CAROLINE HAWERROTH

NEW INSIGHTS INTO THE ROLE PLAYED BY NICKEL AND PICOLINIC ACID IN THE RICE-*Bipolaris oryzae* INTERACTION

Thesis submitted to Universidade Federal de Viçosa as part of the requirements of the Plant Pathology Graduate Program for obtaining the degree of *Doctor Scientiae*.

Advisor: Fabrício de Ávila Rodrigues

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APPROVED: February 21st, 2020.

Assent:

Caroline Hawerroth
Author

Fabricio de Ávila Rodrigues
Advisor

To my parents, Sebastião Hawerroth (in memoriam),
and Renita Dirksen Hawerroth,
I dedicate with all my love.

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I am grateful to God for my life, family, and friends.

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ABSTRACT

HAWERROTH, Caroline, D.Sc., Universidade Federal de Viçosa, February, 2020. **New insights into the role played by nickel and picolinic acid in the rice-B*ipolaris oryzae* interaction.** Advisor: Fabrício de Ávila Rodrigues.

Brown spot, caused by *Bipolaris oryzae*, is one of the most important diseases of rice worldwide. Considering the importance of the micronutrient nickel (Ni) on plant metabolism and the great potential of the non-host selective toxin α-picolinic acid (PA) to be used enhancing plant resistance against diseases, the present study aimed to investigate (*i*) the effect of Ni on increasing rice resistance to brown spot and (*ii*) the effect of PA spray on the photosynthetic performance of rice plants infected by *B. oryzae* and whether high concentrations of PA could affect brown spot development. For the first goal, rice plants were grown in nutrient solution non-amended (-Ni plants) or amended (+Ni plants) with Ni and non-inoculated or inoculated with *B. oryzae*. There were significant increases in foliar Ni concentration and lower disease severity for +Ni in contrast to -Ni plants. The foliar tissues of -Ni plants were massively colonized by hyphae of *B. oryzae* in comparison to the leaf tissues of +Ni plants resulting, therefore, in more cellular damage as indicated by the high malondialdehyde concentration. In general, the activities of the defense enzymes chitinase, β-1,3-glucanase, peroxidase, polyphenoloxidase, and phenylalanine ammonia-lyase showed the same pattern between -Ni inoculated and +Ni inoculated plants during *B. oryzae* infection. High peroxidase activity for +Ni inoculated plants occurred at the earlier stage of fungal infection. High lipoxygenase activity for -Ni plants may have contributed to disease development. The photochemical performance of -Ni inoculated plants (lower maximum photochemical efficiency of photosystem II (PSII) (F_v/F_m) values) was much more affected in comparison to +Ni inoculated plants. High values for effective yield of the PSII (Y(II)) and electron transport rate (ETR) for +Ni plants indicated their efficiency in allocating the absorbed light energy to the photochemical process. For the second goal, rice plants were sprayed with PA at the concentrations of 0 (PA 0), 1 (PA 1), 3 (PA 3), and 5 mg mL^{-1} (PA 5). The *in vitro* assays showed that fungal mycelial growth was inhibited by PA in a dose-dependent manner, conidia germination was not affected, and conidial germ tube length only decreased for PA 5. The higher PA concentrations, PA 3 and PA

5, caused necrotic lesions on non-inoculated plants. The symptoms of brown spot were reduced by PA 1 and PA 3 in comparison to non-sprayed plants. Lesions originated from *B. oryzae* infection and PA toxicity overlapped for inoculated plants sprayed with PA 3 and PA 5. The photochemical performance of non-inoculated plants was hampered by PA 3 and PA 5. The high concentration of photosynthetic pigments and less impairment on the photosynthetic performance of inoculated plants sprayed with PA 1 were noticed based on the values of F_v/F_m, Y(II), yield for dissipation by down-regulation (Y(NPQ)), yield for non-regulated dissipation (Y(NO)), and ETR in comparison to inoculated plants non-sprayed with PA. In conclusion, these studies highlight the potential of Ni to increase rice resistance to brown spot through active participation of some defense enzymes, a robust antioxidative system, and a better photochemical performance and the potential of low concentrations of PA to decrease brown spot severity while preserving the photosynthetic capacity of the infected plants while the highest PA concentration did not seem to benefit *B. oryzae* infection. Therefore, either Ni or PA presents great potential for brown spot management in rice production.

Keywords: Antioxidative metabolism. Brown spot. Foliar disease. Host defense responses. Micronutrient. Necrotrophic pathogen. Non-host selective toxin. Photosynthesis. Plant nutrition.

RESUMO

HAWERROTH, Caroline, D.Sc., Universidade Federal de Viçosa, fevereiro de 2020. **Novas abordagens sobre o papel do níquel e do ácido picolínico na interação arroz-*Bipolaris oryzae*.** Orientador: Fabrício de Ávila Rodrigues.

A mancha parda, causada por *Bipolaris oryzae*, é uma das doenças mais importantes do arroz no mundo. Considerando a importância do micronutriente níquel (Ni) no metabolismo das plantas e o grande potencial da toxina não seletiva ácido α-picolínico (AP) para ser utilizado no aumento da resistência de plantas às doenças, o presente estudo teve como objetivos investigar (1) o efeito do Ni no aumento da resistência do arroz à mancha parda e (2) o efeito da pulverização de PA no desempenho fotossintético de plantas de arroz infectadas por *B. oryzae* e se altas concentrações de AP poderiam afetar o desenvolvimento da mancha parda. Para o primeiro objetivo, plantas de arroz foram cultivadas em solução nutritiva não suprida (plantas -Ni) ou suprida (plantas +Ni) com Ni e não inoculadas ou inoculadas com *B. oryzae*. Houve aumento significativo na concentração foliar de Ni e menor severidade da doença para plantas +Ni em contraste com as plantas -Ni. Os tecidos foliares das plantas -Ni foram colonizados massivamente por hifas de *B. oryzae* em comparação com os tecidos foliares das plantas +Ni resultando, portanto, em maior dano celular conforme indicado pela alta concentração de aldéido malônico. Em geral, as atividades das enzimas de defesa quitinase, β-1,3-glucanase, peroxidase, polifenoloxidase e fenilalanina amônia-liase mostraram o mesmo padrão entre plantas inoculadas -Ni e +Ni durante a infecção por *B. oryzae*. Maior atividade de peroxidase para plantas +Ni inoculadas ocorreu no estágio inicial da infecção. A maior atividade de lipoxigenase para plantas -Ni pode ter contribuído para o desenvolvimento da doença. O desempenho fotoquímico das plantas -Ni inoculadas (menor eficiência fotoquímica máxima do fotossistema II (FSII) (F_v/F_m)) foi muito mais afetado em comparação com as plantas +Ni inoculadas. Valores altos para rendimento efetivo do FSII (Y(II)) e taxa de transporte de elétrons (ETR) para plantas +Ni indicaram sua eficiência na alocação da energia luminosa absorvida no processo fotoquímico. Para o segundo objetivo, as plantas de arroz foram pulverizadas com AP nas concentrações de 0 (AP 0), 1 (AP 1), 3 (AP 3) e 5 mg mL^{-1} (AP 5). Os ensaios *in vitro* mostraram que o crescimento micelial fúngico foi inibido por AP de maneira dependente da dose, a germinação de conídios não foi afetada e o comprimento do tubo

germinativo dos conídios diminuiu apenas para AP 5. As concentrações mais elevadas de AP, AP 3 e AP 5 causaram lesões necróticas em plantas não inoculadas. Os sintomas da mancha parda foram reduzidos por AP 1 e AP 3 em comparação às plantas não pulverizadas com AP. As lesões originadas pela infecção de *B. oryzae* e pela toxicidade do AP se sobrepuseram nas plantas inoculadas e pulverizadas com AP 3 e AP 5. O desempenho fotoquímico das plantas não inoculadas foi prejudicado por AP 3 e AP 5. A alta concentração de pigmentos fotossintéticos e menor comprometimento no desempenho fotossintético das plantas inoculadas pulverizadas com AP 1 foi observado com base nos valores de F_v/F_m, Y(II), rendimento da dissipação não fotoquímica (Y(NPQ)), rendimento da dissipação não regulada (Y(NO)) e ETR em comparação com plantas inoculadas não pulverizadas com AP. Em conclusão, os resultados do presente estudo destacam o potencial do Ni em aumentar a resistência do arroz à mancha parda por meio da participação ativa de algumas enzimas de defesa, um sistema antioxidante robusto e um melhor desempenho fotoquímico. Além disso, o potencial das menores concentrações do AP em diminuir a severidade da mancha parda preservando a capacidade fotossintética das plantas infectadas enquanto a maior concentração de AP não favoreceu a infecção por *B. oryzae*. Assim, tanto o Ni quanto o AP apresentam grande potencial para o manejo da mancha parda na produção de arroz.

