BRUNO DO NASCIMENTO SILVA

THERMAL OSCILLATIONS PROVOKE PHYSIOLOGICAL AND BIOCHEMICAL CHANGES ON WHEAT PLANTS INFECTED BY *Pyricularia oryzae*

Dissertation presented to the Universidade Federal de Viçosa, as part of the requirements of the Graduate Program in Plant Physiology, to obtain the title of *Magister Scientiae*.

Advisor: Fabrício Ávila Rodrigues

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BIOGRAPHY

BRUNO DO NASCIMENTO SILVA was born on October 23, 1989 in the state of Ceará.

In 2017, he obtained the title of Agricultural Engineer from the Federal University of Ceará. At the same institution, she was a scholarship holder of the Programa de Educação Tutorial (PET) developing research on water relations under the guidance of Prof. Rosilene Oliveira Mesquita.

In March 2018, started the Master's degree program in the Postgraduate Program in Plant Physiology of the Department of Plant Biology of the Federal University Viçosa under the guidance of Professor. Fabrício Ávila Rodrigues defending his dissertation on July 25, 2019.

ABSTRACT

SILVA, Bruno do Nascimento, M.Sc., Universidade Federal de Viçosa, July, 2019. **Thermal oscillations provoke physiological and biochemical changes on wheat plants infected by *Pyricularia oryzae***. Advisor: Fabrício Ávila Rodrigues.

Wheat blast, caused by *Pyricularia oryzae*, is the most important wheat disease nowadays. High temperatures, as a consequence of climatic changes, can potentially cause physiological and biochemical changes on plants by altering their resistance to diseases. This study aimed to determine whether thermal oscillations could cause physiological and biochemical changes on wheat plants when infected with *P. oryzae*. Plants were submitted to different thermal acclimations (19ºC and 28ºC) for five days before being inoculated with *P. oryzae*. After inoculation, plants were kept at 25°C for 24 h and then either transferred to the initial temperature conditions or transferred to different temperature combinations (19ºC→28ºC and 28ºC→19ºC) during three days. Non-inoculated plants were submitted to these same conditions. Blast development was reduced on plants submitted to thermal acclimatization of 19ºC→19ºC compared to plants exposed to 28ºC→28ºC. There was no significant difference for blast severity between the thermal acclimations of 19ºC→28ºC and 28ºC→19ºC. Plants submitted to 28ºC before or after inoculation showed impairments on photosynthesis with lower values for maximum photosystem PSII photochemical efficiency and effective PSII quantum yield, but high values for quantum yield of non-regulated energy dissipation besides lower concentration of pigments, reduction on stomatal conductance and an increase in the internal CO_2 concentration. There was high superoxide dismutase and lower ascorbate peroxidase activities for inoculated plants submitted to thermal acclimatization of 28ºC→28ºC. Low and high concentrations of superoxide anion and hydrogen peroxide, respectively, occurred for inoculated plants acclimated at 28ºC→28ºC. High chitinase, phenylalanine ammonia-lyase, and peroxidase activities occurred for inoculated plants acclimated at 28ºC→28ºC and 19ºC→28ºC. Polyphenoloxidase activity was lower for inoculated plants submitted to 28ºC after or before fungal inoculation. Lipoxygenase increased on inoculated plants exposed at thermal acclimatization of 19ºC→19ºC. The malondialdehyde concentration was high for inoculated plants submitted to 28ºC after or before fungal inoculation. In conclusion, blast development was favored on plants pre-acclimated at 28ºC before fungal inoculation and kept at this same temperature during fungal infection process. By contrast, blast development was quite similar on plants pre-acclimated at 19 and 28ºC before inoculation with *P. oryzae* and submitted, respectively, to temperatures of 28 or 19ºC after inoculation. Wheat plants exposed at 28ºC

during the fungal infection process showed damage to the photosynthetic apparatus, a less efficient antioxidative system, and a minor contribution of the enzymes related to host defense.

Key words: Defense enzymes. High temperatures. Host resistance. Photosynthesis. Wheat blast

RESUMO

SILVA, Bruno do Nascimento, M.Sc., Universidade Federal de Viçosa, julho de 2019. **Oscilações térmicas provocam alterações fisiológicas e bioquímicas em plantas de trigo infectadas por *Pyricularia oryzae***. Orientador: Fabrício Ávila Rodrigues.

A brusone do trigo, causada por *Pyricularia oryzae*, é a doença mais importante do trigo hoje em dia. As altas temperaturas, como consequência das mudanças climáticas, podem causar alterações fisiológicas e bioquímicas nas plantas, alterando sua resistência a doenças. Este estudo teve como objetivo determinar como as oscilações térmicas podem causar alterações fisiológicas e bioquímicas em plantas de trigo quando infectadas com *P. oryzae*. Plantas foram submetidas à diferentes aclimatações térmicas (19ºC e 28ºC) por cinco dias e inoculadas com *P. oryzae*. Após a inoculação, as plantas foram mantidas a 25°C durante 24 horas e depois transferidas para as condições iniciais de temperatura ou transferidas para diferentes combinações de temperatura (19°C→28°C e 28°C→19°C) durante três dias. Plantas não inoculadas também foram submetidas à essas mesmas condições. O desenvolvimento da brusone foi menor nas plantas submetidas à aclimatização térmica a 19ºC→19ºC em relação às plantas expostas a 28ºC→28ºC. Não houve diferença significativa para a severidade da brusone entre a aclimatação térmica de 19ºC→28ºC e 28ºC→19ºC. Plantas inoculadas submetidas a 28ºC antes ou após a inoculação, apresentam prejuízos na fotossíntese com menores valores da máxima eficiência fotoquímica do PSII, rendimento quântico efetivo do PSII, mas maiores valores de rendimento quântico da dissipação de energia não regulada, além de menor concentração de pigmentos, redução da condutância estomática e aumento da concentração interna de CO_2. O metabolismo antioxidante foi alterado com maiores atividades de superóxido dismutase e menores de ascorbato peroxidase em plantas inoculadas e submetidas à aclimatização térmica a 28ºC→28ºC. Esses resultados estão diretamente relacionados com menores e maiores concentrações de ânion superóxido e peróxido de hidrogênio, ocorrendo respectivamente em plantas inoculadas e expostas à aclimatações térmicas 28ºC→28ºC. As maiores atividades de quitinase, fenilalanina amônia-liase e peroxidase ocorreram nas plantas inoculadas a 28ºC→28ºC e 19ºC→28ºC. A atividade da polifenoloxidase foi menor nas plantas inoculadas submetidas a 28ºC antes ou após a inoculação do fungo. A atividade da lipoxigenase aumentou nas plantas de trigo inoculadas e expostas a aclimatização térmica a 19ºC→19ºC. A concentração de malondialdeído foi alta em plantas inoculadas submetidas a 28ºC antes ou após a inoculação. Em conclusão, foi demonstrado que o desenvolvimento da brusone foi favorecido em plantas de trigo pré-aclimatadas a 28ºC antes da inoculação com *P. oryzae* e mantidas nesta

mesma temperatura durante o processo de infecção fúngica. Por outro lado, o desenvolvimento da brusone foi bastante similar em plantas pré-aclimatadas a 19 e 28ºC antes da inoculação com *P. oryzae* e submetidas, respectivamente, a temperaturas de 28 ou 19ºC após a inoculação. Plantas de trigo expostas a 28ºC durante o processo de infecção fúngico apresentam prejuízos no aparelho fotossintético, um sistema antioxidante menos eficiente e uma pequena contribuição de enzimas relacionadas à defesa do hospedeiro.

Palavras-chave: Enzimas de defesa. Altas temperaturas. Resistência do hospedeiro. Fotossíntese, Brusone do trigo

SUMMARY

INTRODUCTION

Blast, caused by hemibiotrophic fungi *Pyricularia oryzae* Cavara (teleomorph: *Magnaporthe grisea* (Hebert) (Barr)), is a devastating disease on wheat causing yield losses up to 100% (Kohli et al. 2011; Cruz and Valent 2017; Martínez et al. 2019). Severe epidemics of blast on wheat cultivated in the savanna region of Brazil has been reported (Maciel 2011). Currently wheat blast has been reported on wheat fields in Argentina, Bolivia, Brazil and Paraguay (Kohli et al. 2011). In 2016, blast was reported in Bangladesh and caused losses in about 16% of the area cultivated with wheat in the country (Malaker et al. 2016).

Blast symptoms vary according to the organ of wheat plants. On the leaves, the first symptoms are small green-gray lesions with dark green edges that rapidly expand and become necrotic (Goulart et al. 2007; Cruz et al. 2016). On the spikes, bleaching occurs from the point of fungal penetration and interrupts water translocation to shoots causing alterations on carbohydrates partition that compromises grain quality (Goulart et al. 2007; Rios et al. 2017). Disease management is difficult to achieve due to the absence of cultivars with high level of partial resistance and the low efficacy of available fungicides (Goulart et al. 2007). Wheat resistance to blast depends on the level of aggressiveness of *P. oryzae* isolates and the plant growth stage (Urashima et al. 2004; Arruda et al. 2005; Cruz et al. 2010).

Warm conditions and high humidity are the two major climatic conditions driving the occurrence of severe blast epidemics (Fernandes et al. 2017; Miah et al. 2017). Blast development and intense fungal sporulation occur with great success on plants exposed to temperature greater than 27ºC (Rajput et al. 2017). As a result of climatic changes, heat waves and more warm days will become more frequent worldwide besides the projection that the global temperature can increase in the range of 2.6-4.8ºC by the end of the 21st century (IPCC 2014). In addition, an increase in frequency, intensity, and durations of heatwaves is predicted, besides an increase in the daily minimum temperature increases faster than the average daily maximum. (Davy et al. 2017). This new scenario limits wheat production in certain areas (Rosenzweig et al. 2014), including India and Sudan (Asseng et al. 2017) and Brazilian Savanna, while the average annual temperature ranging from 16ºC to 32ºC (Silva et al. 2008). According to Liu et al (2016), the increase of global temperature at 1ºC, leads to the decline of 4.1 to 6.4% in wheat production. Therefore, the rise in temperature can possible lead to an increase in the reproduction of *P. oryzae* on wheat plants grown in the tropical and subtropical regions of the world (Fernandes et al. 2017).

The increase on temperature may cause changes on the pattern of diseases development and also on the basal level of host resistance due to profound alterations on plant metabolism

resulting, therefore, in great concern regarding food security (Bevitori and Ghini 2014; Onaga et al. 2017). Isolates of *Puccinia striiformis* f. sp. *tritici* became more aggressive on wheat plants exposed to 28ºC as noticed by the great rust development (Milus et al. 200). Tosa et al. (2004) reported that *Lolium* plants submitted to temperatures ranging from 25 to 30ºC during five days exhibited high blast severity. Interestingly, yield losses due to blast coincided with climatic changes and also with the occurrence of *El Niño* (Vales et al. 2014). Pathogens cause physiological changes on their hosts by the action of non-host selective toxins and hydrolytic enzymes (Howlett 2006). According to Debona et al. (2014), wheat plants infected by *P. oryzae* showed lower rates of photosynthesis associated with a decrease in stomatal conductance and biochemical damage to Rubisco.

