Biochemical Changes in the Leaves of Wheat Plants Infected by *Pyricularia oryzae*

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ABSTRACT

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Blast, caused by the fungus *Pyricularia oryzae*, is a major disease of the wheat crop in the Brazilian Cerrado and represents a potential threat to world wheat production. However, information about the wheat–*P. oryzae* interaction is still limited. In this work, the activities of the enzymes superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), glutathione-S-transferase (GST), ascorbate peroxidase (APX), glutathione reductase (GR), and glutathione peroxidase (GPX) and the concentrations of superoxide (O_2^-), hydrogen peroxide (H_2O_2), and malondialdehyde (MDA) as well as the electrolyte leakage (EL) were studied in wheat plants ‘BR 18’ and ‘BRS 229’, which are susceptible and partially resistant, respectively, to leaf blast at the vegetative growth stage, during the infection process of *P. oryzae*. The blast severity in BRS 229 was 50%

lower than in BR 18 at 96 h after inoculation (hai). The activities of SOD, POX, APX, and GST increased for both cultivars in the inoculated plants compared with noninoculated plants and the increases were more pronounced for BRS 229 than for BR 18 at 96 hai. The GR and CAT activities only increased in inoculated plants from BRS 229 at 96 hai. For BR 18, the GR activity was not influenced by plant inoculation, and the CAT activity was lower in inoculated plants. The GPX activity only increased in inoculated plants from BR 18 at 48 and 72 hai. The *P. oryzae* infection increased the O_2^- , H_2O_2 , and MDA concentrations and EL. However, the greater increases of the SOD, POX, APX, GST, GR, and CAT activities for BRS 229 compared with BR 18 contributed to the lower O_2^- , H_2O_2 , and MDA concentrations and EL verified in the former. These results show that a more efficient antioxidative system in the removal of excess of reactive oxygen species generated during the infection process of *P. oryzae* limits the cellular damage caused by the fungus, thus contributing to greater wheat resistance to blast.

Blast, caused by the fungus *Pyricularia oryzae* Cavara (teleomorph: *Magnaporthe grisea* (T. T. Hebert) M. E. Barr), is an agronomically important disease of wheat (*Triticum aestivum* L.) and can cause yield losses $\leq 70\%$ (32). Blast symptoms on leaves begin as gray-green and water-soaked lesions, with dark-green borders after they are completely expanded (67). On glumes, elliptical lesions with a gray center surrounded by brown borders are observed (67). The symptoms on spikes are evidenced as a bright black point on rachis (67). The rachis colonization by *P. oryzae* decreases nutrient translocation to the grains, which become shriveled in appearance, small, misshapen, and underweight (32).

The absence of cultivars with a desirable level of resistance to blast and the low efficiency of the available fungicides (18,32–34,72) makes blast control very difficult to achieve. Today, the release of wheat cultivars with a high level of resistance to blast has been a priority for wheat breeding programs, especially to areas with severe epidemics of blast (18). Some studies have demonstrated the existence of cultivars partially resistant to blast (7,18,73) but the mechanisms involved in this resistance is absent.

Plants under stress conditions, such as pathogen attack, are induced to increase the production of reactive oxygen species (ROS) (50). Hydrogen peroxide (H_2O_2), a type of ROS, is directly involved in the death of both pathogens and host cells and in the oxidative cross-links between proteins in the cell walls, which become more resistant to degradation by lytic enzymes and nonselective toxins released by the pathogen (53). Although H_2O_2 is essential for signaling and plant defense against pathogen infec-

tion, it can react with the superoxide (O_2^-) radical or with metal ions to form a hydroxyl radical ($OH^\bullet$), which is extremely toxic to pathogens in addition to enhancing the cytotoxicity of H_2O_2 (49). Oxidative stress causes lipid peroxidation in the cell membrane and damage to pigments, proteins, and nucleic acids (5).

To remove the excess ROS generated during the host–pathogen interaction, plants have developed nonenzymatic and enzymatic mechanisms (69). Superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), glutathione-S-transferase (GST), ascorbate peroxidase (APX), glutathione reductase (GR), and glutathione peroxidase (GPX) are the enzymes commonly involved in the host defense against oxidative stress (6,22,40,55,60,71). SOD, which is involved in the dismutation of the O_2^- radical to molecular oxygen and H_2O_2 (31), constitutes the first line of defense against oxidative stress (12). The enzymes involved in the removal of H_2O_2 include CAT, POX, APX, GST, GPX, and GR (6,22,40,55,60,73).