Palavras-chave: Doença foliar. Fotossíntese. Mancha parda. Metabolismo antioxidante. Micronutriente. Nutrição de plantas. Patógeno necrotrófico. Respostas de defesa do hospedeiro. Toxina não seletiva.

SUMÁRIO

CHAPTER 1

HISTOPATHOLOGICAL, PHYSIOLOGICAL, AND BIOCHEMICAL ASPECTS OF RICE RESISTANCE TO BROWN SPOT POTENTIATED BY NICKEL

ABSTRACT

Brown spot, caused by *Bipolaris oryzae*, has dramatically decreased rice yield worldwide. Considering the importance of nickel (Ni) on plant metabolism, the present study investigated its effect on increasing rice resistance to brown spot. Histopathological changes due to fungal infection, the photosynthetic performance of plants, the activities of defense and antioxidant enzymes, as well as the concentrations of reactive oxygen species, malondialdehyde, phenolics, and lignin were determined. Rice plants (cultivar Metica-1, susceptible to brown spot) were grown in nutrient solution non-amended (0 mM Ni, -Ni plants) or amended (0.1 mM Ni, +Ni plants) with Ni and non-inoculated or inoculated with *B. oryzae*. Foliar Ni concentration significantly increased for +Ni in comparison to -Ni plants regardless of plant inoculation. Brown spot symptoms were less developed, and disease severity was lower for +Ni in contrast to -Ni plants. The foliar tissues of -Ni plants were massively colonized by hyphae of *B. oryzae* in comparison to the leaf tissues of +Ni plants resulting, therefore, in more cellular damage as indicated by the high malondialdehyde concentration. In general, the activities of the defense enzymes chitinase, β-1,3-glucanase, peroxidase, polyphenoloxidase, and phenylalanine ammonia-lyase showed the same pattern between -Ni inoculated and +Ni inoculated plants during the infection process of *B. oryzae*. High peroxidase activity for +Ni inoculated plants occurred at the earlier stage of fungal infection. High lipoxygenase activity for -Ni plants may have contributed to disease development. The photochemical performance of -Ni inoculated plants (lower maximum photochemical efficiency of photosystem II (PSII) (F_v/F_m) values) was much more affected in comparison to +Ni inoculated plants. The values for effective yield of the photosystem II (Y(II)), yield for other non-photochemical (non-regulated) losses, and apparent electron transport rate (ETR) were lower for inoculated plants in comparison to non-inoculated ones regardless of Ni supply. High values for Y(II) and ETR for +Ni plants indicated their efficiency in allocating the absorbed light energy to the photochemical process. The dissipation of energy through non-photochemical processes, as well as the regulated and non-regulated mechanisms of photoprotection, was not affected on the leaves infected by *B. oryzae* regardless of Ni supply. Taken together, the results of this study highlights the potential of Ni to increase rice resistance to brown spot through active participation of some defense enzymes, a

robust antioxidative system, and a better photochemical performance. Therefore, Ni stands out as a key micronutrient that can not be overlooked in a nutritional strategy for brown spot management in rice production.

Keywords: antioxidative metabolism, foliar disease, host defense responses, micronutrient, necrotrophic pathogen, plant nutrition

INTRODUCTION

Brown spot, caused by the necrotrophic fungus *Bipolaris oryzae* (Breda de Haan) Shoemaker (teleomorph *Cochliobolus miyabeanus* (Iot & Kuribayashi) Drechs ex Dastur), is considered one of the most important diseases affecting rice production worldwide (Ou, 1985). On the leaves, disease symptoms start as small dark brown to reddish-brown oval lesions with yellowish halo and a gray center as they expand (Lee, 1992). Coalescent lesions may take up a considerable part of the leaf blade affecting photosynthesis (Dallagnol et al., 2011a). Black or brown spots are often found on grains, which became of poor quality (Mew & Gonzales, 2002). Non-host selective toxins known as ophiobolins A and B are continuously produced by *B. oryzae* during its infection process on rice leaf tissues and are associated with the development of brown spot (Xiao et al., 1991). The use of certified seeds, seeds treatment with fungicides, balanced fertilization, and application of fungicides are the major control strategies for brown spot management (Barnwal et al., 2013). Some heavy metal salts, including a nickel (Ni) salt, have reduced brown spot development on rice seedlings (Giri & Sinha, 1983).

The Ni is the most newfound discovered essential micronutrient for plant growth (Brown et al., 1987). This micronutrient is a component of urease and involved in the functions of some proteins such as superoxide dismutase, glyoxalase, and hydrogenase enzymes (Brown & Bassil, 2011; Fabiano et al., 2015). The Ni uptake by plants is carried out mainly through the roots via passive diffusion and active transport (Chen et al., 2009; Seregin et al., 2006). The transport of Ni from roots to shoots occurs through the transpiration stream via xylem vessels (Neumann et al., 1986) following retranslocation to young parts of the plants via phloem by metal-ligand proteins that can act as intra-cellular chelators binding it in the cytosol or subcellular compartment for transport, translocation, and accumulation (Chen et al., 2009). Information regarding the effect of Ni on plant disease control is scarce in the literature. Based on the few reports regarding the impact of Ni on diseases development caused viruses, bacteria, nematodes, and fungi of different lifestyles, this micronutrient can either have direct toxicity against pathogens or even be involved on the potentiation of host defense responses (Brown, 2007; Wood & Reilly, 2007). Some Ni salts directly affected the germination of urediniospores of some fungi causing rust (Gerendás et al., 1999). The

indirect effect of Ni occurred at low concentrations without causing a phytotoxic effect on plants but resulted on physiological changes mainly on the activities of antioxidant enzymes and nitrogen metabolism during a short period of time (Brown, 2007; Gerendás et al., 1999; López & Magnitskiy, 2011). Barcelos et al. (2018) reported a reduction in powdery mildew severity and great activities of superoxide dismutase, catalase, peroxidase, and urease in soybean plants sprayed with Ni. The Ni was associated with an increase in phytoalexins concentration on plants of *Brassica* spp. infected by *Leptosphaeria maculans* and wheat seedlings infected by *B. sorokiniana* (Chakraborty & Sinha, 1984; Dahiya & Rimmer, 1989; Wood & Reilly, 2007). The resistance of rice seedlings to brown spot was associated with the production of toxic leaf diffusates in plants supplied with Ni during the penetration stage of *B. oryzae* (Giri & Sinha, 1983).

Considering the positive role played by Ni on plant metabolism, the present study aimed to investigate whether this micronutrient could augment inherent rice resistance to brown spot. To this end, histopathological changes, photosynthetic performance, activities of defense-related and antioxidant enzymes, as well as the concentrations of reactive oxygen species, malondialdehyde, phenolics, and lignin were determined to achieve this goal.