Plants infected by pathogens increase the production of reactive oxygen species (ROS) as a strategy of defense (Magbanua et al. 2007). During the infection process, both pathogens and their hosts are exposed to ROS such hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide anion (O_2^-), and hydroxyl radical ($HO^\bullet$) which, in excess, may cause oxidative stress to the plant cells (Mittler 2002; Lehmann et al. 2015; Moreno and Camejo 2016). Among the ROS, H_2O_2, which due to its uncharged nature, easily cross the plasma membrane and creates a hostile environment against the pathogen besides helping in the strength of cell wall (Wani et al. 2018). Debona et al. (2012) reported an increase on the concentrations of H_2O_2, O_2^-, and malondialdehyde on the leaves of wheat plants infected by *P. oryzae*. Moreover, there was an increase on the activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), glutathione-S-transferase (GST), and ascorbate peroxidase (APX) that contributed to lower the damage caused by *P. oryzae* to leaf cells of wheat plants (Debona et al. 2012). The ROS production can increase considerably on plants exposed to high temperatures (Mittler et al. 2004). Wheat plants exposed to high temperatures show an increase on the production of ROS, extreme damage to the plasmatic membrane, and a decrease on the maximum quantum yield of PSII (Narayanan et al. 2015). According to Almeselmani et al. (2009), plants of wheat cultivars more tolerant to heat showed high activities of SOD, CAT, POX, APX, and glutathione reductase regardless of their growth stage.

The participation of the defense enzymes chitinase (CHI), β-1,3-glucanase (GLU), polyphenoloxidase (PPO), phenylalanine ammonia-lyase (PAL), and lipoxygenase (LOX) is of importance to increase the resistance of plants against pathogens infection (Rodrigues et al. 2017). The fungal cell wall is degraded by CHI and GLU (Sharma et al. 2011); PPO and POX are involved in the detoxification of the ROS and also on the oxidation of phenolics to produce lignin (Madadkhah et al. 2012); PAL is involved with the biosynthesis of phenolics (Rodrigues

et al. 2005), and LOX is involved in the peroxidation of lipids that may result in an increase on the production of jasmonic acid (Porta et al. 2002). Wheat plants infected with *P. oryzae* showed high enzyme activity of CHI, GLU, PAL, and PPO on flag leaves (Silva et al. 2019).

In the context of a race-specific resistance, the temperature plays a detrimental role to affect it. For example, tomato plants carrying the Mi gene, which confer resistance to *Melioidogyne incognita* (race 1), loss their resistance when exposed to soil temperature greater than 32ºC (Ammati et al. 1986). Moreover, *Arabidopsis* plants exposed to temperatures of 28ºC for three days after inoculation with *Pseudomonas syringae* lowered their defense response against bacterial infection (Wang et al. 2009). Wheat cultivars adapted to China's climatic conditions showed great susceptibility to blast when cultivated in regions with average temperature of 26ºC during four days (Nga et al. 2009).

Considering the impact that the climate change may impose on agricultural production worldwide and its potential to alter the plant basal level of resistance against destructive diseases, the present study aimed to investigate the physiological and biochemical alterations on wheat plants exposed to thermal oscillations in response to *P. oryzae* infection.

MATERIAL AND METHODS

Plant growth

Wheat seeds (*Triticum aestivum*) from cultivar BRS-229, moderately resistant to blast (Brunetta et al. 2006), were sowing plastic pots filled with 1 kg of substrate composed of peat, vermiculite, and pine bark in a ratio of 1:1:1 (Tropstrato®, Vida Verde, Mogi Mirim, São Paulo, Brazil). A total of 1.63 g of calcium phosphate monobasic was added to the substrate each pot. A total of 14 seeds were sowed per pot. After seedlings emergence, each pot was thinned to seven plants. Each pot was fertilized with a nutrient solution composed, in g L^{-1}, of: 6.4 KCl, 3.48 K_2SO_4, 5.01 $MgSO_4.7H_2O$, 2.03 $(NH_2)_2CO$, 0.009 $NH_4MO_7O_{24}.4H_2O$, 0.054 H_3BO_3, 0.222 $ZnSO_4.7H_2O$, 0.058 $CuSO_4.5H_2O$, and 0.137 $MnCl_2.4H_2O$ (Xavier-Filha et al. 2011). A volume of 15 mL of nutrient solution containing 0.27 g L^{-1} of $FeSO_4.7H_2O$ and 0.37 g L^{-1} of EDTA bisodic was also applied per pot after seedlings emergence. Plants were watered with deionized water as needed.

Plant acclimatization and inoculation with *P. oryzae*

Wheat plants at growth stage 34 (Lancashire et al. 1991) were transferred to different growth chambers with constant temperatures of 19 and 28ºC, which are optimum and high temperatures for wheat, respectively (Stone and Nicolas, 1994) and 12h photoperiod light /12h dark for five days for thermal acclimatization. Five lamps (Philips MASTER GreenPower Plus 1000W EL/5X6CT; São Paulo, Brazil) positioned at 0.60 m above plants canopies on each growth chamber provided photons emission of 178 μmol photons m^{-2} s^{-1}. After this period, 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 oat-agar 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 25ºC with a 24 h photoperiod for 10 days. After this period, conidia were carefully removed from the Petri dishes with a soft bristle brush using water containing gelatin (1% w v^{-1}). The conidial suspension was calibrated with a hemacytometer to obtain a concentration of 1 × 10^5 conidia ml^{-1}. The conidial suspension was sprayed with an atomizer (Paasche Airbrush Co., Chicago, IL, USA) on the adaxial surface of the leaves. After inoculation, plants were kept in a mist chamber under darkness at 25ºC during 24 h. Thereafter, two groups of five plants each were transferred to their original growth chambers (19 and 28ºC) and other two groups of five plants were transferred to growth chambers with temperatures different from the ones that they were previously acclimatized (19ºC→28ºC and 28ºC→19ºC) for three days. Non-

inoculated plants were also submitted to these same conditions of different temperatures, but were kept apart from the inoculated plants.

Blast severity assessment

Blast severity was evaluated on the third leaf, 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). The area under blast progress curve (AUBPC) was calculated using the trapezoidal integration of disease progress curves according to Shaner and Finney (1977).

Determination of the leaf gas exchange parameters chlorophyll (Chl) *a* fluorescence imaging

The leaf gas exchange parameters were determined on the third leaf, from top to base, at 96 hai. The leaf gas exchange parameters net CO_2 assimilation rate (A), stomatal conductance to water vapor (g_s), internal CO_2 concentration (C_i), and transpiration rate (E) were measured by using a portable open system gas exchange system (LI-6400XT; Li-Cor Inc., Lincoln, NE). Measurements were made from 9:00 am to 11:00 am with 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 and vapor pressure deficit at approximately 1.0 kPa. All measurements were performed by setting the block temperature at 25ºC.

The imaging of the Chl *a* fluorescence parameters were determined by using the Imaging-PAM MAXI chlorophyll fluorometer and the software ImagingWIN (version 2.32) (Heinz Walz GmbH, Effeltrich, Germany). The sensor system consisted of 44 LED's lamp high power (450 nm) required to apply fluorescence excitation, actinic illumination and saturation pulses. These LEDs were arranged in pairs, with each pair featuring a red (660 nm) and a near-infrared (780 nm) LED. A CCD camera with 640 × 480 resolution pixels was located above the plant canopies at a distance of 13.5 cm to give an image area of 10 × 13 cm. Plants from the different treatments and condition were adapted to darkness for 45 min (Baker 2008) and the minimum fluorescence image (F_0) was obtained from the third 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). 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 PSII quantum efficiency (Baker 2008). Leaves were subsequently exposed to actinic photon irradiance (185

µmol m^{-2} s^{-1}) for 300 s to obtain the steady-state fluorescence yield (F_s), after which a saturating white light pulse (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]. 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 96 hai.

Determination of the concentration of photosynthetic pigments

The concentrations of Chl *a*, Chl *b*, and carotenoids were determined using the dimethyl sulfoxide (DMSO) as an extractor (Santos et al. 2008). Five disks of third leaves (1 cm in diameter) were collected at 96 hai, immersed in glass tubes containing five mL of saturated DMSO solution and calcium carbonate ($CaCO_3$) (5 g L^{-1}) (Wellburn 1994), and kept at room temperature during 24 h at darkness. The absorbance of the extracts was read at 480, 649, and 663 nm using a saturated solution of DMSO and $CaCO_3$ as the reference.

Biochemical assays

A bulk sample formed by the third and fourth fully expanded leaves colected from top to base, of each plant per replication of each treatment were collected at 96 hai. the leaves were collected from both inoculated and non-inoculated. Leaves were kept in liquid nitrogen after sampling and stored at -80°C.

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 0.1g 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 4ºC. 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 95ºC. After this period, the reaction was stopped in an ice bath. The samples were centrifuged at 9,000 *g* for 10

min and the specific absorbance was determined at 532 nm. The 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 µmol g^{-1} of fresh weight (FW).

Determination of enzymes activities of the antioxidative metabolism

For determination of the activities of ascorbate peroxidase (APX, EC 1.11.1.11), catalase (CAT, EC 1.11.1.6), superoxide dismutase (SOD, EC 1.15.1.1), and glutathione reductase (EC 1.8.1.7), a total of 0.2 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 4ºC and the supernatant was used as a crude enzyme extract. 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 25ºC. The extinction coefficient of 2.8 mM^{-1} cm^{-1} (Nakano and Asada 1981) was used to calculate APX activity, which was expressed as mmol min^{-1} mg^{-1} of protein. 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 25ºC 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 according to 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 25ºC. The extinction coefficient of 36 M^{-1} cm^{-1} (Anderson et al. 1995) was used to calculate CAT activity, which was expressed as mmol min^{-1} mg^{-1} of protein. The GR activity, the reaction was started after the addition of 100 µl of the crude enzyme extract to a volume of 1.9 ml of a mixture containing 100 mM potassium phosphate (pH 7.5), 1 mM EDTA, 1 mM oxidized glutathione (GSSG), and 0.1 mM NADPH prepared in 0.5 mM Tris-HCl buffer (pH 7.5) according to Carlberg and Mannervik (1985). The decrease in absorbance was determined at 340 nm for 1 min at 30ºC. The extinction coefficient of 6.22 mM^{-1} cm^{-1} (Foyer and Halliwell 1976) was used to calculate GR activity, which was expressed as µmol min^{-1} mg^{-1} of protein. For each enzyme, three 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 defense enzymes activities