Even though changes in the antioxidative system of plants during pathogen infection have been well documented (1,2,21,47), only a few studies have investigated the relationship of these changes to host resistance. To the best of our knowledge, information about the changes in the antioxidative system and the concentration of ROS in the leaves of wheat plants during the infectious process of *P. oryzae* is missing. Therefore, this study aimed to determine the activity of antioxidative enzymes, the concentration of ROS, and damage to the cell membrane in two cultivars with different levels of resistance to blast.

MATERIALS AND METHODS

Plant growth. Wheat seed from ‘BR 18’ and ‘BRS 229’ were surface-sterilized in 10% (vol vol⁻¹) NaOCl for 2 min, rinsed in sterilized water for 3 min, and sown in plastic pots (20 cm in

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diameter) (Ecovaso, Jaguariúna, São Paulo, Brazil) filled with 1 kg of substrate made from a 1:1:1 mixture of pine bark, peat, and expanded vermiculite (Tropstrato; Vida Verde, Mogi Mirim, São Paulo, Brazil). A previous study showed that BR 18 and BRS 229 were susceptible and partially resistant, respectively, to leaf blast at the vegetative growth stage (18). In total, 1.63 g of calcium phosphate monobasic was added to each plastic pot. In all, 12 seeds were sown per pot and, 5 days after seedling emergence, each pot was thinned to six seedlings. During seed germination, the temperature was $25 \pm 5^\circ\text{C}$, the relative humidity was $65 \pm 5\%$, and sunlight conditions were provided inside the greenhouse. The substrate in each pot was fertilized with a nutrient solution containing the following: KCl at 6.4 g liter^{-1} , K_2SO_4 at $3.48 \text{ g liter}^{-1}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ at $5.01 \text{ g liter}^{-1}$, $(\text{NH}_2)_2\text{CO}$ at $2.03 \text{ g liter}^{-1}$, $\text{NH}_4\text{MO}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ at $0.009 \text{ g liter}^{-1}$, H_3BO_3 at $0.054 \text{ g liter}^{-1}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ at $0.222 \text{ g liter}^{-1}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ at $0.058 \text{ g liter}^{-1}$, and $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ at $0.137 \text{ g liter}^{-1}$ (75). A volume of 15 ml of nutrient solution containing $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ at $0.27 \text{ g liter}^{-1}$ and ethylenediaminetetraacetic acid (EDTA) bisodic at $0.37 \text{ g liter}^{-1}$ was also applied after seedlings emergence. The nutrient solution was prepared using deionized water. After seedling emergence, 30 ml of the nutrient solution was applied to each pot every week. The plants were watered as needed with deionized water.

Inoculum production and inoculation procedure. All the leaves from each plant were inoculated with a conidial suspension of *P. oryzae* (10^5 conidia ml^{-1}) at 30 days after emergence (growth stage 19) (76). Suspension (25 ml) was applied as a fine mist to the adaxial leaf blades of each plant until runoff using a VL Airbrush atomizer (Paasche Airbrush Co., Chicago). Gelatin (1%, wt vol^{-1}) was added to the suspension to aid conidial adhesion to the leaf blades. Immediately after inoculation, the plants were transferred to a growth chamber with a temperature of $25 \pm 2^\circ\text{C}$ and a relative humidity of $90 \pm 5\%$ and were subjected to an initial 24-h dark period. After this period, the plants were transferred to a plastic mist growth chamber (MGC) inside a greenhouse for the duration of the experiment. The MGC was made of wood (2 m wide, 1.5 m high, and 5 m long) and covered with 100-mm-thick transparent plastic. The temperature inside the MGC ranged from $25 \pm 2^\circ\text{C}$ (day) to $20 \pm 2^\circ\text{C}$ (night). The relative humidity was maintained at $92 \pm 3\%$ using a misting system in which nozzles (model NEB-100; KGF Company, São Paulo, Brazil) sprayed mist every 30 min above the plant canopy. The relative humidity and temperature were measured with a thermohygrograph (TH-508; Impac, São Paulo, Brazil). The maximum natural photon flux density at plant canopy height was approximately $900 \mu\text{mol m}^{-2} \text{ s}^{-1}$.

Assessment of blast severity. The fourth and fifth leaves from the base of each plant per replication of each treatment were marked and used to evaluate the blast severity at 72 and 96 h after inoculation (hai) using the scale proposed by the International Rice Research Institute (43).

Determination of enzyme activities. Samples from the fourth and fifth leaves from the base of each plant (a total of 12 leaves per replication of each treatment) were collected at 48, 72, and 96 hai. The leaf samples were kept in liquid nitrogen during sampling and then stored at -80°C until further analysis.