MATERIAL AND METHODS

Plant growth and Ni application

Rice seeds from cultivar Metica-1, susceptible to brown spot (Moreira et al., 2013), were surface disinfected in 0.5% (v/v) NaOCl for 3 min, rinsed in current water, and germinated on sand substrate. The 7-day old seedlings were transferred to plastic pots (five plants per plastic pot) containing 5 liters of one-half strength of a nutrient solution prepared according to Hoagland & Arnon (1950) with some modification as follow: 1.0 mM KNO_3, 0.25 mM $NH_4H_2PO_4$, 0.1 mM NH_4Cl, 0.5 mM $MgSO_4$.7H2O, 1.0 mM $Ca(NO_3)_2$, 0.3 µM $CuSO_4.5H_2O$, 0.33 µM $ZnSO_4.7H_2O$, 11.5 µM H_3BO_3, 3.5 µM $MnCl_2.4H_2O$, 0.1 µM $(NH_4)_6Mo_7O_{24}.4H_2O$, 25µM $FeSO_4.7H_2O$, and 25 µM EDTA disodium. The nutrient solution strength was increased over time during the 40 days and changed every four days. The pH was checked daily and maintained at approximately 5.5 by adding NaOH or HCl (1 M) solutions as needed. Plants at the V_5 growth stage (Counce et al., 2000) were supplied with 0.1 mM of Ni ($NiSO_4$) three days before being inoculated with *B. oryzae*. Plants non-supplied with Ni served as the control treatment.

Inoculum production and plant inoculation with *B. oryzae*

Plants were inoculated with *B. oryzae* following the procedures of Dallagnol et al. (2011b). The isolate UFV/DFP *Bo*-01 of *B. oryzae* was preserved on filter paper in glass vials with silica gel at 4°C. Pieces of filter paper containing the fungus were transferred to Petri dishes with potato-dextrose-agar (PDA) medium. After 3 days, PDA plugs containing fungal mycelia were transferred to new Petri dishes containing oat-agar medium. The Petri dishes were kept in a growth chamber at 25°C with 12 h photoperiod for 10 days. After this period, conidia were carefully removed from the dishes with a soft bristle brush using water containing gelatin 1% (w/v). The conidial suspension was calibrated using a hemacytometer to obtain a concentration of 5×10^3 conidia mL^{-1}. The adaxial surface of the leaves was sprayed with the conidial suspension until runoff using a VL Airbrush atomizer (Paasche Airbrush Co.). Immediately after inoculation, plants were transferred to a mist chamber (temperature of 25 ± 2°C and relative humidity of 90 ± 5%) for an initial 24 h dark period. After this period, plants were transferred to a growth chamber with 12 h photoperiod of photon irradiance of 350 µmol m^{-2} s^{-1} provided by cool-white fluorescent lamps. Plants were kept inside the mist chamber for

the duration of the experiments. The non-inoculated plants were kept in a separate growth chamber but exposed to the same environmental conditions.

Assessment of brown spot severity

The third and fourth leaves, from the main tiller of each plant, were marked and used to evaluate brown spot severity at 48, 72, and 96 hours after inoculation (hai) using a scale based on the percentage of diseased leaf area (IRRI, 1996).

Microscopical observations

Processing infected leaf tissues for light microscopy

A total of 30 leaf fragments (≈ 25 mm^2) were collected from the third leaf of each plant per replication of each treatment at 48 and 96 hai. Leaf fragments from non-inoculated plants were also sampled and served as the control treatment. Leaf fragments were carefully transferred to glass vials containing 10 mL of a fixative composed of 3% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.1 mol L^{-1} sodium cacodylate buffer (pH 7.2). Vials were covered with aluminum foil and stored at 4°C until being processed for microscopical observations. Leaf fragments were removed from the fixative and washed with sodium cacodylate buffer (0.1 mol L^{-1}), subsequently dehydrated through a graded alcohol series, and embedded in methacrylate resin (Historesin, Leica Microsystems®, Nussloch/Heidelberg, Germany). The leaf fragments were placed in a vacuum chamber for 2 h during the pre-infiltration and infiltration steps twice a day for 3 weeks for a better resin infiltration into the leaf fragments (Araujo et al., 2016). The fragments were stored at 4°C after each procedure. A total of six blocks of resin, each one containing two leaf fragments, were obtained for each treatment at each sampling time. A total of 36 transversal series sections (4 µm thick), which were cut from each block using a Leica RM 2245 rotary microtome (Leica Microsystems®), were randomly divided and placed on three glass slides and stained with 0.5% toluidine blue in 2% sodium borate for 2 min. Toluidine blue is a metachromatic dye commonly used for staining plant tissue sections. When the specimen samples stained with toluidine blue are viewed under the light microscope, distinct different cell components produce different colors: DNA is bluish-green; RNA is violet; the middle lamella is red; non-lignified cell walls and soluble phenolics are red-violet, blue-violet, blue or purple; and polymerized phenolics such as lignin become

green or bluish-green (Vermerris & Nicholson, 2006). The images of the details regarding fungal infection and host defense responses were acquired digitally (Axio Cam HR, Carl Zeiss, Jena, Thuringia, Germany) using a Carl Zeiss Axio Imager A1 microscope (Carl Zeiss, Germany) in the bright-field mode and further processed using AXION VISION v.4.8.1 software.

Processing infected leaf tissues for fluorescence microscopy

Hyphae of *B. oryzae* were stained using the chitin-specific dye wheat germ agglutinin-alexa fluor conjugate (WGA-AF) 488 (Molecular Probes, Karlsruhe, Germany) following the procedures of Doehlemann et al. (2007) with a few modifications. A total of 50 leaf fragments obtained from the third leaf of each plant per replication of each treatment were collected at 48 and 96 hai. Leaf fragments from non-inoculated plants were also sampled and served as the control treatment. Leaf fragments were placed in plastic tubes containing 50 mL of potassium phosphate buffer (pH 7.2), covered with aluminum foil, and stored at -20°C until further analysis. Leaf fragments were destained with ethanol for at least 48 h. A total of 30 handmade transverse sections (40 to 60 µm thick) were obtained from both non-inoculated and inoculated leaf fragments using a scalpel blade at each sample time. Sections were incubated in staining solution (10 µg mL^{-1} WGA-AF 488 prepared with 0.02% Tween 20 in 1 × phosphate-buffered saline (PBS) (137 mM NaCl, 27 mM KCl, 100 mM Na_2HPO_4, and 2 mM K_2HPO_4 adjusted to pH 7.4) for 30 min. During this period, the solution was vacuum infiltrated three times for 1 min at 25 mm Hg. After rinsing in 1 × PBS (pH 7.4), leaf fragments were mounted on glass slides. Fluorescence images were recorded on a Carl Zeiss Axio Imager A1 microscope using the Zeiss filter set 9 (Alexa; 450 to 490 nm excitation, 510 nm beam splitter, and 515 nm emission). All digital images were acquired with an AxioCam HRc Rev. 2 and further processed with the AXION VISION v.4.8.1 software.

Determination of chlorophyll (Chl) *a* fluorescence

The fourth leaf of each plant per replication of each treatment were collected at 24, 48, 72, and 96 hai to obtain the images of the parameters of Chl *a* fluorescence using the MAXI version of the Imaging-PAM chlorophyll fluorometer and the Imaging Win software (Heinz Walz GmbH, Effeltrich, Germany) according to Tatagiba et al. (2016). Leaves from non-inoculated plants were sampled at these evaluation times to serve as

the control treatment. Leaves were individually fixed in the holder at a distance of 18.5 cm from the recording camera CCD ('charge-coupled device') coupled to the fluorescence device and then exposed to a weak, modulated measuring beam (0.5 $\mu molm^{-2}$ s^{-1}, 100 μs, 1 Hz) to determine the initial fluorescence (F_0) when all the PS II reaction centers were 'open'. Next, a saturating white light pulse of 2400 μmol m^{-2} s^{-1} (10 Hz) was applied for 0.8 s to ensure the maximum fluorescence emission (F_m) when all the PS II reaction centers were 'closed'. Based on these initial measurements, the maximum PS II photochemical efficiency of the dark-adapted leaves was estimated through the variable-to-maximum Chl fluorescence ratio, $F_v/F_m = [(F_m - F_0)/F_m)]$. The leaves were subsequently exposed to actinic photon irradiance (185 μmol m^{-2} s^{-1}) for 300 s to obtain the steady-state fluorescence yield (F_s), after which a saturating white light pulse (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 & Baker (1997). Following the calculations of Kramer et al. (2004), the energy absorbed by PS II for the following three yield components for dissipative processes was determined: the yield of photochemistry [$Y(II) = (F_m' - F_s)/F_m')$], the yield for dissipation by down-regulation [$Y(NPQ) = (F_s/F_m') - (F_s/F_m)$], and the yield for other non-photochemical (non-regulated) losses [$Y(NO) = F_s/F_m$]. The apparent electron transport rate was calculated as $ETR = Y(II) \times PPFD \times f \times \alpha$ according to Baker (2008). The rectangle option on the Imaging Win software was used to determine the parameters of Chl *a* fluorescence.