For determination of the activities of chitinase (CHI, EC 3.2.1.14), *β*-1,3- 24 glucanase (GLU, EC 3.2.1.39), phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), polyphenoloxidase (PPO, EC 1.10.3.1), peroxidase (POX, EC 1.11.1.7), and lipoxygenase (LOX, EC 1.13.11.12), a total of 0.2 g of leaf tissue was macerated with liquid nitrogen in a mortar with the addition of polyvinylpyrrolidone (PVP) 1% (w v^{-1}) to obtain a fine powder. The powder was homogenized in 2 mL of 50 mM sodium phosphate (pH 6.5) containing 1 mM phenylmethylsulfonicfluoride (PMSF) and 0.1 mM acid etilenodiaminotetracetic (EDTA). The homogenized material was centrifuged at 20,000 *g* for 25 min at 4ºC and the supernatant was used for enzyme determination. The enzyme extracts for CHI were prepared following the method of Harman et al. (1993) with a few modifications. The reaction was started by the addition of 20 µl of crude enzyme extract to a mixture containing 480 µl 50 mM sodium acetate buffer (pH 5.0) and *p*-nitrofenil *β*-D-N-N'-diacetilquitobiose (Sigma-Aldrich) at 2 mg ml^{-1}. The reaction mixture was incubated in a water bath at 37ºC for 2 h. Adding 500 µL of 0.2 M sodium carbonate terminated the reaction. The absorbance of the end products released by the CHI present in the crude enzyme extract was recorded at 410 nm. An extinction coefficient of 7×10^4 mM^{-1} cm^{-1} was used to calculate CHI activity, which was expressed as mmol of *p*-nitrophenyl min^{-1} mg^{-1} protein. The GLU activity was determined as described by Lever (1972). The reaction was initiated by the addition of 20 µL aliquots of the supernatant to a mixture of 230 µL of 100 mM sodium acetate (pH 5.0) and 250 µL of the substrate laminarin (Sigma-

Aldrich) in a concentration of 4 mg mL^{-1}. The reaction mixture was incubated in a water bath for 30 min at 45ºC. After the incubation period, the amount of reducing sugars was determined by adding 500 µL of dinitrosalicylic acid to the mixture and then incubating the resulting mixture in a water bath for 15 min at 100ºC. The reaction was interrupted by cooling the samples in an ice bath. In the control samples, the reaction mixture was the same, except that the extract was added after heating the mixture at 100ºC. The absorbance of the product released by GLU was measured at 540 nm and the activity of GLU was expressed in Δ absorbance min^{-1} mg^{-1} of protein. The POX and PPO activities were determined by the oxidation of pyrogallol according to the method of Kar and Miashra (1976). For POX activity, a mixture of 300 µL of distilled water, 250 µL of 100 mM potassium phosphate buffer (pH 6.8), 200 µL of 100 mM pyrogallol, and 200 µL of 100 mM hydrogen peroxide was added to 50 µL of the extract. For PPO activity, the mixture was composed of 300 µL of distilled water, 250 µL of 100 mM potassium phosphate buffer (pH 6.8), and 200 µL of 100 mM pyrogallol, which was added to 50 µL of the extract. The absorbance was measured in spectrophotometer at 420 nm every 10 seconds for 1 min after addition of the extract to the mixture in a total of five readings. A molar extinction coefficient of 2.47 mM^{-1} cm^{-1} was used to calculate POX and PPO activities, which were expressed in mmol purpurogallin produced mmol min^{-1} mg^{-1} protein. The PAL activity was determinate adding 0.1 mL of the extract to a mixture containing 0.5 mL of Tris-HCl (pH 8.8, 25 mM) and 0.4 mL of 100 mM *L*-phenylalanine. The reaction mixture was incubated in a water bath at 30ºC for 3 h. In the control samples, *L*-phenylalanine was replaced with Tris-HCl buffer. The reaction was finalized by adding 0.1 mL of 6 N HCl. The absorbance of the *trans*-cinnamic acid derivatives was measured in a spectrophotometer at 290 nm and a molar extinction coefficient of 10^4 mM^{-1} cm^{-1} (Zucker 1965) was used to calculate PAL activity, which was expressed in µmol min^{-1} mg^{-1} protein. The LOX activity was determined adding 0.3 mL of the extract to a mixture containing 2 mL of buffer, 50 mM sodium phosphate (pH 6.0), and 0.3 mL of a 10 mM sodium linoleate substrate. The mixture was incubated in a water bath for 4 min at 25ºC. The LOX activity was determined according to the method described by Axelrod et al. (1981). The absorbance of the product released by LOX was measured in a spectrophotometer at 234 nm. The molar extinction coefficient of 25 000 M^{-1} cm^{-1} was used to determine LOX activity, which was expressed as mmol min^{-1} mg^{-1} of protein. For each enzyme, five 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.

Superoxide anion (O_2^-) concentration

A total of 0.2 g of leaf tissue was ground into a fine powder in a mortar and pestle with liquid nitrogen. The fine powder was homogenized in 2 ml of a solution containing 100 mM sodium phosphate buffer (pH 7.2) and 1 mM sodium diethyldithiocarbamate (SDD). The homogenate was centrifuged at 22,000 *g* for 20 min at 4ºC. After centrifugation, 0.1 ml of the supernatant was reacted with 1.9 ml of a solution containing 100 mM sodium phosphate buffer (pH 7.2), 1 mM SDD, and 0.25 mM NBT. The O_2^- concentration was determined by subtracting the absorbance of the final product from the initial absorbance at 540 nm (Chaitanya and Naithani 1994).

Hydrogen peroxide (H_2O_2) concentration

A total of 0.2 g of leaf tissue was ground into a fine powder in a mortar and pestle with liquid nitrogen. The fine powder was homogenized in 2 ml of a solution containing a mixture of 50 mM potassium phosphate buffer (pH 6.5) and 1 mM hydroxylamine. The homogenate was centrifuged at 12,000 *g* for 15 min at 4ºC and the supernatant was used as the crude extract to determine the H_2O_2 concentration (Kuo and Kao 2003). The supernatant was added to a reaction mixture containing 100 µM ferric ammonium sulfate ($FeNH_4[SO_4]$), 25 mM sulphuric acid, 250 µM xylenol orange, and 100 mM sorbitol in a final volume of 250 µl (Gay and Gebicki 2000). After 30 min of dark incubation at room temperature, the absorbance of the samples was determined at 560 nm. Blanks were prepared under the same conditions and subtracted from the samples. A standard curve for H_2O_2 (Sigma-Aldrich, São Paulo, Brazil) was used to determine the H_2O_2 concentration.

Experimental design and data analyzes

A 4 × 2 factorial experiment, consisting of four different thermal acclimatization (TA) (19ºC→19ºC, 28ºC→28ºC, 19ºC→28ºC, and 28ºC→19ºC) and two plant inoculation conditions (IC) (non-inoculated or inoculated plants), was arranged in a completely randomized design with four replications. Each replication corresponded to a plastic pot containing seven plants. The experiment was repeated once. Data were subjected to analysis of variance (ANOVA) and treatments means were compared by Tukey's test ($P \leq .05$). Data were analyzed using the Minitab software (version 18; Minitab Corporation).

RESULTS

Analysis of variance

There were significant effect of the treatments thermal acclimatization (TA), inoculation conditions (IC), and their interaction for the most of the variables and parameters evaluated (Table 1).

Blast severity

At 96 hai, small water-soaked lesions were noticed on the leaves of plants from the 19ºC→19ºC TA treatment. By contrast, on the leaves of plants from the 28ºC→28ºC, 19ºC→28ºC, and 28ºC→19ºC TA treatments, there were many and bigger oval-shaped necrotic lesions of grayish coloration surrounded by intense chlorosis and coalescence (Fig. 1A-D). Blast severity progressed much faster on the leaves of plants from the 28ºC→28ºC TA treatment from 72 to 96 hai in comparison to plants submitted to the other TA treatments (Fig. 2A). The AUBPC was significantly lower by 71, 46, and 48% for plants from the 19ºC→19ºC, 28ºC→19ºC, and 19ºC→28ºC TA treatments, respectively, in comparison to the 28ºC→28ºC TA treatment (Fig. 2B). For the 28ºC→19ºC and 19ºC→28ºC TA treatments, AUBPC was significantly higher by 45 and 46% in comparison to the 19ºC→19ºC TA treatment (Fig. 2B).

Leaf gas exchange parameters

The A, g_s, and E (56, 44, and 41%; 90, 76, and 73%; 78, 50, and 53% as well as 70, 21, and 29%, respectively, for 19ºC→19ºC, 28ºC→28ºC, 19ºC→28ºC, and 28ºC→19ºC TA treatments) significantly decreased for inoculated plants in comparison to the non-inoculated ones (Fig. 3A, B, and D). The C_i (9, 12, 13, and 14%, respectively, for 19ºC→19ºC, 28ºC→28ºC, 19ºC→28ºC, and 28ºC→19ºC TA treatments) significantly decreased for non-inoculated plants in comparison to the inoculated ones (Fig. 3C). The A significantly decreased by 42 and 84% for 28ºC→28ºC, by 28 and 63% for 19ºC→28ºC, and by 32 and 54% for 28ºC→19ºC TA treatments in comparison to the 19ºC→19ºC TA treatment, respectively, for non-inoculated and inoculated plants (Fig. 3A). For non-inoculated plants, there was no significant difference for g_s among the treatments (Fig. 3B). For inoculated plants, g_s was significantly higher by 61 and 62%, respectively, for the 19ºC→19ºC and 28ºC→19ºC TA treatments in comparison to the 28ºC→28ºC TA treatment (Fig. 3B). For non-inoculated plants, C_i was significantly lower by 7 and 5% for the 19ºC→19ºC and 19ºC→28ºC TA treatments, respectively, in comparison to the 28ºC→28ºC TA and by 11% for the 19ºC→19ºC TA treatment in comparison to the 28ºC→19ºC TA treatment (Fig. 3C). Significant decreases of 6

and 3% on C_i for 19ºC→19ºC and 19ºC→28ºC TA treatments in comparison to the 28ºC→28ºC TA treatment and of 12% for 19ºC→19ºC TA treatment in comparison to the 28ºC→19ºC TA treatment occurred for inoculated plants (Fig. 3C). The *E* significantly increased by 82, 50, and 53% for the 19ºC→19ºC, 28ºC→28ºC, and 28ºC→19ºC TA treatments, respectively, in comparison to the 19ºC→28ºC TA treatment for non-inoculated plants. For inoculated plants, *E* significantly decreased by 62 and 63% for the 28ºC→28ºC TA treatment and by 56 and 57% for the 19ºC→28ºC TA treatment in comparison, respectively, to 19ºC→19ºC and 28ºC→19ºC TA treatments (Fig. 3D).

Chl *a* fluorescence parameters

On inoculated leaves at 96 hai, images of Chl *a* fluorescence evidenced expressive changes in the F_v/F_m, Y(II), Y(NPQ), and Y(NO) in comparison to the leaves of non-inoculated plants. The most notable loss of the photosynthetic capacity occurred for the leaves of plants from the 28ºC→28ºC TA treatment in comparison to the other TA treatments (Fig. 4).