To determine the activities of SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), POX (EC 1.11.1.7), GST (EC 2.5.1.18), APX (EC 1.11.1.11), and GPX (EC 1.11.1.9), 300 mg of leaf tissue (mix of the 12 leaves collected per replication of each treatment) 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 50 mM potassium phosphate buffer (pH 6.8), 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2% (wt vol^{-1}) polyvinylpyrrolidone (PVPP). The homogenate was centrifuged at $12,000 \times g$ for 15 min at 4°C , and the supernatant was used as a crude enzyme extract. To determine the GR (EC 1.8.1.7) activity, 300 mg of leaf tissue was

ground as described above. The fine powder was homogenized in an ice bath in 2 ml of a solution containing 100 mM potassium phosphate buffer (pH 7.5), 0.1 mM EDTA, 1 mM DL-dithiothreitol, 1 mM PMSF, and 2% (wt vol^{-1}) PVPP. The homogenate was centrifuged as described previously.

The SOD activity was determined by measuring its ability to photochemically reduce the *p*-nitrotetrazole blue (NTB) (20). The reaction was started after the addition of 60 μl of the crude enzyme extract to 1.94 ml of a mixture containing 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NTB, 0.1 mM EDTA and 2 μM riboflavin. The reaction occurred at 25°C under a 15-W 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 (Evolution 60; Thermo Fisher Scientific Inc., MA) (31). 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% (10).

The CAT activity was determined following the method of Cakmak and Marschner (15). The reaction mixture consisted of 50 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 $36 \text{ M}^{-1} \text{ cm}^{-1}$ (3) was used to calculate the CAT activity, which was expressed as millimoles per minute per milligram of protein.

The POX activity was assayed following the colorimetric determination of pyrogallol oxidation according to Kar and Mishra (45). The reaction mixture contained 25 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 $2.47 \text{ mM}^{-1} \text{ cm}^{-1}$ (17) was used to calculate the POX activity, which was expressed as micromoles of purpurogallin produced per minute per milligram of protein.

The GST activity was determined using the methodology proposed by Habig et al. (36). In total, 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 $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$ (36) was used to determine the GST activity, which was expressed as micromoles per minute per milligram of protein.

The APX activity was determined according to method of Nakano and Asada (59). 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 $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ (59) was used to calculate the APX activity, which was expressed as micromoles per minute per milligram 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 (16). The decrease in absorbance was determined at 340 nm for 1 min at 30°C . An extinc-

tion coefficient of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ (27) was used to calculate the GR activity, which was expressed as micromoles 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 (58). The decrease in absorbance was measured at 340 nm for 1 min at 30°C . An extinction coefficient of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ (4) was used to calculate the GPX activity, which was expressed as nanomoles per minute per milligram of protein.

For each enzyme activity, 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 concentrations of the extracts were measured by the method of Bradford (13) using bovine serum albumin as the standard protein.

Determination of superoxide concentration. In total, 100 mg of leaf tissue was incubated in a volume of 2 ml of a mixture containing 100 μM ethylenediaminetetraacetic acid disodium salt (Na_2EDTA), 20 μM β -nicotinamide adenine nucleotide reduced (NADH), and 20 mM sodium phosphate buffer (pH 7.8) (57). The mixture was prepared in septum-stoppered flasks. The reaction was initiated after the addition of 100 μl of 25.2 mM epinephrine in 0.1 N HCl using a chromatography syringe. The samples were incubated for 5 min at 28°C under shaking. Then, the leaf tissues were carefully removed using a plain sterile wood stem and, after 7 min, the absorbance was measured at 480 nm for 5 min. The controls corresponded to the mixtures without leaf tissue. The O_2^- production was determined by the rate of adrenochrome accumulation (54). An extinction coefficient of $4.0 \times 10^3 \text{ M}^{-1}$ (11) was used to calculate the O_2^- concentration, which was expressed as millimoles per kilogram of fresh weight.

Determination of hydrogen peroxide concentration. In total, 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 an ice bath in a volume of 2 ml of a mixture containing 50 mM potassium phosphate buffer (pH 6.5) and 1 mM hydroxylamine. The homogenate was centrifuged at $10,000 \times g$ for 15 min at 4°C (46). In total, 100 μl of the supernatant was added to a reaction mixture containing 100 μM ferric ammonium sulfate ($\text{FeNH}_4[\text{SO}_4]$), 25 mM sulfuric acid, 250 μM xylenol orange, and 100 mM sorbitol in a volume of 2 ml (30). The samples were kept in the dark for 30 min and then the absorbance was determined at 560 nm. The controls for the reagents and crude extracts were prepared under the same conditions and subtracted from the sample. The H_2O_2 concentration, estimated based on a standard curve of H_2O_2 , was expressed as micromoles per kilogram of fresh weight.