Additionally, leaves sampled at 96 hai were used to obtain the F_v/F_m values using the transect function of the Imaging Win software (Honorato Júnior et al., 2015). A total of 60 lesions (transect of 4 mm for each lesion) were used per replication of each treatment to obtain the F_v/F_m values. The pixel values were adjusted to represent the distance of 4 mm in the true leaf image. The standard error was calculated for each point, and the line graph was drawn for each treatment.

Biochemical assays

The third and fourth leaves of each plant per replication of each treatment were collected at 12, 24, 48, and 96 hai using liquid nitrogen and stored at -80°C until further analysis. Leaves from non-inoculated plants were sampled at these same evaluation times to serve as the control treatment.

Determination of the activities of antioxidant enzymes

The activities of ascorbate peroxidase (APX) (EC 1.11.1.11), catalase (CAT) (EC 1.11.1.6), glutathione reductase (GR) (EC 1.8.1.7), and superoxide dismutase (SOD) (EC 1.15.1.1) were determined using a total of 0.2 g of leaf tissue. Frozen leaf tissue was ground into a fine powder in a mixer mill MM 400 (Retsch GmbH/Haan, Germany). The fine powder was homogenized in a 2 mL solution containing 100 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, and 1 mM phenylmethylsulphonyl fluoride (PMSF). The homogenized material was centrifuged at 13,000 *g* at 4°C for 15 min, and the supernatant was used to determine enzymes activities. The APX activity was determined following the procedures of Nakano & Asada (1981) with a few modifications. A total of 2 µL of the crude enzyme extract was added to 248 µL of a mixture containing 100 mM phosphate buffer (pH 7.0), 0.1 mM H_2O_2, and 0.5 mM ascorbic acid. The rate of ascorbate oxidation was measured by recording the absorbance at 290 nm for 1 min. The CAT activity was determined according to Azevedo et al. (2002) with some modifications. The reaction mixture was composed of 3 µL of the crude enzyme extract and 247 µL of a mixture containing 100 mM potassium phosphate buffer (pH 7.0) and 12.5 mM hydrogen peroxide (H_2O_2). The absorbance was recorded at 240 nm for 1 min. The GR activity was assayed by determining the NADPH oxidation as proposed by Cakmak et al. (1993). The reaction was started after the addition of 30 µL of the crude extract to 220 µL of a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.8), 1 mM oxidized glutathione (GSSG), and 0.075 mM NADPH. The absorbance was recorded at 340 nm for 2 min. The SOD activity assay was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) (Giannopolitis & Ries, 1977) by adding 12.5 µL of the crude enzyme extract to 237.5 µL of a mixtures containing 50 mM potassium phosphate buffer (pH 7.8), 14 mM methionine, 75 µM NBT, 0.1 µM EDTA, and 2 µM riboflavin. Samples were light-exposed for 10 min and the production of formazan blue, resulting from the photoreduction of NBT, was measured at 560 nm. Samples kept in the dark for 7 min served as a blank. One unit of SOD was defined as the amount of enzyme necessary to inhibit NBT photoreduction by 50%. Enzyme activities were expressed in a protein basis whose concentration was determined according to the method of Bradford (1976).

Determination of the activities of defense-related enzymes

To determine the activities of chitinase (CHI) (EC 3.2.1.14), *β*-1,3-glucanase (GLU) (EC 3.2.1.39), peroxidase (POX) (EC 1.11.1.7), polyphenoloxidase (PPO), lipoxygenase (LOX) (EC 1.13.11.12), and phenylalanine ammonia-lyase (PAL) (EC 4.3.1.5), a total of 0.2 g of leaf tissue was ground into a fine powder in a mixer mill MM 400 (Retsch GmbH/Haan, Germany). The fine powder was homogenized in 2 mL of a solution containing 50 mM potassium phosphate buffer (pH 6.8), 1 mM EDTA, and 1 mM PMSF. The homogenate was centrifuged at 13,000 *g* for 15 min at 4°C, and the supernatant was collected to be used for enzymes activities assays. The GLU activity was determined according to Lever (1972). The reaction mixture composed of 5 µL of the crude enzyme extract, 50 mM sodium acetate buffer (pH 5.0), and laminarin (1 mg mL^{-1}) was incubated in a water bath at 45°C for 1 h. Afterward, 125 µL of this mixture was added to a reaction mixture of dinitrosalicylic acid (DNS). This reaction mixture was then incubated in a water bath for 8 min at 100°C and then cooled in an ice bath until it reached 30°C. The absorbance was measured at 540 nm. A similar procedure was used for the control samples except that the first incubation was excluded. The CHI activity was determined by adding 5 µL of the crude enzyme extract to a reaction mixture containing 50 mM sodium acetate buffer (pH 5.0) and 0.1 mM *p*-nitrophenyl-β-*D*-*N*-*N*'-diacetylchitobiose. The reaction mixture was incubated in a water bath at 37°C for 2 h and the reaction was terminated by adding 500 µL of 0.2 M sodium carbonate. For the control samples, the sodium carbonate was added soon after the addition of the crude enzyme extract to the reaction mixtures (Harman et al., 1998; Yedidia et al., 1999). The absorbance of the product released by CHI was measured at 410 nm. For POX, the pyrogallol oxidation was determined at 420 nm for 3 min in a reaction mixture containing 2 µL of the crude enzyme extract, 25 mM potassium phosphate (pH 6.8), 20 mM pyrogallol, and 20 mM H_2O_2 (Chance & Maehly, 1955; Kar & Mishra, 1976). The PPO activity was determined using the same procedure described for POX, but H_2O_2 was omitted from the reaction mixture. The LOX activity assay was determined by measuring the pyrogallol oxidation as described by Axelrod et al. (1981) by adding 10 µL of the crude enzyme extract to a reaction mixture containing 50 mM sodium phosphate buffer (pH 6.5) and 50 µM sodium linoleate. The reaction mixture was incubated at 25°C, and the absorbance of the product released by LOX was measured at

234 nm for 3 min. The PAL activity was determined according to Guo et al. (2007). The reaction mixture was composed of 140 µL of crude enzyme extract and 150 µL of a reaction mixture containing 40 mM sodium borate buffer (pH 8.8) and 25 mM L-phenylalanine. The reaction mixture was incubated at 40°C for 3 h. For the control samples, the extract was replaced by borate buffer. The reaction was stopped by adding 200 µL of 6 N HCl. The absorbance of the *trans*-cinnamic acid derivatives was recorded at 290 nm.

Determination of concentration of malondialdehyde (MDA)

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 & Horst, 1991). A total of 0.1 g of leaf tissue was ground into a fine powder using a mixer mill MM 400 (Retsch GmbH/Haan, Germany). The fine powder was homogenized in 2 mL of 0.1% (w/v) trichloroacetic acid (TCA) solution in an ice bath, and the homogenate was centrifuged at 12,000 *g* for 15 min at 4°C. After that, 250 µL of the supernatant was added to 750 µL of TBA solution (0.5% in 20% TCA) and kept for 30 min in a boiling water bath at 95°C. Then, the reaction was stopped in an ice bath. The samples were centrifuged at 10,000 *g* for 10 min at 4°C, and the absorbance of the supernatant was read at 532 nm discounting the non-specific absorbance at 600 nm (Hodges et al., 1999).

Determination of concentration of superoxide (O_2^-)

A total of 0.2 g of leaf tissue was ground into a fine powder in a mixer mill MM 400 (Retsch GmbH/Haan, Germany). 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 4°C. After centrifugation, 100 µL 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 & Naithani, 1994).