The F_v/F_m and Y(II) (9 and 24%, 38 and 80%, 14 and 55% as well as 16 and 65%, respectively, for 19ºC→19ºC, 28ºC→28ºC, 19ºC→28ºC, and 28ºC→19ºC TA treatments) significantly decreased for inoculated plants in comparison to the non-inoculated ones (Fig. 5A and B). There was no significant difference among the treatments for F_v/F_m, Y(II), Y(NPQ), and Y(NO) obtained from non-inoculated plants (Fig. 5A-D). The Y(NO) (11, 42, 17, and 24%, respectively, for 19ºC→19ºC, 28ºC→28ºC, 19ºC→28ºC, and 28ºC→19ºC TA treatments) significantly decreased for non-inoculated plants in comparison to the inoculated ones (Fig. 5D). For inoculated plants, F_v/F_m was significantly lower by 32, 28, and 27%, respectively, for the 19ºC→19ºC, 19ºC→28ºC, and 28ºC→19ºC TA treatments in comparison to the 28ºC→28ºC TA treatment. The F_v/F_m for inoculated plants was significantly lower by 3 and 7%, respectively, for 19ºC→28ºC and 28ºC→19ºC TA treatments in comparison to the 19ºC→19ºC TA treatment (Fig. 5A). The Y(II) for inoculated plants was significantly lower by 76, 49, and 63%, respectively, for 28ºC→28ºC, 19ºC→28ºC, and 28ºC→19ºC TA treatments in comparison to 19ºC→19ºC TA treatment (Fig. 5B). For inoculated plants, Y(NPQ) was significantly lower by 34% for 28ºC→28ºC TA treatment in comparison to 19ºC→28ºC TA treatment (Fig. 5C). The Y(NO) for inoculated plants was significantly lower by 44 and 23%, respectively, for 19ºC→19ºC and 19ºC→28ºC TA treatments in comparison to 28ºC→28ºC TA treatment. The inoculated plants displayed significantly lower Y(NO) for 19ºC→19ºC TA treatment in comparison to 28ºC→19ºC TA treatment (Fig. 5D).

Photosynthetic pigments

The Chl *a*, Chl *b*, and Chl *a+b* concentrations (39, 24, and 36%; 37, 22, and 38%; 40, 44, and 41% as well as 44, 24, and 39%, respectively, for 19ºC→19ºC, 28ºC→28ºC, 19ºC→28ºC, and 28ºC→19ºC TA treatments) significantly decreased for inoculated plants in comparison to the non-inoculated ones (Fig. 6A, B, and D). For 19ºC→19ºC TA treatment, inoculated plants displayed significant lower carotenoids concentration of 26% in comparison to the non-inoculated plants (Fig. 6C). There was no significant difference among the treatments for Chl *b* and carotenoids concentrations from inoculated plants (Fig. 6B and C). For non-inoculated plants, Chl *a* concentration was significantly lower by 32, 23, and 36%, respectively, for 28ºC→28ºC, 19ºC→28ºC, and 28ºC→19ºC TA treatments in comparison to 19ºC→19ºC TA treatment (Fig. 6A). The concentration of Chl *a* was significantly lower by 23 and 35%, respectively, for 28ºC→28ºC and 28ºC→19ºC TA treatments in comparison to 19ºC→19ºC TA treatment (Fig. 6A). For non-inoculated plants, Chl *b* concentration was significantly higher by 26 and 30%, respectively, for 19ºC→19ºC and 19ºC→28ºC TA treatments in comparison to 28ºC→28ºC TA treatment (Fig. 6B). The carotenoids concentration was significantly lower by 30% for 28ºC→28ºC TA treatment in comparison to 19ºC→19ºC TA treatment (Fig. 6C). The Chl *a+b* concentration for non-inoculated plants was significantly lower by 33, 16, and 25%, respectively, for 28ºC→28ºC, 19ºC→28ºC, and 28ºC→19ºC TA treatments in comparison to 19ºC→19ºC TA treatment. For non-inoculated plants, the Chl *a+b* concentration was significantly lower by 35% for 19ºC→19ºC TA treatment in comparison to the 28ºC→28ºC TA treatment. For inoculated plants, the Chl *a+b* concentration was significantly lower by 35 and 29% for 28ºC→28ºC and 28ºC→19ºC TA treatments in comparison to 19ºC→19ºC TA treatment (Fig. 6D).

Activities of antioxidant enzymes

The activities of SOD for 28ºC→28ºC TA treatment (86%), APX for 19ºC→19ºC, 19ºC→28ºC, and 28ºC→19ºC TA treatments (47, 43, and 48%, respectively), and CAT for 28ºC→28ºC and 19ºC→28ºC TA treatments (33 and 45%, respectively) significantly decreased for non-inoculated plants in comparison to the inoculated ones (Fig. 7A-C). The activities of APX for 28ºC→28ºC TA treatment (54%), CAT for 19ºC→19ºC TA treatment (8%), and GR for 19ºC→19ºC and 28ºC→19ºC TA treatments (23 and 33%, respectively) significantly decreased for inoculated plants in comparison to the non-inoculated ones (Fig. 7B-D). There was no significant difference among treatments for APX and GR activities from non-inoculated plants and also for CAT activity from inoculated ones (Fig. 7B-D). For non-inoculated plants,

SOD activity was significantly lower by 72% for 28ºC→28ºC TA treatment in comparison to 28ºC→19ºC TA treatment. For inoculated plants, SOD activity was lower by 55, 67, and 68%, respectively, for 19ºC→19ºC, 19ºC→28ºC, and 28ºC→19ºC TA treatments in comparison to 28ºC→28ºC TA treatment (Fig. 7A). The APX activity was significantly higher by 47, 28, and 46%, respectively, for 19ºC→19ºC, 19ºC→28ºC, and 28ºC→19ºC TA treatments in comparison to 28ºC→28ºC TA treatment for inoculated plants (Fig. 7B). The CAT activity for inoculated plants was significantly lower by 34 and 45%, respectively, for 28ºC→28ºC and 19ºC→28ºC TA treatments in comparison to 19ºC→19ºC TA treatment (Fig. 7C). For inoculated plants, GR activity was significantly lower by 26% for 28ºC→19ºC TA treatment in comparison to 19ºC→28ºC TA treatment (Fig. 7D).

Activities of defense enzymes

The activities of CHI for 28ºC→19ºC TA treatment (34%), PAL for 19ºC→19ºC, 28ºC→28ºC, and 19ºC→28ºC TA treatments (73, 93, and 94%, respectively), POX for 28ºC→28ºC, 19ºC→28ºC, and 28ºC→19ºC TA treatments (63, 78, and 57%, respectively), and LOX for 19ºC→19ºC and 19ºC→28ºC TA treatments (67 and 50%, respectively) significantly decreased for non-inoculated plants in comparison to the inoculated ones (Fig. 8A, C, E, and F). The activities of GLU for 19ºC→28ºC and 28ºC→19ºC TA treatments (65 and 62%, respectively) and of PPO for 28ºC→28ºC, 19ºC→28ºC, and 28ºC→19ºC TA treatments (54, 45, and 58%, respectively) were significantly lower for inoculated plants in comparison to the non-inoculated ones (Fig. 8B and D). There was no significant difference among treatments for CHI activity for inoculated plants and also for POX and LOX activities from non-inoculated plants (Fig. 8A, E, and F). For non-inoculated plants, CHI activity was significantly higher by 197, 116 and 67%, respectively, for the 28ºC→28ºC 19ºC→28ºC and 28ºC→19ºC TA treatments in comparison to the 19ºC→19ºC TA treatment (Fig. 8A). For non-inoculated plants, GLU activity was significantly higher by 16 and 29% for 28ºC→28ºC and 19ºC→28ºC TA treatments, respectively, in comparison to the 19ºC→19ºC TA treatment. The GLU activity was significantly lower by 58% for 28ºC→19ºC TA treatment in comparison to 28ºC→28ºC TA treatment (Fig. 8B). For non-inoculated plants, PAL activity was significantly lower by 86 and 87% for 28ºC→28ºC and 19ºC→28ºC TA treatments, respectively, in comparison to 28ºC→19ºC TA treatment. For inoculated plants, PAL activity was significantly lower by 47 and 66%, respectively, for 19ºC→19ºC and 28ºC→19ºC TA treatments in comparison to 28ºC→28ºC TA and by 54 and 13%, respectively, for 19ºC→19ºC and 28ºC→19ºC TA treatments in comparison to 19ºC→28ºC TA treatment (Fig. 8C). The PPO activity was

significantly lower by 28 and 18%, respectively, for 19ºC→19ºC and 19ºC→28ºC TA treatments in comparison to 28ºC→19ºC TA treatment for non-inoculated plants. The PPO activity for inoculated plants was significantly lower by 50, 44, and 48%, respectively, for 28ºC→28ºC, 19ºC→28ºC, and 28ºC→19ºC TA treatments in comparison to 19ºC→19ºC TA treatment (Fig. 8D). For inoculated plants, POX activity was significantly lower by 53 and 37%, respectively, for 19ºC→19ºC and 28ºC→19ºC TA treatments in comparison to 28ºC→28ºC TA treatment and by 135 and 25%, respectively, for 19ºC→19ºC and 28ºC→19ºC TA treatments in comparison to 19ºC→28ºC TA treatment (Fig. 8E). The LOX activity for inoculated plants was significantly lower by 34, 37, and 41%, respectively, for 28ºC→28ºC, 19ºC→28ºC, and 28ºC→19ºC TA treatments in comparison to 19ºC→19ºC TA treatment (Fig. 8F).

Concentrations of MDA, O_2^-, and H_2O_2

The concentrations of MDA for cxºC→28ºC, 19ºC→28ºC, and 28ºC→19ºC TA treatments (70, 43, and 58%, respectively), O_2^- for 19ºC→19ºC, 19ºC→28ºC, and 28ºC→19ºC TA treatments (32, 24, and 56%, respectively), and H_2O_2 for 19ºC→19ºC, 28ºC→28ºC, 19ºC→28ºC, and 28ºC→19ºC TA treatments (15, 30, 15, and 16%, respectively) significantly decreased for non-inoculated plants in comparison to the inoculated ones (Fig. 9A-C). For non-inoculated plants, there was no significant difference among treatments for MDA and H_2O_2 concentrations (Fig. 9A and C). For inoculated plants, MDA concentration was significantly lower by 50, 15, and 11% for 28ºC→28ºC, 19ºC→28ºC, and 28ºC→19ºC TA treatments, respectively, in comparison to 19ºC→19ºC TA treatment (Fig. 9A). For non-inoculated plants, O_2^- concentration was significantly lower by 34 and 32%, respectively, for 19ºC→19ºC and 28ºC→19ºC TA treatments in comparison to 28ºC→28ºC TA treatment and by 34 and 33%, respectively, for 19ºC→19ºC and 28ºC→19ºC TA treatments in comparison to 19ºC→28ºC TA treatment. For inoculated plants, O_2^- concentration was significantly lower by 26 and 30%, respectively, for 19ºC→19ºC and 28ºC→28ºC TA treatments in comparison to 19ºC→28ºC TA treatment and by 36 and 66%, respectively, for 19ºC→19ºC and 28ºC→28ºC TA treatments in comparison to 28ºC→19ºC TA treatment (Fig. 9B). The H_2O_2 concentration was significantly lower by 15, 14, and 20%, respectively, for 19ºC→19ºC, 19ºC→28ºC, 28ºC→19ºC TA treatments in comparison to 28ºC→28ºC TA treatment for inoculated plants (Fig. 9C).