Determination of malondialdehyde concentration. Oxidative damage in the leaf cells was estimated as the content of total 2-thiobarbituric acid (TBA) reactive substances and expressed as equivalents of malondialdehyde (MDA) according to Cakmak and Horst (14). In total, 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 $^{-1}$) trichloroacetic acid (TCA) solution in an ice bath. The homogenate was centrifuged at $12,000 \times g$ for 15 min at 4°C . After centrifugation, 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 $9,000 \times g$ 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 $155 \text{ mM}^{-1} \text{ cm}^{-1}$ (38) was used to calculate the MDA concentration, which was expressed as micromoles per kilogram of fresh weight.

Determination of electrolyte leakage. The electrolyte leakage (EL) was determined according to the methodology of Lima et al. (48), with a few modifications. In total, 22 leaf discs (8 mm in diameter) were collected from the fourth and fifth leaves from the base of each plant per replication and treatment at 72 and 96 hai. The leaf discs were thoroughly washed in deionized water immediately after being sampled. Then, the leaf discs were left to float on 60 ml of deionized water in sealed glass for 4 h at 25°C . After this period, the first value of conductivity (reading one) was obtained using a conductivity meter (Tecnopon mCA-150; MS Tecnopon Instrumentação Científica, São Paulo, Brazil). Next, the vials were transferred to an oven for 2 h at 90°C to obtain a new value for conductivity (reading two). The EL, given as a percentage, was obtained by dividing the value of reading one by the value of reading two.

Experimental design and data analysis. A two-by-two factorial experiment, consisting of two wheat cultivars (BR 18 and BRS 229) and plants that were noninoculated or inoculated with *P. oryzae*, was arranged in a completely randomized design with four replications. Each experimental unit consisted of one plastic pot with six plants. The experiment was repeated once. The data from all variables were analyzed by the analysis of variance (ANOVA), and the means from the noninoculated and inoculated plant treatments for each cultivar were compared by the *t* test ($P \leq 0.05$) using SAS (version 6.12; SAS Institute, Inc., Cary, NC). For the ANOVA, the design was considered to be a two-by-two factorial experiment, consisting of two wheat cultivars and two evaluation times (72 and 96 hai) for the blast severity. For the SOD, CAT, POX, GST, APX, GR, and GPX activities as well as the O_2^- , H_2O_2 , and MDA concentrations, the design was considered to be a two-by-two-by-three factorial experiment for the ANOVA, consisting of two wheat cultivars, noninoculated or inoculated plants, and three sampling times (48, 72, and 96 hai). For EL, it was considered to be a two-by-two-by-two factorial experiment for ANOVA, consisting of two wheat cultivars, noninoculated or inoculated plants, and two sampling times (72 and 96 hai).

RESULTS

Only the factors cultivars and evaluation times were significant for the blast severity (Table 1). The blast severity was significantly lower for BRS 229 than for BR 18, regardless of the evaluation time. For both cultivars, there was a significant increase in the blast severity from 72 to 96 hai. The blast severity increased from 10 to 13% for BRS 229 and from 24 to 32% for BR 18 from 72 to 96 hai. The blast severity showed a similar trend for the treatments, as determined in a preliminary experiment (data not shown).

At least one of the factors (cultivars, plant inoculation, or sampling times) and some of their interactions were significant for the activities of SOD, CAT, POX, GST, APX, GR, and GPX (Table 2), concentrations of O_2^- , H_2O_2 , and MDA (Table 3), and EL (Table 4). Alone, plant inoculation was the most important factor (higher *F* values) that explained the variation in all the variables evaluated, except for CAT activity (Table 2) and the H_2O_2 concentration (Table 3). The cultivars–plant inoculation–sampling times interaction was not significant only for the CAT and GR activities (Table 2) and the H_2O_2 concentration (Table 3).

TABLE 1. Analysis of variance of the effects of cultivars and evaluation time for blast severity

Sources of variation	df	<i>F</i> values ^a
Cultivars (C)	1	162.12*
Evaluation time (ET)	1	34.97*
C $\times$ ET	1	1.25 ns

^a Levels of probability: ns = not significant and * = 0.01.

The SOD activity significantly increased in the inoculated plants of BR 18 and BRS 229 compared with the noninoculated plants at 48, 72, and 96 hai (Fig. 1A and B). The SOD activity increased by 40 and 28% at 48 hai, 44 and 53% at 72 hai, and 79 and 167% at 96 hai for the inoculated plants of BR 18 and BRS 229, respectively, relative to the noninoculated plants.

