaf gas exchange parameters (net CO_2 assimilation rate [*A*], stomatal conductance to water vapor [g_s], internal CO_2 concentration [C_i] and transpiration rate [*E*]), chlorophyll fluorescence imaging parameters (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)]), enzymes of the antioxidant system (superoxide dismutases [SOD], glutathione reductases [GR], ascorbate peroxidases [APX], catalases [CAT], peroxidases [POX], glutathione peroxidases [GPX] and glutathione-*S*-transferases [GST]) as well as concentration of malondialdehyde [MDA] determined in the leaves of wheat plants that were sprayed with water (−PA/−*Po*), sprayed with 0.1 mg of picolinic acid (+PA/−*Po*) ml^{-1}(PA), only inoculated with *Pyricularia oryzae* (−PA/+*Po*), and sprayed with 0.1 mg PA ml^{-1} and inoculated with *P. oryzae* (+PA/+*Po*) at 48 (A and B), 72 (C and D) and 96 (E and F) hours after inoculation.

acid (a non-host selective toxin produced by all *Fusarium* species) at a concentration of 200 µg ml^{-1} (Dong et al. 2012). We additionally showed that impairments to photosynthesis caused by PA were also associated with photochemical constraints as denoted by the reductions in both F_v/F_m and q_P that were accompanied by increases in Y(NO), which altogether indicate damage to the PSII photochemistry. Based on these results, the indirect or direct effects of exogenous PA spray to wheat leaves and further inoculation with *P. oryzae* were next tested.

Based on the *in vitro* tests, fungal mycelial growth, conidia production and germination were affected by PA suggesting, therefore, that this non-host selective toxin interfered on fungal physiology. In this regard, Pasechnik et al. (1993) demonstrated that concentrations ranging from 0.12 to 0.37 mg PA ml^{-1} caused a drastic decrease on conidial germination of *P. oryzae*. Although the concentration of 0.3 mg PA ml^{-1} caused alterations in the pattern of fungal growth, fungal sporulation and conidial germination, this concentration was not selected due to the photosynthetic alterations and the occurrence of necrotic lesions.

A more effective antioxidative metabolism of the plants sprayed with PA, mainly for the enzymes related to the H_2O_2 metabolism, enhanced their resistance to blast. This suggests alterations in cellular homeostasis due to high production of ROS associated with host defense responses and the restoration of cellular oxidative balance in agreement with the findings of Torres (2010) and Torres et al. (2006). Additionally, this response was more pronounced for plants sprayed with a solution of 0.1 mg PA ml^{-1}, especially for SOD, APX, GR, POX, GPX and GST activities at 48 hai, which suggests that different ROS detoxification pathways were operating in response to PA spray. Therefore, PA favored a more efficient antioxidant metabolism of the infected plants as denoted through the less accumulation of H_2O_2 and $O_2^{\bullet-}$. Previous

studies have shown that an efficient antioxidant system, especially based on the high activities of APX, POX, SOD and GST involved in the removal of ROS, limited cell damages caused by *P. oryzae* infection on wheat leaves, thus contributing to a reduced blast severity (Debona et al. 2012).