DISCUSSION

The present study provides novel information from physiological and biochemical perspectives regarding on how thermal oscillations could influence wheat resistance to blast. In nature, plants and their pathogens are constantly exposed to different environmental variables, which temperature is one of the environmental factors that determines the development of the disease, as well as the expression of symptoms (Pandey et al. 2015). According to Rajput et al. (2017), temperatures at 27ºC influence spore germination and appressorium formation, favoring the infection process**.** According to Cardoso et al. (2008), blast incidence increased on wheat plants exposed at 30ºC. In the present study, blast development was lesser on plants submitted to the thermal acclimatization of 19ºC→19ºC in comparison to plants exposed to the thermal acclimatization of 28ºC→28ºC. Curiously, plants kept at 28ºC before being inoculated with *P. oryzae* and exposed to low temperature after inoculation thereafter (thermal acclimatization of 28ºC→19ºC) showed blast development similarly to plants exposed to the thermal acclimatization of 19ºC→28ºC. *Arabidopsis* plants exposed to temperature of 30ºC instead of 27ºC, for three days, were more susceptible to *Pseudomonas syringae* pv. tomato (Huot et al. 2017). Lettuce plants exposed to temperatures of 30ºC for eight days after inoculation with *Fusarium equiseti* were more susceptible than plants exposed to 15ºC (Garibaldi et al. 2016).

Photosynthesis and respiration are responsible for carbon balance in wheat plants and important determinants of their growth (Lohraseb et al. 2017). Wheat plants submitted to 30ºC showed decrease in *A* because of an increase on both photorespiration and mitochondrial respiration (Silva-Pérez et al. 2017). In the present study, non-inoculated plants exposed to 28ºC showed a decrease in the *A* and concentration of Chl *a*. High temperature increased the expression of chlorophyllase, which is responsible for chlorophyll degradation (Yang et al. 2018). The deleterious effect of exposing the non-inoculated wheat plants at 28°C on Chl *a* concentration persisted even after submitting them to the thermal acclimatization of 28°C→19°C. Thermal stress damage the thylakoid membranes on chloroplasts that lower the concentration of pigments as well as the oxygen evolution complex of PSII (Komayama et al. 2007; Yamasaki et al. 2002). Wheat plants exposed to 30ºC for six hours showed reduced concentration of chlorophylls (Sarkar et al. 2016).

Photosynthesis is the main physiological process on plants infected by pathogens and has been deeply investigated for the wheat-*Bipolaris sorokiniana* (Rios et al. 2016), rice-*Monographella albescens* (Tatagiba et al. 2016), common bean-*Sclerotinia sclerotiorum* (Fagundes-Nacarath et al. 2018), and soybean-*Corynespora cassiicola* (Fortunato et al. 2015)

interactions. During the colonization process, *P. oryzae* releases non-host selective toxins that cause leaf tissues disintegration that compromises the photosynthetic apparatus (Bastiaans 1993). The infection by *P. oryzae* reduced the photosynthesis rates on plants exposed to the thermal acclimatizations of 19ºC→19ºC, 28ºC→28ºC, 19ºC→28ºC, and 28ºC→19ºC in comparison to non-inoculated ones. In the present study, plants exposed to 28ºC showed lower *A* due to reduction in the concentration of Chl *a*. Rios et al. (2014) reported a reduced concentration of photosynthetic pigments on the leaves of wheat plants due to the massive colonization by *P. oryzae*. The infection by *P oryzae* greatly affected the photosynthesis of wheat plants as noticed by the lower *A*, g_s, and *E* values, great C_i values, and lower Chl *a* concentration. The decrease in *A* was associated with biochemical damage, observed by the high C_i. Plants exposed at 28ºC after or before showed more susceptible to blast, as noticed for higher severity and lower photosynthesis. Debona et al. (2014) showed that wheat plants infected by *P. oryzae* showed impairment on photosynthesis due to decreases in g_s and damage to Rubisco. The concentration of photosynthetic pigments decreased on common bean and rice plants infected by *Colletotrichum lindemuthianum* and *Bipolaris oryzae*, respectively that lower the photosynthesis (Lobato et al. 2010; Dallagnol et al. 2011).

The fluorescence image of Chl *a* is a very useful tool to detect thermal stress on plants (Jedmowski and Brüggemann 2015). In the present study, there was no effect of thermal acclimatization on chlorophyll *a* fluorescence in non-inoculated plants tested to decrease F_v/F_m**.** According to Sharma et al. (2012), wheat plants exposed to 37.5ºC showed lower F_v/F_m values. Wheat plants infected by *P. oryzae* and exposed to the thermal acclimation of 28ºC→28ºC showed lower F_v/F_m values but without any impact on *A* possibly because other mechanisms responsible for CO_2 fixation were compromised. Impairments on photosynthesis caused by *P. oryzae* infection could be also associated with photochemical constraints as noticed by the decreases in F_v/F_m and Y(II) and increase in Y(NO). No changes were observed in Y(NPQ) which represents the photoprotection of the thylakoid ΔpH lumen and the xanthophyll cycle (Demmig-Adams et al. 2015). The non-host selective toxins released by *P. oryzae* are directly linked to blast lesions expansion besides inhibiting the PSII and compromising the flow of electrons between the Q_A and Q_B and, consequently, contributing to decrease F_v/F_m (Chen et al. 2007). In general, leaves infected by pathogens show high Y(NPQ) and Y(NO) values and lower Y(II) values (Rolfe and Scholes 2010). Lower Y(II) and Y(NPQ) values and high Y(NO) values were obtained from wheat leaves infected by *P. oryzae* (Rios et al. 2017). According to Rios et al. (2016), reductions on Y(II) was due to damage on electron transport rate (Rios et al. 2016) and can be more impacted when plants are exposed to high temperatures as noticed in

the present study. Photooxidative damage in plant tissues may be associated with an increase in Y(NO) values reflecting, therefore, in the mechanisms of energy dissipation in the non-regulated form that negatively impact the photoprotection mechanisms (Klughammer and Schreiber 2008; Huang et al. 2012; Sekulska-Nalewajko et al. 2019).

The ROS (*e.g.*, hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide anion (O_2^-), and hydroxyl radical (OH)) acts both as signaling molecules to potentiate host defense response against pathogens infection or causing cellular damage (Vellosillo et al. 2010; Camejo et al. 2016). The generation of H_2O_2 and O_2^- at the sites of infection is one of the earliest and strongest biochemical defense of plants to avoid infection (Yarden et al. 2014). The process of shift biotrophic to necrotrophic phase, involves the massive accumulation of H_2O_2 (Shetty et al. 2007; Wang et al. 2019a). Plants have an efficient enzymatic system to protect them against the toxic effects caused by ROS (You and Chan 2015). High SOD, APX, CAT, and GR activities were noticed on plants of wheat cultivars more tolerant to high temperatures (Balla et al. 2009; Bavita et al. 2012). The SOD activity was high on infected wheat plants exposed to thermal acclimatization of 28ºC→28ºC. The high activity of SOD in plants inoculated at 28ºC→28ºC reduced O_2^- concentration and led to high severity therefore, possibly acting negatively in resistance to disease. Debona et al. (2012) showed that wheat plants from a cultivar more resistant to blast exhibited high SOD, APX, CAT, and GR activities. The SOD acts in the first line of host defense against the ROS by converting O_2^- into H_2O_2 and water whereas the APX, POX, and CAT act in the detoxification of H_2O_2 (Das and Roychoudhury 2014). The removal of H_2O_2 is the first step of the ascorbate-glutathione cycle that comprises a series of redox reactions involving APX and GR (Kramarenko et al. 2006; Keramat et al. 2017). The APX is responsible for the removal of H_2O_2 in the chloroplasts, peroxisomes, and mitochondria by using ascorbate as a specific electron donor to reduce H_2O_2 to water (Miyake et al. 2006; Quan et al. 2008). In general, APX activity was higher in inoculated wheat plants than in non-inoculated ones. The low APX activity on infected wheat plants exposed to thermal acclimatization of 28ºC→28ºC was linked to a high production of H_2O_2 by these plants. The CAT, found mainly in peroxisomes, catalyzes the reaction that converts H_2O_2 into H_2O (Apel and Hirt 2004). Unlike APX, CAT does not require a reducing agent being therefore a more efficient form on H_2O_2 removal (Choudhury et al. 2013). The CAT activity was of great importance for wheat plants exposed to high temperatures (Sarkar et al. 2016). The CAT activity was lower on non-inoculated wheat plants and exposed to thermal acclimatizations of 28ºC→28ºC and 19ºC→28ºC than inoculated ones. Thus, plants exposed at 28ºC after inoculation showed minor contribution of CAT leading higher severity. Cucumber plants

exposed to 48ºC for two hours and infected by *Pseudoperonospora cubensis* showed high CAT, APX, and SOD activities (Ding et al. 2016). The GR catalyzes the NADP-dependent reduction of GSSG to generate reduced glutathione which plays an important role during stress conditions such as pathogens infection (Noctor et al. 2015; Kusvuran and Dasgan 2017; Pittner et al. 2019). In the present study, GR activity on non-inoculated plants was quite similar among the thermal acclimatizations tested. On the other hand, for plants exposed to thermal acclimatizations of 19ºC→19ºC and 28ºC→19ºC, GR activity was great for non-inoculated plants in comparison to inoculated ones. Infection caused by *P. oryzae* on wheat showed reduction GR (Debona et al. 2012). There was no difference between inoculated and non-inoculated plants at 28ºC→28ºC and 19ºC→28ºC can be affect high temperature. Wheat plants exposed to 30ºC for six hours showed high GR activity in comparison to plants exposed to 25ºC (Sarkar et al. 2016). Some studies report that GR activity on plants increases in response to infection by pathogens. Rice and soybean plants infected by *Monographella albescens* and *Corynespora quercus*, respectively, showed high GR activity (Tatagiba et al. 2016; Fortunato et al. 2015).

The CHI are important defense enzymes that degrades chitin on fungal cell wall (Sanchez-Vallet et al. 2015). The GLU are important lytic enzymes acting directly on fungal cells by increasing β-1,3/1,6-glucans resulting in mycelia lysis (Farrakh et al. 2018). The GLU and CHI played an important role on wheat resistance to blast (Rios et al. 2014). In the present study, CHI activity was high for inoculated and non-inoculated wheat plants exposed to thermal acclimatizations of 28ºC→28ºC and 19ºC→28ºC, which are condition of temperature favorable of infection. On the other hand, wheat plants exposed at 19ºC after inoculation noticed lower QUI activity, being this condition unfavorable of fungi development. Wheat plants exposed to 12ºC showed high CHI activity than plants exposed to 4ºC (Żur et al. 2013). The GLU activity was lower for non-inoculated wheat plants exposed to thermal acclimatizations of 19ºC→28ºC and 28ºC→19ºC. Melon plants exposed to 50ºC for 12 h showed an increase on the expression of CHI and GLU genes (Widiastuti et al. 2013). Silva et al. (2019) reported high CHI, GLU, PPO, and PAL activities on wheat plants infected by *P. oryzae*. Wheat plants infected by *Puccinia triticina* showed high expression of the GLU gene (Casassola et al. 2015).