The CAT activity significantly decreased in the inoculated plants of BR 18 compared with the noninoculated plants at 72 and 96 hai (Fig. 1C). For BRS 229, the CAT activity was higher for the inoculated plants than the noninoculated plants at both 48 and 96 hai (Fig. 1D).

The POX activity was higher in the inoculated plants of BR 18 and BRS 229 compared with the noninoculated plants at 48, 72, and 96 hai (Fig. 1E and F). The inoculated plants of BR 18 and BRS 229 showed increases of 239 and 189% at 48 hai, 400 and 368% at 72 hai, and 437 and 678% at 96 hai, respectively, for the POX activity compared with the noninoculated plants.

The inoculated plants of BR 18 and BRS 229 showed a significant increase in the GST activity compared with the noninoculated plants at 48, 72, and 96 hai (Fig. 1G and H). Increases of 92 and 102% at 48 hai, 50 and 140% at 72 hai, and 61 and 849% at 96 hai were obtained for the inoculated plants of BR 18 and BRS 229, respectively, compared with the noninoculated plants.

There was a significant increase in the APX activity for the inoculated plants of BR 18 and BRS 229 at 48, 72, and 96 hai compared with the noninoculated plants (Fig. 1I and J). For the inoculated plants of BR 18 and BRS 229, there were increases of 70 and 44% at 48 hai, 29 and 79% at 72 hai, and 30 and 120% at 96 hai, respectively, for the APX activity compared with the noninoculated plants.

The GR activity was not affected by plant inoculation at 48, 72, and 96 hai for BR 18 and at 72 hai for BRS 229 (Fig. 1K and L). For BRS 229, the GR activity was higher for the inoculated plants than for the noninoculated plants at 48 and 96 hai.

For BR 18, the differences in the GPX activity between the inoculated and noninoculated plants were observed at 48 and 72 hai, with higher values of activity obtained for the former (Fig. 1M). For BRS 229, the GPX activity was not affected by plant inoculation with *P. oryzae* regardless of the sampling time (Fig. 1N).

Differences between the inoculated and noninoculated plants for the O_2^- concentration were found at 72 and 96 hai for BR 18 and only at 96 hai for BRS 229, with the highest values occurring for the inoculated plants (Fig. 2A and B). The O_2^- concentration increased by 153 and 72% at 96 hai for the inoculated plants of BR 18 and BRS 229, respectively, compared with the noninoculated plants.

For the H_2O_2 concentration, there was a difference between the inoculated and noninoculated plants only for BR 18 at 72 and 96 hai, with the higher values occurring for the noninoculated and inoculated plants, respectively (Fig. 2C and D).

Regarding the MDA concentration, significant differences between the inoculated and noninoculated plants of BR 18 occurred at 48, 72, and 96 hai and only at 48 hai for BRS 229 (Fig. 2E and

F). For BR 18 and BRS 229, the MDA concentration was higher in the inoculated plants than in the noninoculated plants.

The inoculated plants of BR 18 and BRS 229 showed significant increases in EL at 72 and 96 hai compared with the noninoculated plants (Fig. 3). The EL increased by 124 and 87% at 72 hai and by 323 and 188% at 96 hai for the inoculated plants of BR 18 and BRS 229, respectively, compared with the noninoculated plants.

A preliminary experiment to determine the enzyme activities and the O_2^- , H_2O_2 , and MDA concentrations was performed and showed a similar trend for the treatments discussed above (data not shown).

DISCUSSION

The lower blast severity in the leaves of plants of BRS 229 compared with BR 18 confirmed the variation in resistance previously reported by Cruz et al. (18), which ranked them as moderately resistant (BRS 229) and susceptible (BR 18) to blast. Supporting the data provided by Cruz et al. (18), the present study brings novel evidence that a more efficient antioxidative system in the leaves of wheat plants from the moderately resistance BRS 229 that removes the excess ROS helps to limit the cellular damage that occurs during the infection process of *P. oryzae* and may be linked to the resistance of this cultivar. These results are supported by the data obtained in a series of preliminary experiments that showed similar trends (data not shown).