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

A total of 0.1 g of leaf tissue was ground into a fine powder in a mixer mill MM 400 (Retsch GmbH/Haan, Germany) and homogenized in 1 mL of 80% (v/v) methanol solution. The crude extract was shaken at 300 rpm at 25°C for 12 h, and the mixture was centrifuged at 13,000 *g* for 30 min. The TSP concentration was determined in the methanolic extract, and the pellet was kept at 20°C to determine the LTGA derivatives concentration according to Fortunato et al. (2015).

Histochemical localization of H_2O_2

A total of 15 leaf fragments of the third and fourth leaves (10 cm of length) of each plant per replication of each treatment were collected at 96 hai. Leaves from non-inoculated plants collected at this same evaluation time served as control. Leaf fragments were placed in glass vials containing a solution of 3,3'-diaminobenzidine tetrahydrochloride (1mg mL^{-1}) (Sigma-Aldrich, São Paulo, Brazil) and kept in the dark at 25°C for 12 h. After this period, leaf fragments were cleared in boiling aqueous ethanol (80%) for 1 h and then stored in glycerol solution (70%) (Aucique-Pérez et al., 2019).

Determination of foliar Ni concentration

The third and fourth leaves of each plant per replication of each treatment used to evaluate brown spot severity were collected at the end of the experiment. The leaves were dried for 72 h at 65°C and ground to pass through a 40-mesh screen. The foliar Ni concentration was determined by inductively coupled plasma optical emission spectrometry analysis of 0.5 g of dried and nitric perchloric acid digestion (Sarruge & Haag, 1974).

Experimental design and statistical analysis

A 2 × 2 factorial experiment, consisting of two Ni concentrations (0 and 0.1 mM; hereafter referred to as -Ni and +Ni plants, respectively) and non-inoculated or inoculated plants, was arranged in a completely randomized design with five replications. Each replication consisted of a plastic pot with five plants. The experiment was repeated once. Data from all the variables and parameters evaluated were subjected

to analysis of variance (ANOVA) and treatments means were compared by *F* test ($P \leq 0.05$) using the Minitab software (version 18; Minitab Corporation). For brown spot severity, ANOVA was considered to be a 2 × 2 × 3 factorial experiment consisting of two Ni concentrations, non-inoculated or inoculated plants, and three sampling times. For the Chl *a* parameters, enzymes activities as well as H_2O_2, O_2, MDA, TSP and LTGA derivatives concentrations, ANOVA was considered to be a 2 × 2 × 4 factorial experiment consisting of two Ni concentrations, non-inoculated or inoculated plants, and four sampling times.

RESULTS

Foliar Ni concentration

The foliar concentration of Ni significantly increased by 94% for +Ni in comparison to -Ni on non-inoculated and inoculated plants (Fig. 1).

Brown spot development

At 96 hai, circular to oval dark brown lesions with yellowish halos surrounding them were noticed on the leaves of -Ni plants in contrast to small brown necrotic lesions that were formed on the leaves of +Ni plants (Fig. 2A). For +Ni plants, brown spot severity significantly decreased by 22, 26, and 38% at 48, 72, and 96 hai, respectively, in comparison to -Ni plants (Fig. 2B).

Microscopical observations

The bulliform, epidermal, and mesophyll cells as well as the bundle sheath and vascular bundles on the leaf tissues of -Ni plants were massively colonized by hyphae of *B. oryzae* (Figs. 3 and 4). Fungal hyphae were easily noticed in the deformed mesophyll cells on the leaf tissues of -Ni plants in comparison to the leaf tissues from +Ni plants at 48 hai (Fig. 3C-F). At 96 hai, the bulliform and mesophyll cells at the necrotic leaf tissues of -Ni plants showed extensive disorganization due to the intense colonization by fungal hyphae (Fig. 4 A, C, and E), while the integrity of the leaf tissues from +Ni plants was preserved in large extension (Fig. 4 B, D, and F). Even though fungal hyphae were found on the different tissues on the leaves of +Ni plants, the colonization was much more reduced in contrast to the leaf tissues of -Ni plants, particularly at 96 hai (Fig. 4). The abundant presence of fungal hyphae at the necrotic leaf tissues of -Ni plants was noticed by using the chitin-specific dye WGA-AF 488 in contrast to what was observed in the leaf tissues of +Ni plants at both 48 and 96 hai (Figs. 3G-J and 4G-J).

Chl *a* fluorescence parameters

The visual changes in the images for F_v/F_m, Y(II), Y(NPQ), and Y(NO) parameters were noticeable on the leaves of inoculated plants at 48 hai regardless of Ni supply (Fig.

5). Changes in the images of these parameters occurred as the lesions expanded on the leaves of -Ni plants, especially at 96 hai (Fig. 5). By contrast, on the leaves of +Ni plants the variations were less noticeable in the images for F_v/F_m, Y(II), Y(NPQ), and Y(NO) (Fig. 5). According to the semi-quantitative analysis of the Chl *a* fluorescence parameters, there was no significant difference for F_v/F_m and Y(NO) between non-inoculated -Ni and +Ni plants regardless of the sampling time (Fig. 6A and G). For non-inoculated plants, Y(II) and ETR significantly decreased, respectively, by 23 and 25% while Y(NPQ) increased by 22% at 24 hai for +Ni plants in comparison to -Ni plants (Fig. 6C, E, and I). For inoculated plants, there were significant increases, respectively, of 5, 7, and 8% at 48, 72, and, 96 hai for F_v/F_m, of 26% at 24 hai for Y(II), and of 27% at 24 hai for ETR in comparison to -Ni plants (Fig. 6B, D, and J). There was no significant difference between inoculated -Ni and +Ni plants for Y(NPQ) and Y(NO) regardless of the sampling time (Fig. 6F and H). For inoculated -Ni plants, there were significant decreases, respectively, of 8 and 9% at 48 and 72 hai for F_v/F_m, of 51, 59, and 34% at 24, 48, and 72 hai for Y(II), and of 51, 40, 38, and 34% at 24, 48, 72, and 96 hai for ETR in comparison to non-inoculated -Ni plants (Fig. 6A-D, I, and J). For inoculated -Ni plants, there were significant increases for Y(NPQ) by 41% at 24 hai and for Y(NO) by 26, 25, and 22% at 48, 72, and 96 hai, respectively, in comparison to their non-inoculated counterparts (Fig. 6E-H). There were significant decreases, respectively, for F_v/F_m by 3, 1, and 3% at 48, 72, and 96 hai, for Y(II) by 31 and 28% at 48 and 72 hai, and ETR by 31 and 29% at 48 and 72 hai for inoculated +Ni plants in comparison to non-inoculated +Ni plants (Fig. 6A-D, I, and J). Y(NO) significantly increased by 16, 18, and 13% at 48, 72, and 96 hai, respectively, for inoculated +Ni plants in comparison to non-inoculated +Ni plants (Fig. 6G-H). Values for F_v/F_m at the center of the brown spot lesions were lower for -Ni plants (0.40 ± 0.097) in comparison to those obtained for +Ni plants (0.75 ± 0.020) (Fig. 7).

Activities of antioxidant enzymes

SOD activity significantly decreased for inoculated +Ni plants by 26% at 96 hai in comparison to inoculated -Ni plants (Fig. 8B). For inoculated -Ni plants, there were significant increases, respectively, of 36, 31, 30, 57% for SOD at 12, 24, 48, and 96 hai, of 43, 28, 47% for GR at 12, 48, and 96 hai, and for APX of 22, 25, and 28% at 24, 48, and 96 hai in comparison to non-inoculated -Ni plants (Fig. 8A-F). For +Ni plants, there

were significant increases, respectively, of 34, 33, 35, and 42% at 12, 24, 48, and 96 hai for SOD, of 23, 20, and 41% at 24, 48, and 96 hai for APX, and of 25 and 41% at 48 and 96 hai for GR in comparison to their control counterparts (Fig. 8A-F).