The PAL catalyzes a reaction converting the amino acid *L*-phenylalanine to *trans*-cinnamic acid in the phenylpropanoid pathway leading to the synthesis of phenolics and lignin (Campbell and Sederoff 1996; Rodrigues et al. 2005). The PAL, considered to be one of the major enzymes related to the acclimatization of plants to thermal stress, increased its activity on hard fescue plants submitted at 33ºC/28ºC (day/night) (Wang et al. 2019b). In the present study, inoculated plants exposed to 28ºC after inoculation (thermal acclimatizations of

28ºC→28ºC and 19ºC→28º) showed high PAL and POX activities. The high PAL and PPO activities were linked to an increase in the lignin-thioglycolic acid (LTGA) derivatives resulting, therefore, in great lignification of wheat leaves and more resistance to blast (Silva et al. 2019). The POX uses H_2O_2 as an oxidant in the production of phenolics and cell wall lignification (Almagro et al. 2008; Podgórska et al. 2017). Proteomic analyzes showed that plants of chickpea kept at 45ºC/25ºC (day/night) for three days showed an increase on PAL activity (Parankusam et al. 2017). Tomato plants kept at both high and low temperatures showed high PAL activity and great concentration of phenolics but reduced POX and PPO activities (Rivero et al. 2001). Tobacco plants exposed to a combination of water-deficit and high temperatures showed high PAL activity in comparison to plants exposed to these stresses separately (Rizhsky et al. 2002). Wheat plants exposed at 28ºC before or after inoculation showed less activity of PPO, while contributed for higher severity of blast. Rice plants infected by *Bipolaris oryzae* showed an increase on PPO activity (Debona et al. 2018).

The LOX catalyzes the oxygenation of fatty acids to produce oxylipins, jasmonic acid (JA), methyl-JA as well as lipid peroxidases (Hammond-Kosack et al. 1996; Turner et al. 2002). The LOX is also important for plants tolerance to high temperatures (Wang et al. 2016) and resistance to pathogens (Rancé et al. 1998). In the present study, LOX activity was high for inoculated plants exposed to thermal acclimatization of 19ºC→19ºC reducing, therefore, lipid peroxidation and contributing for wheat resistance to blast. Wheat plants inoculated and exposed to thermal acclimatization of 28ºC→28ºC, 19ºC→28ºC, and 28ºC→19ºC showed lower LOX activity, resulting in high severity. Rios et al. (2014) showed that wheat plants infected by *P. oryzae* showed high LOX activity. Thus, the results in presents study showed that high severity of blast coincides with lower contribution of PPO and LOX, therefore both enzymes are important role in resistance of disease. The results from the present study contrast with those reported by Hu et al. (2013) which showed that tomato plants exposed to 50ºC for 2 h and infected by *Cladosporium fulvum* showed high LOX activity. It is plausible to postulate that the combination of high temperatures and *P. oryzae* infection, especially at advanced stage of fungal infection, may have impaired LOX activity leading, therefore, to lower the resistance of wheat plants to blast.

One of the earliest defense of plants against pathogens infection is the generation of extracellular ROS at the infection sites (Yarden et al. 2014; Zhang et al. 2019). The lipids peroxidation, which is linked with increased on MDA concentration, not only is triggered by ROS, consequences of infection by *P. oryzae* on wheat plants, (Debona et al. 2012), but also result from LOX activity (Farmer and Mueller 2013). Studies on the wheat-*Septoria tritici*

interaction revealed that H_2O_2 is important in the defence during infection (Yang et al. 2013). In the present study, non-inoculated plants exposed to thermal acclimatizations of 28ºC→28ºC and 19ºC→28ºC showed high O_2^- concentration. Wheat plants exposed to maximum/minimum daytime temperature of 35ºC/25ºC showed high concentrations of MDA, H_2O_2, and O_2^- after three days of stress (Djanaguiraman et al. 2018). On the meantime, inoculated wheat plants exposed to the thermal acclimatization of 28ºC→28ºC showed high MDA concentration as a consequence of great H_2O_2 concentration in a scenario where SOD activity was maximum.

Based on the results from the present study, it was demonstrated that blast development was favored on wheat plants pre-acclimated at 28ºC before inoculation with *P. oryzae* and kept at this same temperature during the fungal infection process. By contrast, blast development was quite similar on plants pre-acclimated at 19 and 28ºC before inoculation with *P. oryzae* and submitted, respectively, to temperatures of 28 or 19ºC after inoculation. The photosynthesis, as demonstrated by the gas exchange and Chl *a* fluorescence parameters, was negatively impaired on wheat plants exposed to 28ºC during the infection process of *P. oryzae* in association with lower chloroplastidic pigments concentration, a less efficient antioxidative system, and a minor contribution of the enzymes related to host defense. This information can be translated into practical knowledge for the development of wheat cultivars with a high level of basal resistance to blast to be grown in environments with favorable climatic conditions for severe blast epidemics in a scenario of climate change.

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TABLES AND FIGURES

Table 1. Analysis of variance of the effects of thermal acclimatization (TA), inoculation conditions (IC), and their interactions for area under disease progress curve (AUDPC), 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)), fluorescence parameters of chlorophyll *a* (maximal photosystem II quantum yield (F_v/F_m), effective PS II quantum yield (Y(II)), quenching for dissipation by down-regulation (Y(NPQ)), and quantum yield of non-regulated energy dissipation (Y(NO))), concentrations of photosynthetic pigments (chlorophyll *a* (Chl *a*), chlorophyll *b*, carotenoids, and total chlorophylls (Chl *a*+*b*)), enzymes activities ((superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), chitinase (CHI), β-1,3-glucanase (GLU), polyphenoloxidase (PPO), peroxidase (POX), phenylalanine ammonia-lyase (PAL), and lipoxygenase (LOX)) and concentrations of malondialdehyde (MDA), superoxide anion (O_2^-), and hydrogen peroxide (H_2O_2).

Variables/Parameters	TA	IC	TA × IC
AUDPC	<0.001	-	-
A	<0.001	<0.001	0.489
g_s	0.064	<0.001	0.003
C_i	<0.001	<0.001	0.068
E	0.002	<0.001	<0.001
F_V/F_m	<0.001	<0.001	<0.001
Y(II)	<0.001	<0.001	<0.001
Y(NPQ)	0.025	0.351	0.539
Y(NO)	<0.001	<0.001	0.005
Chl *a*	<0.001	<0.001	0.393
Chl *b*	0.001	<0.001	0.028
Carotenoids	0.118	0.616	0.032
Chl *a*+*b*	<0.001	<0.001	0.441
SOD	0.012	0.001	<0.001
APX	0.039	0.001	0.023
CAT	0.751	0.164	0.002
GR	0.093	<0.001	0.124
QUI	<0.001	0.264	0.084

GLU	0.009	<0.001	<0.001
PAL	0.066	<0.001	<0.001
PPO	0.011	<0.001	<0.001
POX	0.002	<0.001	<0.001
LOX	0.099	<0.001	0.003
MDA	0.009	<0.001	0.053
O_2^-	<0.001	<0.001	<0.001
H_2O_2	<0.001	<0.001	0.001

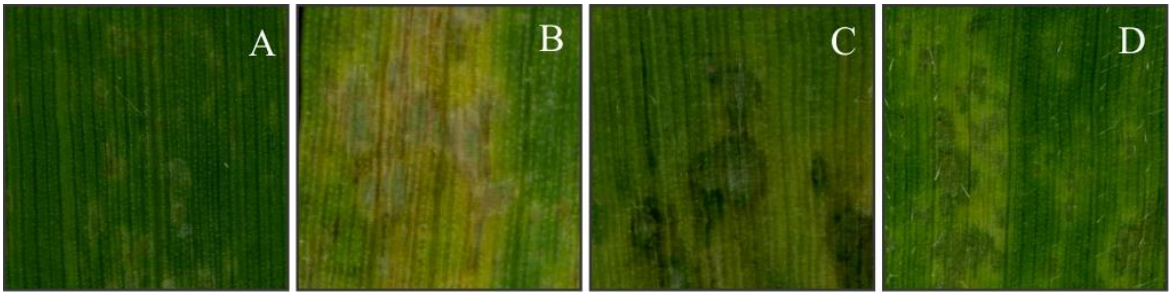

Figure 1. Blast symptoms on leaves of wheat plants submitted to thermal acclimatizations of 19ºC→19ºC (A), 28ºC→28ºC (B), 19ºC→28ºC (C), and 28ºC→19ºC (D) at 96 hours after inoculation with *Pyricularia oryzae*.

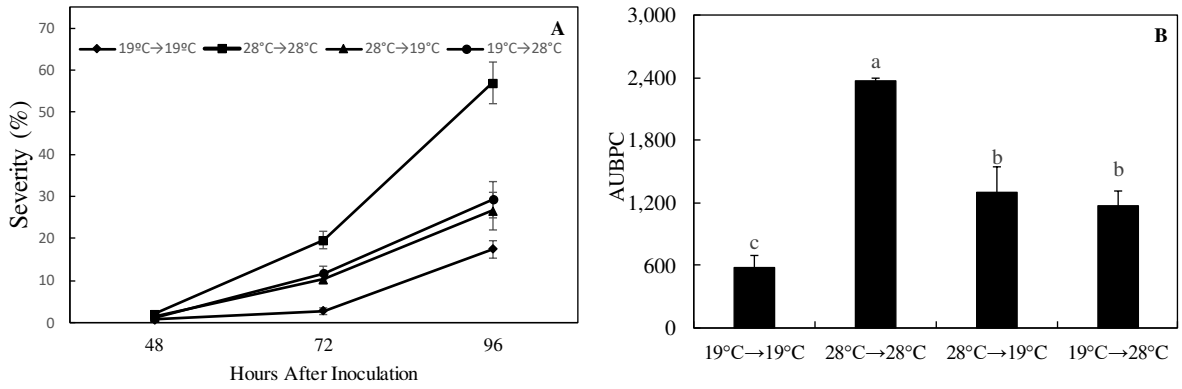

Figure 2. Blast progress curves (A) and area under blast progress curve (AUBPC) (B) for wheat plants submitted to different thermal acclimatizations. For AUBPC, means followed by different letters are significantly different according to Tukey's test ($P \leq 0.05$). The error bars represent the standard error of the mean. $n = 4$.

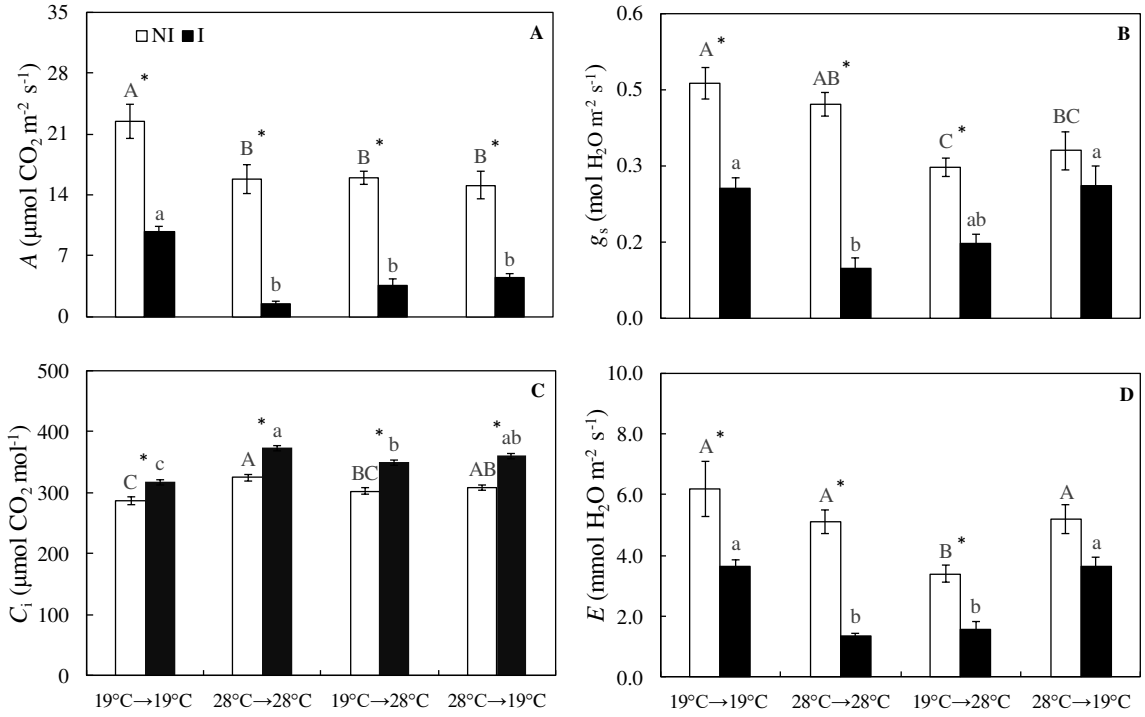

Figure 3. Leaf gas exchange parameters: net carbon assimilation rate (*A*) (A), stomatal conductance to water vapor (g_s) (B), internal CO_2 concentration (C_i) (C), and transpiration rate (*E*) (D) determined on the leaves of wheat plants submitted to different thermal acclimatizations and noninoculated (NI) or inoculated (I) with *Pyricularia oryzae*. Means followed by the same uppercase and lowercase letters are not significantly different according to Tukey´s test ($P \leq 0.05$), respectively, for NI and I plants. Means of NI and I plants for each thermal acclimatization followed by an asterisk (*) are significantly different according to Tukey's test ($P \leq 0.05$). The error bars represent the standard error of the mean. $n = 4$.