TABLE 3. Analysis of variance of the effects of cultivars (C), plant inoculation (PI), and sampling time (ST) on the concentration of superoxide (O_2^-), hydrogen peroxide (H_2O_2), and malondialdehyde (MDA)

Sources of variation	df	F values ^a		
		O_2^-	H_2O_2	MDA
C	1	3.21 ns	21.22**	15.27**
PI	1	19.32**	0.36 ns	51.82**
ST	2	2.02 ns	3.80*	39.46**
C × PI	1	2.69 ns	0.74 ns	30.62**
C × ST	2	4.15*	0.58 ns	9.17**
PI × ST	2	13.63**	4.68*	4.49*
C × PI × ST	2	7.11**	0.83 ns	16.82**

^a Levels of probability: ns = not significant, * = 0.05, and ** = 0.01.

TABLE 4. Analysis of variance of the effects of cultivars, plant inoculation, and sampling time on the electrolyte leakage

Sources of variation	df	F values ^a
Cultivars (C)	1	13.74**
Plant inoculation (PI)	1	286.49**
Sampling time (ST)	1	98.87**
C × PI	1	15.66**
C × ST	1	8.25**
PI × ST	1	68.55**
C × PI × ST	1	7.13*

^a Levels of probability: * = 0.05 and ** = 0.01.

TABLE 2. Analysis of variance of the effects of cultivars (C), plant inoculation (PI), and sampling time (ST) on the activity of superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), glutathione-S-transferase (GST), ascorbate peroxidase (APX), glutathione reductase (GR), and glutathione peroxidase (GPX)

Sources of variation	df	F values ^a						
		SOD	CAT	POX	GST	APX	GR	GPX
C	1	103.30**	0.34 ns	39.08**	39.80**	2.73 ns	2.68 ns	17.12**
PI	1	1,649.08**	3.93 ns	3,416.66**	241.32**	150.97**	13.08**	29.82**
ST	2	18.61**	25.76**	206.24**	33.08**	4.75*	4.31*	1.45 ns
C × PI	1	11.60**	115.56**	29.20**	18.59**	7.61**	5.17*	23.13**
C × ST	2	111.95**	3.98*	56.52**	13.99**	4.62*	5.62**	4.87*
PI × ST	2	115.78**	22.66**	212.82**	24.52**	0.77 ns	3.85*	1.28 ns
C × PI × ST	2	56.28**	1.79 ns	55.95**	26.45**	8.33**	2.29 ns	11.90**

^a Levels of probability: ns = not significant, * = 0.05, and ** = 0.01.

The ROS production is an important plant defensive response against pathogenic infection (50). However, ROS accumulation caused by the imbalance between its production and the host defense mechanisms in favor of the former results in oxidative

stress that can damage the host tissue (48). Therefore, plants possess nonenzymatic and enzymatic mechanisms to keep the ROS levels approximately constant in the cells (69). The SOD activity increased in the leaves of wheat plants in response to *P. oryzae*

Fig. 1. Activities of **A and B**, superoxide dismutase (SOD); **C and D**, catalase (CAT); **E and F**, peroxidase (POX); **G and H**, glutathione-S-transferase (GST); **I and J**, ascorbate peroxidase (APX); **K and L**, glutathione reductase (GR); and **M and N**, glutathione peroxidase (GPX) in leaves of wheat plants from 'BR 18' (A, C, E, G, I, K, and M) and 'BRS 229' (B, D, F, H, J, L, and N) noninoculated (NI) or inoculated (I) with *Pyricularia oryzae*. Means from NI and I treatments followed by an asterisk (*), for each sampling time, are significantly different ($P \leq 0.05$) by *t* test. Bars represent the standard error of the means. Two experiments were conducted with consistent results and the data presented represent the best experiment.

infection. In tomato, the peroxisomal SOD activity increased during the initial stage of *Botrytis cinerea* infection but decreased as the necrotic lesions appeared (47). However, in the present study, there was an increase in the SOD activity in the leaves of the inoculated plants as blast symptoms developed, which contrasted with the response in the leaves of noninoculated plants. Although the SOD activity increased in the leaves of the inoculated plants of both cultivars, its activity in BRS 229 showed a more prominent increase than in BR 18, resulting in a lower O_2^- concentration in the former. The SOD activity was also shown to increase in strawberry leaves infected by *Mycosphaerella fragariae* but the SOD activity for the resistant cultivars was greater than for the susceptible ones (24). Some fungal pathogens could benefit from an increase in ROS levels generated in the host cells during the defense against their infection as a facilitating factor to maximize their tissue colonization and nutrient uptake (35). Thus, higher SOD activity in resistant cultivars could be a strategy to restrict pathogen colonization because the excess ROS can be removed (24).