Activities of defense-related enzymes

Activities of GLU and PAL significantly increased for non-inoculated +Ni plants by 64% at 96 hai and by 59 and 58% at 12 and 96 hai, respectively, in comparison to non-inoculated -Ni plants (Fig. 9A and K). For inoculated plants, there were significant increases for GLU activity of 41% at 24 hai and for POX activity of 19% at 12 hai and significantly decreases for CHI and PPO of 38 and 24% at 96h, respectively, for +Ni plants in comparison to -Ni plants (Fig. 9B, D, F, and H). LOX activity significantly decreased by 40% at 12 hai and increased significantly by 40% at 96 hai for inoculated +Ni plants in comparison to inoculated -Ni plants (Fig. 9J). For inoculated -Ni plants, there were significantly increases, respectively, of 90, 92, and 98% at 24, 48, and 96 hai for GLU, of 37 and 82% at 48 and 96 hai for CHI, of 25 and 64% at 48 and 96 hai for PPO, and of 37, 90, and 87% at 12, 48, and 96 hai for LOX in comparison to non-inoculated -Ni plants (Fig. 9A-F, I, and J). POX activity significantly increased by 30, 60, 77, and 86% while PAL increased by 80, 73, 86, and 73%, respectively at 12, 24, 48, and 96 hai for -Ni inoculated plants in comparison to -Ni non-inoculated plants (Fig. 9G-H, K, and L). For inoculated +Ni plants, there were significant increases of 38 and 74% for CHI, of 30 and 52% for PPO at 48 and 96 hai, of 80, 93, and 91% for LOX, and of 55, 71, and 54% for PAL at 24, 48, and 96 hai, respectively (Fig. 9C-F and I-L). There were significantly increases of 75, 92, 91, and 93% for GLU activity and 94, 61, 78, and 83% for POX activity at 12, 24, 48, and 96 hai, respectively for +Ni inoculated plants in comparison to their non-inoculated counterparts (Fig. 9A-B, G and H).

Concentrations of MDA, O_2^-, TSP, and LTGA derivatives

The MDA concentration significantly increased for inoculated -Ni and +Ni plants by 76 and 53%, respectively, at 96 hai in comparison to their non-inoculated counterparts (Fig. 10A and B). For inoculated plants, MDA concentration significantly decreased by 39% at 96 hai for +Ni plants in comparison to -Ni plants (Fig. 10B). TSP concentration increased significantly by 44, 45, and 29% at 12, 24, and 48 hai, respectively, for non-inoculated +Ni plants in comparison to non-inoculated -Ni plants (Fig. 12A). There was

no significant different between -Ni and +Ni plants for O_2^- and LTGA derivatives regardless of fungal inoculation and sampling time (Figs. 10C-D and 12C-D).

DISCUSSION

The present study brings novel insights regarding the effect of Ni on the potentiation of rice resistance in response to *B. oryzae* infection at the histopathological, physiological, and biochemical levels. Besides its role as a component of important plant enzymes, this micronutrient is also involved in the resistance of plants against some pathogens' infection by having either a direct effect against them or on the activation of some mechanisms of host defense (Brown, 2007). Previous studies demonstrated a reduction in the incidence of some important rice diseases such as blast and brown spot using low concentrations of Ni salts (Giri & Sinha, 1983; Kamalakannan et al., 2001). Despite the knowledge of Ni's involvement in plant diseases control, the mechanisms by which this micronutrient operates on plants are still poorly understood and deserve further investigation.

The higher foliar Ni concentration gained by suppling this micronutrient to the rice roots reduced brown spot severity. The symptoms of brown spot in the leaves of +Ni plants were reduced to small necrotic spots, whereas typical circular dark brown lesions surrounded by yellow halos were observed in the leaves of -Ni plants. The histopathological investigation revealed that hyphae of *B. oryzae* colonized the bulliform, epidermal, and mesophyll cells in addition to the bundle sheath and vascular bundles. Although the vessel bundles are prone to form a physical barrier to avoid the lateral colonization of rice leaf tissues by hyphae of *B. oryzae* (Tullis, 1935), numerous hyphae were observed in the leaf tissues of -Ni plants at advanced stages of fungal infection. Following other necrotrophic fungi, *B. oryzae* also produces non-host selective toxins (known as ophiobolins A and B) that contribute to its aggressiveness during the colonization of rice leaf tissues (Xiao et al., 1991). The leaf tissues of -Ni plants showed intense disorganization due to the massive presence of fungal hyphae in comparison to the leaf tissues of +Ni plants. Furthermore, the MDA concentration for -Ni plants was high at 96 hai, indicating great cellular damage caused by *B. oryzae* infection. According to Dallagnol et al. (2011a), an increase in MDA concentration was owing to the toxins produced by *B. oryzae* on rice leaves.

The CHI and GLU are host defense-related enzymes involved in the hydrolyze of chitin and β-1,3-glucan, respectively, and directly restrict fungal colonization on host tissues (Shetty et al., 2009). Moreover, the oligomers of chitin and β-1,3-glucan released

from the hydrolysis of fungal cell walls may act as elicitors of host defense reactions (Jia & Martin, 1999). The activities of both CHI and GLU were enhanced in the leaves of rice plants in response to *B. oryzae* infection regardless of Ni supply. This finding is in agreement with what was reported by other studies with the rice-*B. oryzae* interaction (Dallagnol et al., 2011a; Debona et al., 2018). While the high CHI activity for -Ni plant at 96 hai was not sufficient to slow down the colonization of the leaf tissues by *B. oryzae*, GLU activity was higher for +Ni plants at earlier stages of fungal infection and also for +Ni non-inoculated plants. Interestingly, based on proteomic analysis, the GLU was one of the defense-related proteins accumulated in rice leaves infected by *B. oryzae* suggesting its importance for rice resistance against brown spot (Kim et al., 2014). The activities of LOX, PAL, POX, and PPO increased in the leaf tissues infected by *B. oryzae* regardless of Ni supply. Ni plays a role in the shikimic acid pathway, which is responsible for the biosynthesis of the aromatic amino acids phenylalanine, tryptophan, and tyrosine (Kutman et al., 2013). The PAL catalyzes the conversion of phenylalanine to *trans*-cinnamic acid and ammonia, which is the first step in the biosynthesis of phenolics (MacDonald & D'Cunha, 2007). The PAL activity was high for non-inoculated +Ni plants at 12 and 96 hai while for inoculated plants such response was not observed, suggesting, therefore, less participation of this enzyme on rice resistance potentiated by Ni. Accordingly, the concentration of shikimic acid remained constant on the leaves of soybean plants sprayed with Ni during the infection process of *Microsphaera diffusa* (Barcelos et al., 2018). However, high POX activity for +Ni inoculated plants at 12 hai may have played a role in the reduction of fungal colonization on rice leaf tissues. The POX is a key enzyme in the production of lignin due to its participation in the polymerization of phenolics that may increase the physical resistance of plant cell walls (Jwa et al., 2005). In the present study, high POX activity was not linked to the great concentration of LTGA derivatives suggesting that for +Ni plants in particular, POX may have played a more prominent role in rice resistance to brown spot due to its antioxidant activity than acting on the polymerization of phenolics. For inoculated plants, LOX activity decreased in the absence of Ni at 12 hai but increased for +Ni plants at 96 hai. The LOX catalyzes the conversion of polyunsaturated fatty acids into conjugated unsaturated hydroperoxy fatty acids and the oxygenated fatty acid derivatives and may participate in the response of plants against pathogens infection (Kyoungwon et al., 2011). The oxylipins produced by LOX activity are involved in the jasmonic acid (JA) synthesis, which is an important plant hormone

involved in induced systemic resistance of plants to counteract the infection of some pathogens particularly those of a necrotrophic lifestyle (Pieterse et al., 2012). The high LOX activity for -Ni plants may have contributed to the development of brown spot symptoms. It is known that high LOX activity can damage the cell membranes by increasing their permeability and the leakage of cell contents (Brash, 1999). High LOX activity was linked to an increase in white mold severity on the leaflets of common bean plants (Fagundes-Nacarath et al., 2018). On the other hand, high LOX activity may have limited the colonization of leaf tissues of +Ni plants by *B. oryzae*. In contrast, even though the colonization of rice leaf tissues by *B. oryzae* was linked to great LOX activity, exogenous JA application was unable to reduce brown spot severity (Ahn et al., 2005; De Valeesschauwer et al., 2010).