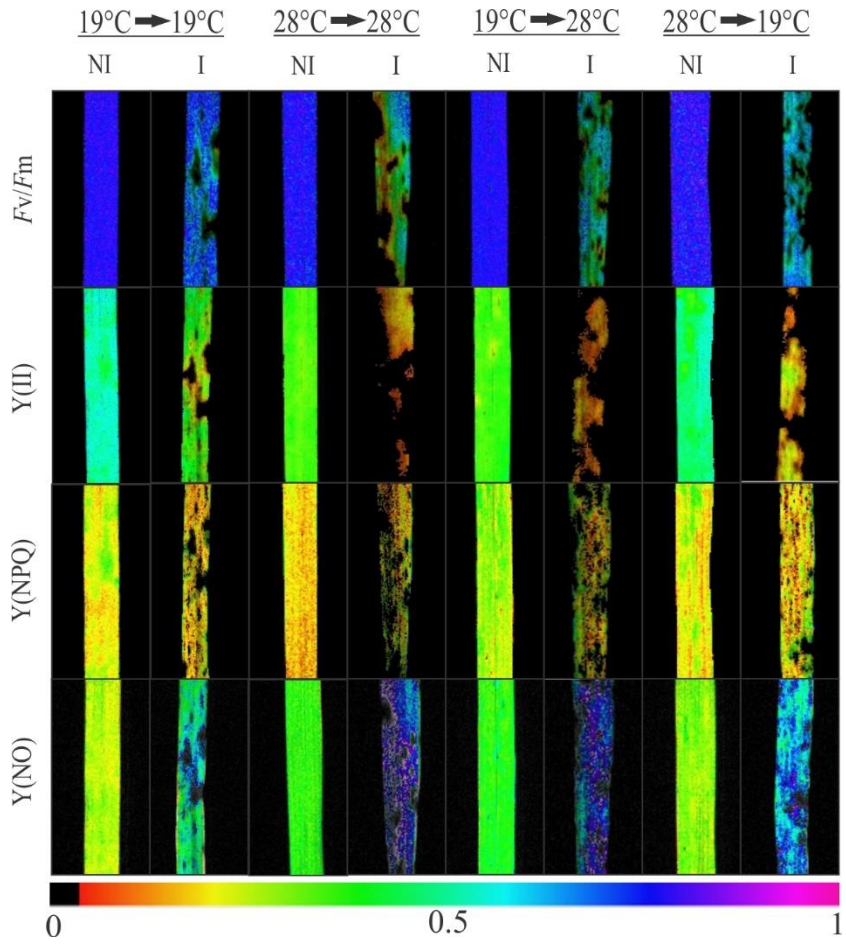

Figure 4. Images of chlorophyll *a* fluorescence parameters: maximal photosystem II quantum yield (F_v/F_m), effective PS II quantum yield (Y(II)), quenching for dissipation by down-regulation (Y(NPQ)), and quantum yield of non-regulated energy dissipation (Y(NO)) determined on the leaves of wheat plants submitted to different thermal acclimatizations and noninoculated (NI) or inoculated (I) with *Pyricularia oryzae.* Scale bar shows numbers from 0 to 1 which represent the pixel value of a particular color.

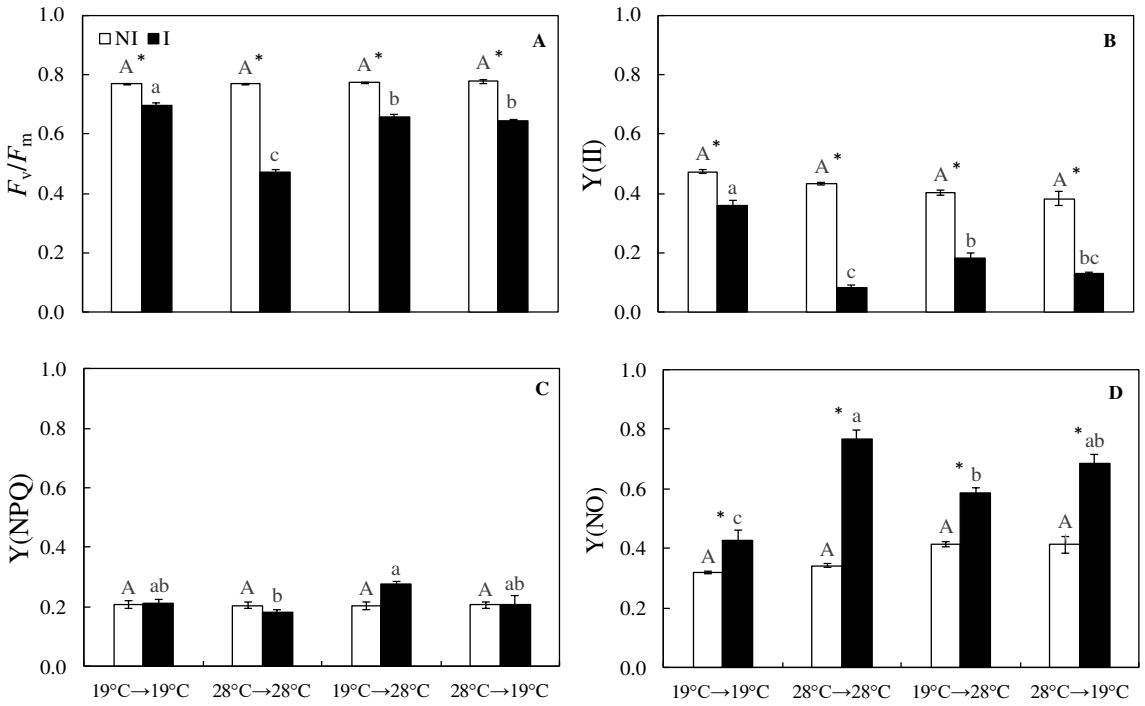

Figure 5. Parameters of chlorophyll *a* fluorescence: maximal photosystem II quantum efficiency ($F\checkmark F_m$) (A), effective photosystem II quantum yield (Y(II)), quantum yield of regulated energy dissipation Y(NPQ), and quantum yield of non-regulated energy dissipation Y(NO) (D) determined on the leaves of wheat plants submitted to different thermal acclimatizations and noninoculated (NI) or inoculated (I) with *Pyricularia oryzae.* Means followed by the same uppercase and lowercase letters are not significantly different according to Tukey´s test ($P \leq 0.05$), respectively, for NI and I plants. Means of NI and I plants for each thermal acclimatization followed by an asterisk (*) are significantly different according to Tukey's test ($P \leq 0.05$). The error bars represent the standard error of the mean. $n = 4$.

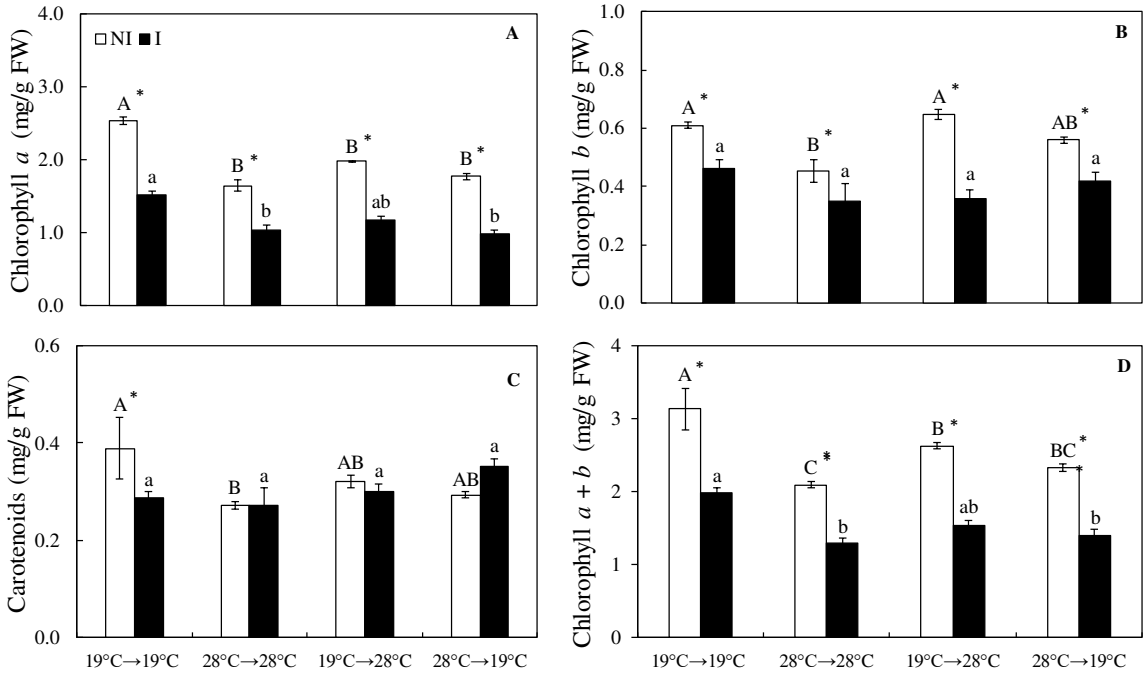

Figure 6. Concentrations of chlorophyll *a* (A), chlorophyll *b* (B), carotenoids (C), and chlorophyll *a* + *b* (D) determined in the leaves of wheat plants submitted to different thermal acclimatizations and noninoculated (NI) or inoculated (I) with *Pyricularia oryzae*. Means followed by the same uppercase and lowercase letters are not significantly different according to Tukey´s test ($P \leq 0.05$), respectively, for NI and I plants. Means of NI and I plants for each thermal acclimatization followed by an asterisk (*) are significantly different according to Tukey's test ($P \leq 0.05$). The error bars represent the standard error of the mean. $n = 4$.