Among the enzymes involved in the removal of the excess H_2O_2 generated spontaneously or by O_2^- dismutation via SOD,

CAT plays a key role (55). In the present study, the increase in the H_2O_2 concentration observed in the leaves of the inoculated plants of BR 18 compared with the noninoculated plants at 96 hai was associated with a reduction in CAT activity, which confirms reports for other pathosystems. In the pea-*Plum pox virus* interaction, susceptible plants showed a decrease in the CAT activity and a concomitant increase in the H_2O_2 concentration only after the symptoms caused by this virus were evident (21). In the present study, the CAT activity only increased on leaves with intense blast symptoms at 48 hai. Based on a previous study in tomato (47), CAT and SOD activities were increased during the initial stage of *B. cinerea* infection and decreased after the necrotic lesion formation. The reduction in CAT activity could be a consequence of enhanced proteolysis caused by peroxisomal endopeptidases, which are induced by oxidative stress (63), as is the case of *P. oryzae* infection. Therefore, these results can explain the reduction in CAT activity in the leaves of wheat plants of BR 18 during the advanced stages of fungal infection.

Although the CAT activity decreased as the blast progressed in the leaves of the plants of BR 18, this result was not true for BRS

Fig. 2. Concentrations of **A and B**, superoxide (O_2^-); **C and D**, hydrogen peroxide (H_2O_2); and **E, and F**, malondialdehyde (MDA) in leaves of wheat plants from 'BR 18' (A, C, and E) and 'BRS 229' (B, D, and F) noninoculated (NI) or inoculated (I) with *Pyricularia oryzae*. Means from NI and I treatments followed by an asterisk (*), for each sampling time, are significantly different ($P \leq 0.05$) by *t* test. Bars represent the standard error of the means. FW = fresh weight. Two experiments were conducted with consistent results and the data presented represent the best experiment.

Fig. 3. Electrolyte leakage (EL) in leaves of wheat plants from **A**, 'BR 18' and **B**, 'BRS 229' noninoculated (NI) or inoculated (I) with *Pyricularia oryzae*. Means from NI and I treatments followed by an asterisk (*), for each sampling time, are significantly different ($P \leq 0.05$) by *t* test. Bars represent the standard error of the means. Two experiments were conducted with consistent results and the data presented represent the best experiment.

229. In this case, the CAT activity was higher in the inoculated than noninoculated leaves at 48 hai; however, no difference between the inoculated and noninoculated plants occurred for the H_2O_2 concentration. Therefore, the difference in the CAT activity between cultivars suggests that this enzyme plays a major role in the wheat resistance to blast. This result is in agreement with Magbanua et al. (50), who demonstrated that the CAT activity was higher in the leaves of maize lines resistant to *Aspergillus flavus* than in susceptible ones. However, the reduction of CAT activity usually increases plant resistance to pathogenic attack because plants can maintain high concentrations of H_2O_2 (50). Thus, the role of CAT in the plant–pathogen interaction seems to be more complex than for abiotic stress (47), which involves an association between CAT activity and plant tolerance (56,61,74).

For the inoculated plants of both cultivars, there was a consistent increase in the POX, GST, and APX activities during the infectious process of *P. oryzae*. However, the increase was more pronounced for BRS 229 than for BR 18, suggesting that, in addition to CAT, those enzymes played key roles to maintain the H_2O_2 concentration constant in the inoculated plants of BRS 229.

In addition to the involvement of POX in the removal of H_2O_2 , this enzyme plays an important role in plant defense against pathogens due to its participation in lignin biosynthesis (66). Hong-xia et al. (41) verified that the POX activity was more important for resistant than for susceptible wheat cultivars when infected by *Rhizoctonia cerealis*. The higher POX activity in the leaves of wheat plants during the late stages of *P. oryzae* infection was detrimental to the resistance against blast (75). These reports are in agreement with the data from the present study, which showed that the POX activity was maintained at a higher level in the leaves of wheat plants of BRS 229 than in BR 18 during the late stages of *P. oryzae* infection.

The GST is a large and diverse group of enzymes that catalyze the conjugation of reduced GSH to a variety of hydrophobic, electrophilic, and usually cytotoxic substrates (22,64). GST reduces H_2O_2 and phospholipid hydroperoxides (26). The expression of the *gst* gene is responsive to biotic and abiotic stresses, suggesting its importance in plant defense (23,52). Although whether the increase in GST activity confers increased tolerance to abiotic stresses is unknown (71), few studies have shown the activity of this enzyme in plants infected by pathogens, although the GST gene expression is induced in a wide range of plant–pathogen interactions. The higher GST activity at 96 hai in the leaves of the plants of BRS 229 compared with BR 18 highlights its importance in ROS removal and, consequently, in the wheat resistance to blast.