The colonization of rice leaf tissues by *B. oryzae* negatively affected their photochemical performance as indicated by the lower F_v/F_m values resulting, therefore, in the photoinhibition of photosynthesis. The values for Y(II), Y(NO), and ETR were lower for infected plants in comparison to those obtained from the non-inoculated plants regardless of Ni supply. Horino & Akai (1970) reported changes occurring in the chloroplasts on the cells of rice leaves infected by *B. oryzae,* which became swelled and of abnormal shape with a disintegrated double membrane of the envelope. In addition to the ultrastructural changes noticed in the chloroplasts, *B. oryzae* infection reduced the concentration of chloroplastic pigments affecting the photosynthesis process by impairing light capture ability and decreasing the mesophyll capacity to fix CO_2 (Dallagnol et al., 2011b). In the present study, the F_v/F_m values were less impacted on the leaf tissues of +Ni plants in comparison to -Ni plants during the infection process of *B. oryzae*, suggesting the positive effect of this micronutrient to attenuate the photooxidative damage. In addition, the high values of Y(II) and ETR for +Ni plants also indicated a better efficiency to allocate the absorbed light energy to the photochemical process on their leaves. The dissipation of energy through non-photochemical processes as well as the regulated and non-regulated mechanisms of photoprotection based on the Y(NPQ) and Y(NO) values was not affected on the leaves infected by *B. oryzae* regardless of Ni supply.

The production of reactive oxygen species (ROS) is one of the earliest events occurring in the plant tissues in response to pathogens infection. ROS are involved in signaling events but are also cytotoxic and highly reactive, therefore, plants require different mechanisms for ROS scavenging either by modulating the low levels of ROS

for signaling purposes or by detoxifying the excess of ROS during the stress events such as pathogens attack (Mittler, 2002). Among the endogenous plant systems to protect the cells from the deleterious effect of ROS, the action of the enzymes SOD, APX, POX, GR, and CAT are of detrimental importance (Hao et al., 2011). SOD is the primary scavenger, which is responsible to converts superoxide to H_2O_2 and O_2, while CAT, POX, and APX detoxify the H_2O_2 produced spontaneously or by SOD activity (Lee et al., 2001). In the present study, *B. oryzae* infection was associated with an increase in SOD, APX, and GR activities regardless of Ni supply. Interestingly, Ni supply did not affect the activities of the antioxidant enzymes studied regardless of plant infection by *B. oryzae*. For being not a redox-active metal, Ni is not involved in ROS production, but its toxicity to the plant metabolism has been associated with oxidative stress and ROS production (Chen et al., 2009; Shahzad et al., 2018). Some studies showed that plants exposed to low concentrations of Ni showed increases on SOD, POX, GR, and guaiacol peroxidase (GOPX) activities to enhance the removal of ROS while high concentration of ROS reduced the activities of antioxidant enzymes resulting in ROS accumulation and an extreme oxidative stress (Chen et al., 2009). The exposure of wheat plants to Ni for up to 9 days increased H_2O_2 concentration and decreased SOD and CAT activities, whereas the activities of APX and POX increased during this same period of time (Gajewska & Sklodowska, 2007). The activities of antioxidant enzymes may vary with the environment, plant species, cultivar, and part of the plant exposed to any condition of stress (Anjum et al., 2017). Such changes in antioxidant enzymes activities and ROS concentration were not observed in the present study due to Ni supply suggesting that rice plants were not under Ni stress and also that these enzymes did not seem to play a role in rice resistance to brown spot modulated by Ni.

In conclusion, rice plants supplied with Ni showed less brown spot symptoms and less cellular damage due to the constraint on fungal colonization. The higher resistance of the rice plants was regulated mostly by the defense enzymes. Besides, Ni supply also ensured a better photochemical performance of the rice plants during *B. oryzae* infection. Taken together, the results from this study present novel evidence of Ni potential as a nutritional strategy to manage brown spot in rice through the potentiation of defense mechanisms.

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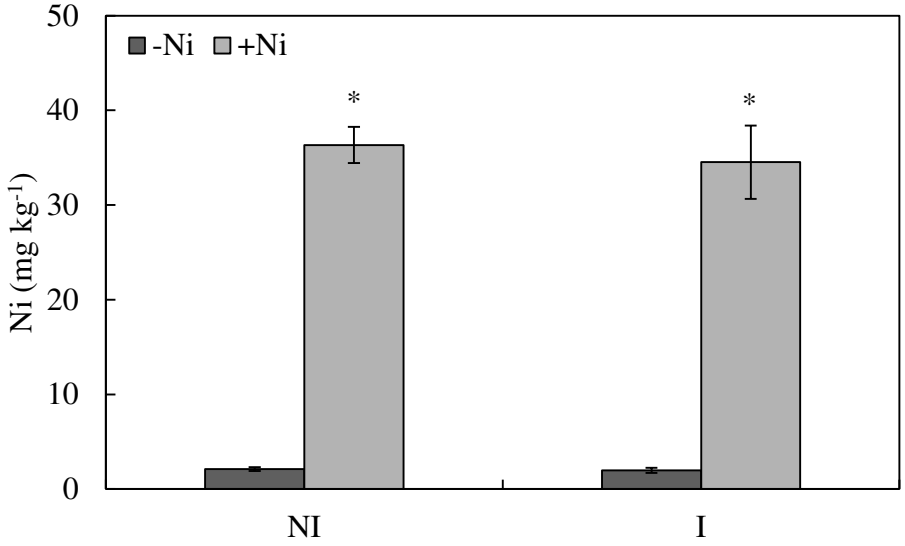
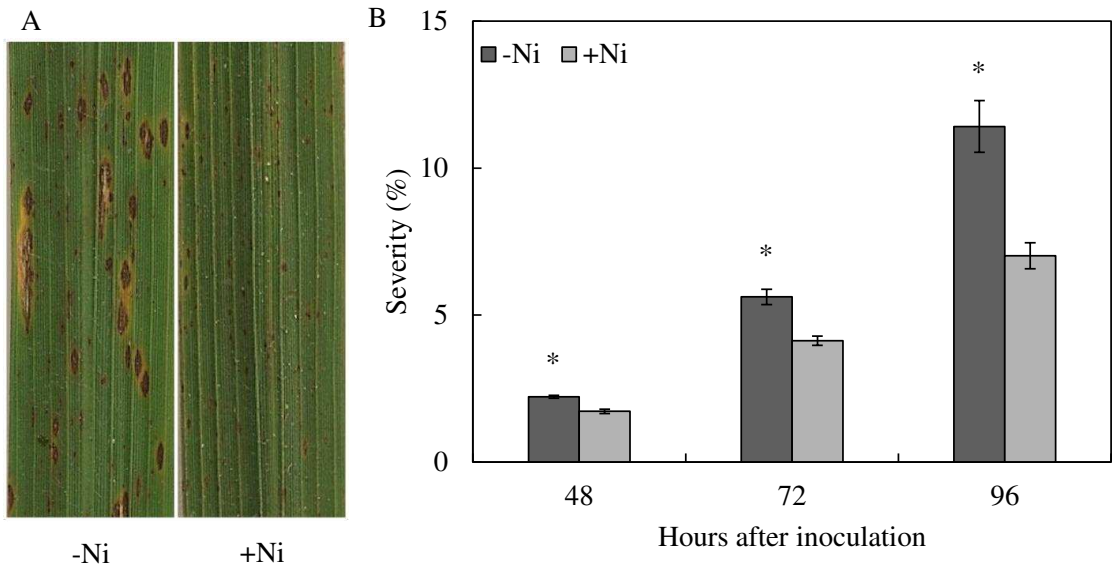


Figure 1. Foliar nickel (Ni) concentration for rice plants supplied with 0 (-Ni) or with 0.1 mM (+Ni) nickel (Ni) and non-inoculated (NI) or inoculated (I) with *Bipolaris oryzae*. Means for -Ni and +Ni treatments followed by an asterisk (*) are significantly different ($P \leq 0.05$) according to *F* test. Bars represent the standard error of the means. $n = 6$.