This results reinforce the importance of APX and SOD as the major ROS-scavenging enzymes and that a balance on their activities is of paramount importance to determine the state of H_2O_2 and $O_2^{\bullet-}$ scavenging (Asada and Takahashi 1987, Bowler et al. 1992, Willekens et al. 1997). Other enzymes also played a role in scavenging ROS, as was the case of CAT, whose activity was higher for inoculated plants regardless of PA spray indicating its importance for the ROS metabolism. Interestingly, CAT activity increased on wheat plants sprayed with abscisic acid, salicylic acid, calcium or H_2O_2 (Agarwal et al. 2005). The POX, another enzyme involved in the antioxidant system of plants infected by pathogens (Milavec et al. 2001), was the one that showed the greatest increase throughout fungal infection (+PA/+*Po* and −PA/+*Po* treatments) compared to the non-inoculated plants. Similar results were reported for the *Cucurbita pepo*-zucchini yellow mosaic virus interaction using the salicylic acid as a plant inducer (Radwan et al. 2007). The GPX and GST, which use GSH to reduce H_2O_2 and other hydroperoxides (Noctor et al. 2002, Gill and Tuteja 2010), displayed increased activities during the early stage of *P. oryzae* infection on plants sprayed with PA. According to Debona et al. (2012), GST activity was considered the most important antioxidant enzyme on wheat cultivars resistant to blast. Therefore, it is tempting to suggest 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, a typically chloroplastidic enzyme, plays an essential role in the plant defense system against ROS by sustaining the reduced status of GSH (Gill and Tuteja 2010); it displayed a high activity at 48 hai for plants submitted to the +PA/+*Po* treatment, suggesting a possible effect of PA in protecting cells against oxidative damage by sustaining a high GSH/GSSG ratio. In some plant–pathogen interactions, GR activity, in addition to controlling the H_2O_2 concentration, contributes to avoid lesion expansion, accumulation of salicylic acid, activation of genes related to pathogenesis and signaling pathways of ethylene and jasmonic acid (Mhamdi et al. 2010). Most importantly, the orchestrated action of the antioxidative system of wheat plants sprayed with PA proved to be successful to counteract fungal infection as judged from the lower MDA concentration, which suggests a reduction of cell damage caused by the oxidative stress.

The gas exchange and Chl *a* fluorescence imaging parameters were virtually uncompromised on infected wheat plants sprayed with PA in contrast to the dramatic alterations on their values on leaves of non-sprayed plants. All of these findings are consistent with Debona et al. (2014), who reported that the efficient 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 often decreases g_s that in turn lead to increased resistance to CO_2 diffusion from the atmosphere into the leaf (Erickson et al. 2003, Resende et al. 2012, Aucique-Pérez et al. 2014, Bispo et al. 2014). In this study, g_s and E were drastically reduced coupled with increments in C_i for inoculated plants in comparison to the non-inoculated ones. However, the greatest impact of *P. oryzae* occurred in g_s, C_i and E for plants non-sprayed with PA. In the leaves of wheat plants infected with *P. oryzae*, reduction 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 that the non-sprayed leaves with PA were more affected by *P. oryzae* infection than their sprayed counterparts. The F_v/F_m, Y(II), Y(NPQ) and Y(NO) can increase or decrease as reported for the pathosystems *Arabidopsis thaliana-Pseudomonas syringae* (Bonfig et al. 2006), coffee-*Hemileia vastatrix* (Honorato et al. 2015), maize-*Stenocarpella macrospora* (Bermúdez-Cardona et al. 2015a, 2015b) and rice-*Monographella albescens* (Tatagiba et al. 2014). Decreases in F_v/F_m on plants infected by pathogens indicate damage to the reactions at the PSII level (Baker 2008). Additionally, reductions in Y(II) and Y(NPQ) coupled with an increase in Y(NO) at 96 hai for infected plants non-sprayed with PA in contrast to their sprayed counterparts indicated that the ability of leaves on the photochemical energy conversion was significantly compromised at the necrotrophic phase of *P. oryzae*. Decrease in Y(NPQ) suggest impairments in the dissipation of excess of the energy of excitation at the level of the complex antenna (Su et al. 2017), whereas the increase in Y(NO) suggests that both photochemical energy conversion and protective regulatory mechanisms were inefficient (Bonfig et al. 2006). Taken together, these results suggest that *P. oryzae* infection caused drastic alterations in the photosynthetic machinery of the wheat plants by compromising both the dissipation and photo-protective mechanisms, thus ultimately leading to oxidative stress. Nevertheless, these effects were relieved by an efficient antioxidant metabolism on plants sprayed with PA at the earlier stages of fungal infection.

The lesions formed on the leaves of plants sprayed with PA, mainly at the highest concentrations, dramatically compromised their photosynthetic performance. However, a low PA concentration unable to impair photosynthesis potentiated the antioxidative metabolism of leaves infected by *P. oryzae* for a rapid removal of the ROS and a less oxidative damage that culminated to an increase on wheat resistance to blast.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Lesions and images of the chlorophyll *a* fluorescence parameters

Fig. S2. Images of the parameters of chlorophyll *a* fluorescence

Edited by C. Foyer