The removal of H_2O_2 is the first step of the ascorbate-glutathione cycle (9,27). This cycle comprises a series of redox reactions involving, in addition to APX, the enzymes monodehydroascorbate reductase, dehydroascorbate reductase, and GR (27,59). APX is responsible for H_2O_2 removal in chloroplasts, peroxisomes, and mitochondria (65), using ascorbate as a specific electron donor to reduce H_2O_2 to water (8). The increases in the level of the *apx* transcripts and in the enzyme activity during the plant–pathogen interactions have been well documented (1,2,25,37,42,68). In the present study, the APX activity increased in response to *P. oryzae* infection in both wheat cultivars but its increase was higher in BRS 229 than in BR 18. Hükelhoven et al. (42) reported that the expression of the *apx* gene increased in the leaves of barley plants inoculated with *Blumeria graminis* f. sp. *hordei*. Harrach et al. (37) observed that the activities of POX, APX, and GST were similarly altered in the leaves of barley plants inoculated with *B. graminis* f. sp. *hordei*.

The GR constitutes a group of nicotinamide dinucleotide (phosphate) NAD(P)H-dependent enzymes that catalyze the reduction of GSSG to form two GSH molecules (16,70). Hernández et al. (39) found that the GR activity in the resistant plants was higher than in the susceptible plants of apricot after inoculation with the

Plum pox virus. In the present study, the GR activity increased in the leaves of the inoculated plants of BRS 229 and BR 18 but was slightly greater for BR 229, confirming its high level of resistance to blast. The role of GR in plant resistance against pathogens is still unclear because no studies have demonstrated a close relationship between GR activity and an increase in host resistance. El-Zahabi et al. (25) did not find any difference in the GR activity in the leaves from three barley cultivars infected by virulent and avirulent isolates of *B. graminis* f. sp. *hordei*. In contrast, the GR activity only increased in the roots of chickpea plants infected by a virulent isolate of *Fusarium oxysporum* f. sp. *ciceris* (29).

The GPX comprises a family of isoenzymes using GSH to reduce H_2O_2 and lipid hydroperoxides to protect the cells against oxidative damage (65). According to Asada (9), the main functions of GPX in plants are lignin biosynthesis, degradation of indole-3-acetic acid, and resistance to pathogens. In the present study, the GPX activity increased only in the leaves of the plants of BR 18 inoculated with *P. oryzae*. The activity of peroxisomal GPX in the leaves of tomato plants increased during the early stages of infection by *B. cinerea* and decreased thereafter, as the severity of the gray mold reached high values (47).

An increase in both the O_2^- and H_2O_2 concentrations in the leaves of BR 18 plants during the infectious process of *P. oryzae* contributed to the higher concentration of MDA and high EL values. The high concentration of ROS in the roots of tomato plants infected by *F. oxysporum* f. sp. *lycopersici* resulted in increased lipid peroxidation (51). According to Mandal et al. (51), a less efficient antioxidative system, particularly a decrease in CAT activity, explained the high level of damage caused by *F. oxysporum* f. sp. *lycopersici*. In the present study, the CAT activity was lower only in plants of BR 18 infected by *P. oryzae*.

The extent of the cellular damage caused by the oxidative stress related to the plant response against the pathogen infection can be estimated by the products of the peroxidation of membrane lipids (51). The MDA produced during lipid peroxidation is an indicator of cellular damage that results in an increase in EL (19). Any type of cellular stress, such as the action of nonselective toxins produced by *P. oryzae*, which are highly cytotoxic to rice (62) and possibly also to wheat, can cause rapid lipid peroxidation of the plasma membrane by ROS (28,44). In the present study, the MDA concentration was higher in the leaves of the inoculated plants of BRS 229 than the noninoculated plants only at 48 hai. As a result of the lower lipid peroxidation, the increase in EL was less pronounced in the plants from this cultivar than in plants from BR 18, which can be linked to the reduced blast severity observed on the leaves of plants of the former cultivar. The higher activities of SOD, POX, APX, GST, and GR in the leaves of the inoculated plants of BRS 229 compared with inoculated plants of BR 18 at 96 hai resulted in lower concentrations of O_2^- and H_2O_2 , thereby limiting the cellular damages possibly caused by these ROS.

The results of the present study indicate that a more efficient antioxidative system in the removal of the excess of ROS generated during the infectious process of *P. oryzae* limits the cellular damage, thereby contributing to a greater resistance to blast. Further research that aims to investigate the antioxidative system in the most productive wheat cultivars will play a pivotal role in the development of biochemical markers that can be used in breeding programs to select resistant or tolerant cultivars that can be grown in areas in which severe epidemics of blast occur.

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