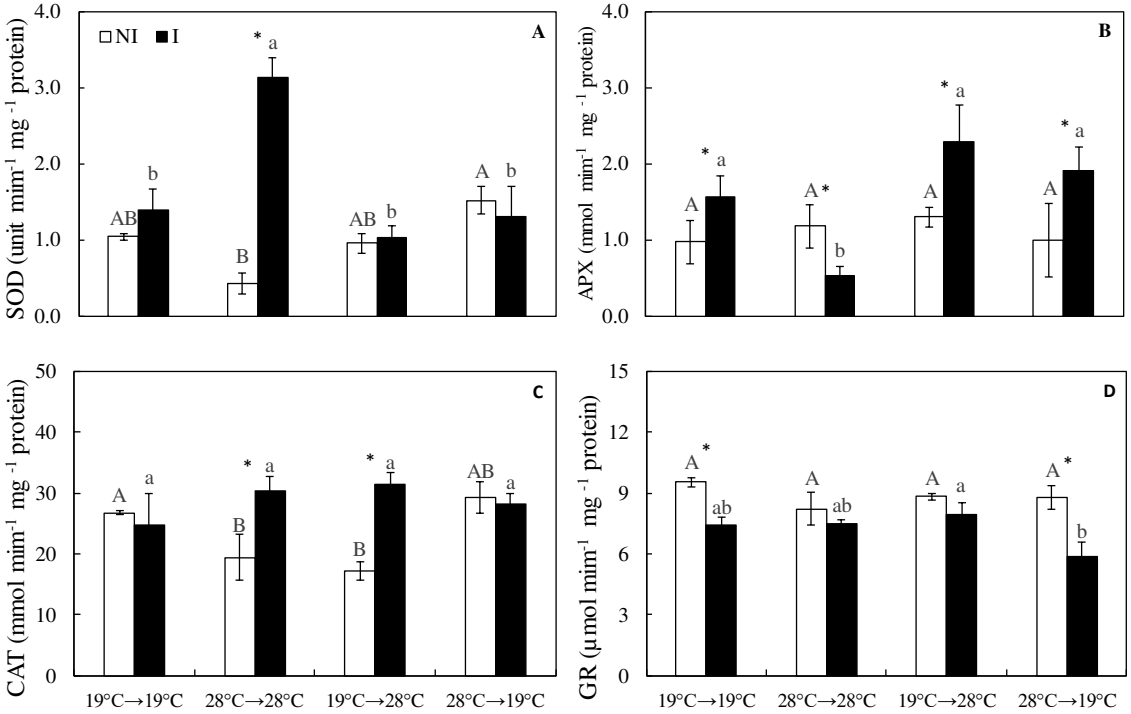

Figure 7. Activities of superoxide dismutase (SOD) (A), ascorbate peroxidase (APX) (B), glutathione reductase (GR) (C), and catalase (CAT) (D) determined on the leaves of wheat plants at different thermal acclimatization and noninoculated (NI) and inoculated (I) with *Pyricularia oryzae*. Means followed by the same uppercase and lowercase letters are not significantly different according to Tukey´s test ($P \leq 0.05$), respectively, for NI and I plants. Means followed by the same uppercase and lowercase letters are not significantly different according to Tukey´s test ($P \leq 0.05$), respectively, for NI and I plants. Means of NI and I plants for each thermal acclimatization followed by an asterisk (*) are significantly different according to Tukey's test ($P \leq 0.05$). The error bars represent the standard error of the mean. $n = 4$.

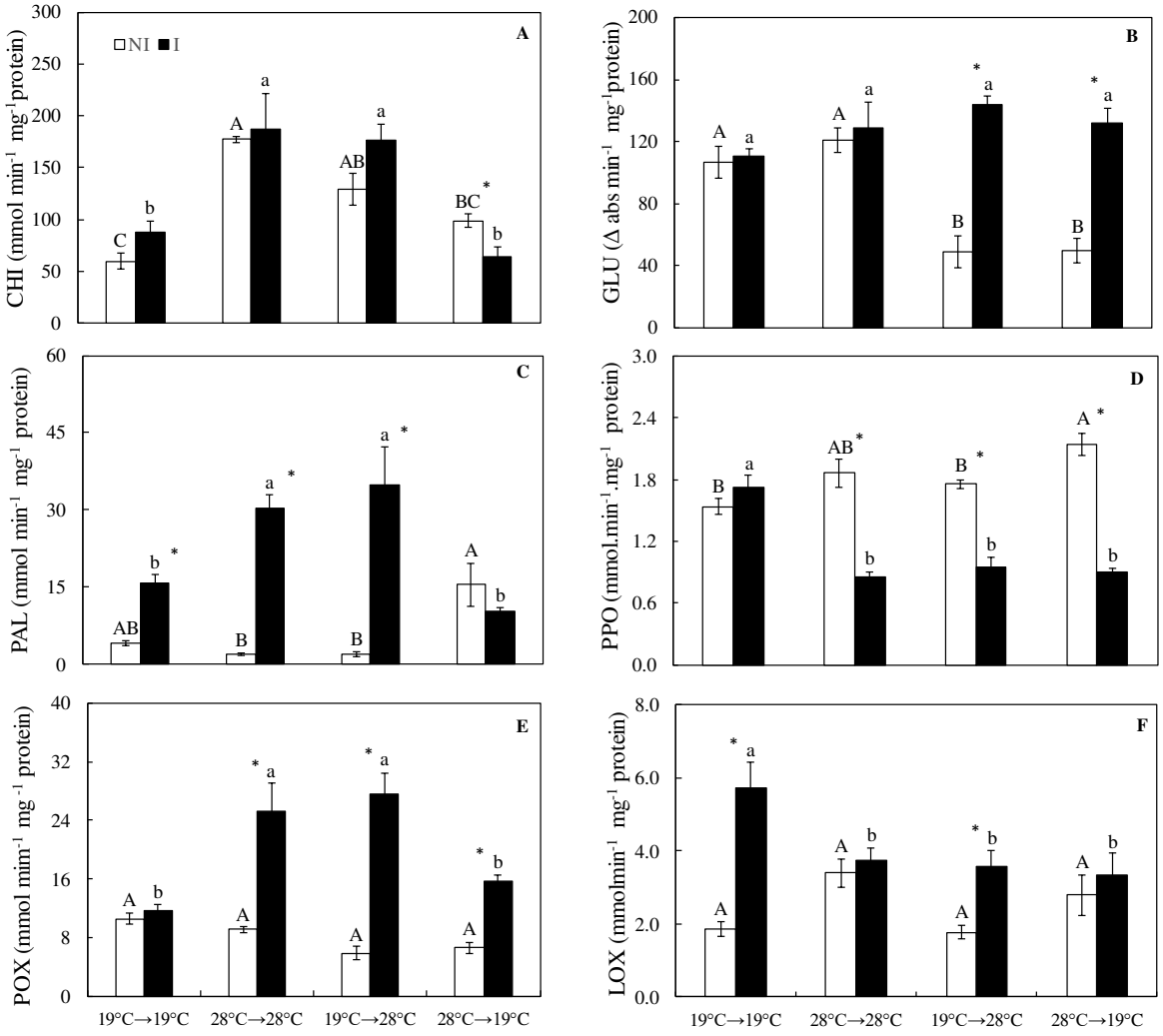

Figure 8: Activities of chitinase (CHI) (A), *β*-1,3-glucanase (GLU) (B), phenylalanine ammonia-lyase (PAL) (C), polyphenoloxidase (PPO) (D), peroxidase (POX) (E), and lipoxygenase (LOX) (F) determined on the leaves of wheat plants at different thermal acclimatization and noninoculated (NI) and inoculated (I) with *Pyricularia oryzae*. Means followed by the same uppercase and lowercase letters are not significantly different according to Tukey´s test ($P \leq 0.05$), respectively, for NI and I plants. Means of NI and I plants for each thermal acclimatization followed by an asterisk (*) are significantly different according to Tukey's test ($P \leq 0.05$). The error bars represent the standard error of the mean. $n = 4$.

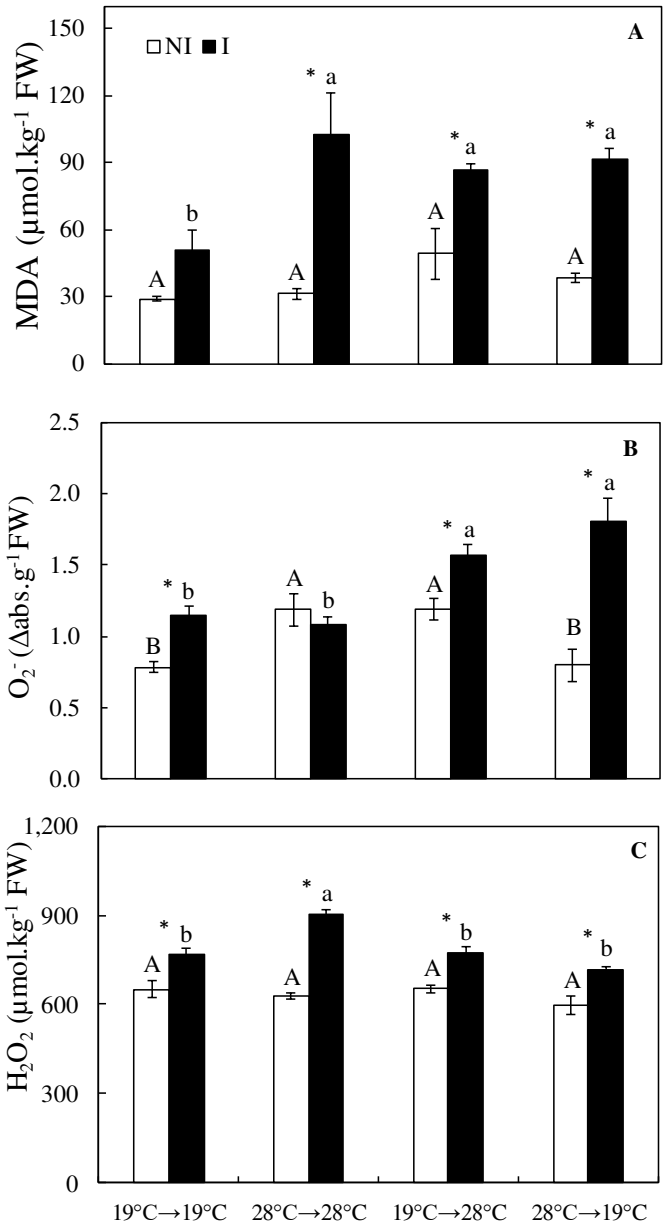

Figure 9. Concentrations of malondialdehyde (MDA) (A), superoxide (O_2^-) (B), and hydrogen peroxide (H_2O_2) (C) determined on the leaves of wheat plants at different thermal acclimatization and noninoculated (NI) or inoculated (I) with *Pyricularia oryzae*. Means followed by the same uppercase and lowercase letters are not significantly different according to Tukey´s test ($P \leq 0.05$), respectively, for NI and I plants. Means of NI and I plants for each thermal acclimatization followed by an asterisk (*) are significantly different according to Tukey's test ($P \leq 0.05$). The error bars represent the standard error of the mean. $n = 4$.