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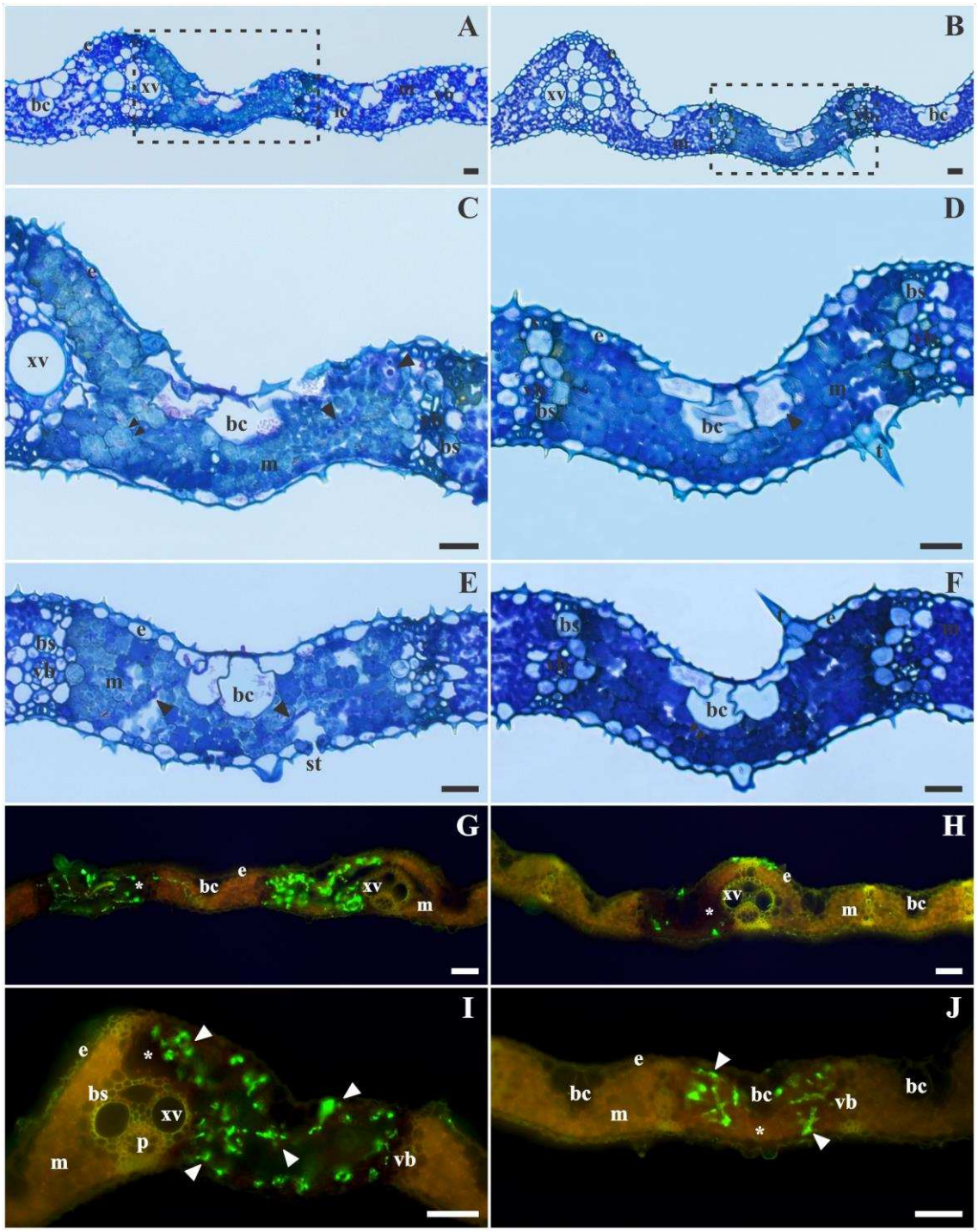

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hyphae that were stained with WGA-AF 488 at the necrotic leaf tissue (asterisks). Many fungal hyphae were observed in the leaf tissues of -Ni plants. Bulliform cell (bc), bundle sheath (bs), epidermis (e), intercellular space (ic), mesophyll cells (m), phloem (p), sclerenchyma (s), stomata (st), trichome (t), vascular bundle (vb), and xylem vessels (xv). Scale bars: 20 µm (A-F) and 50 µm (G-J).

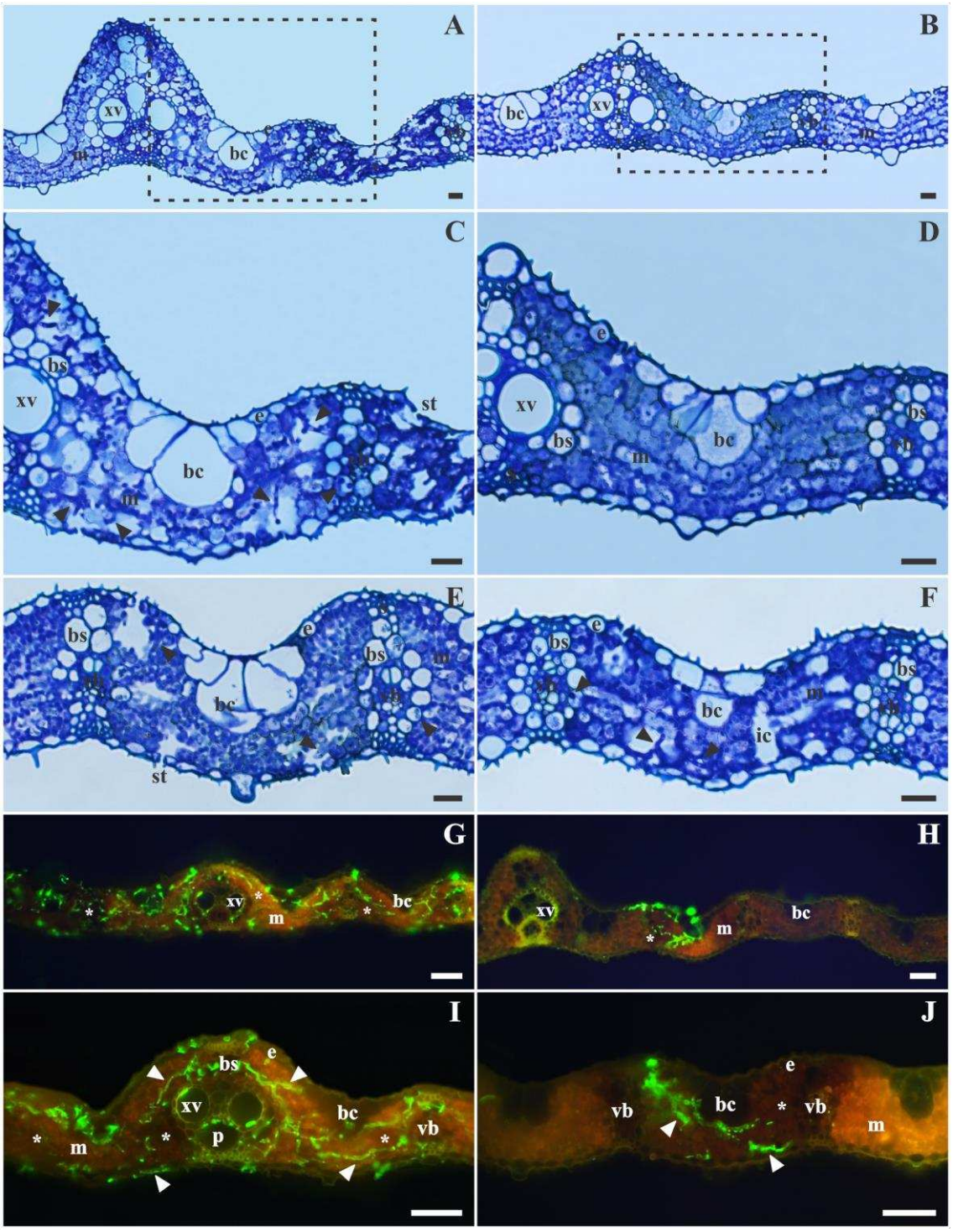
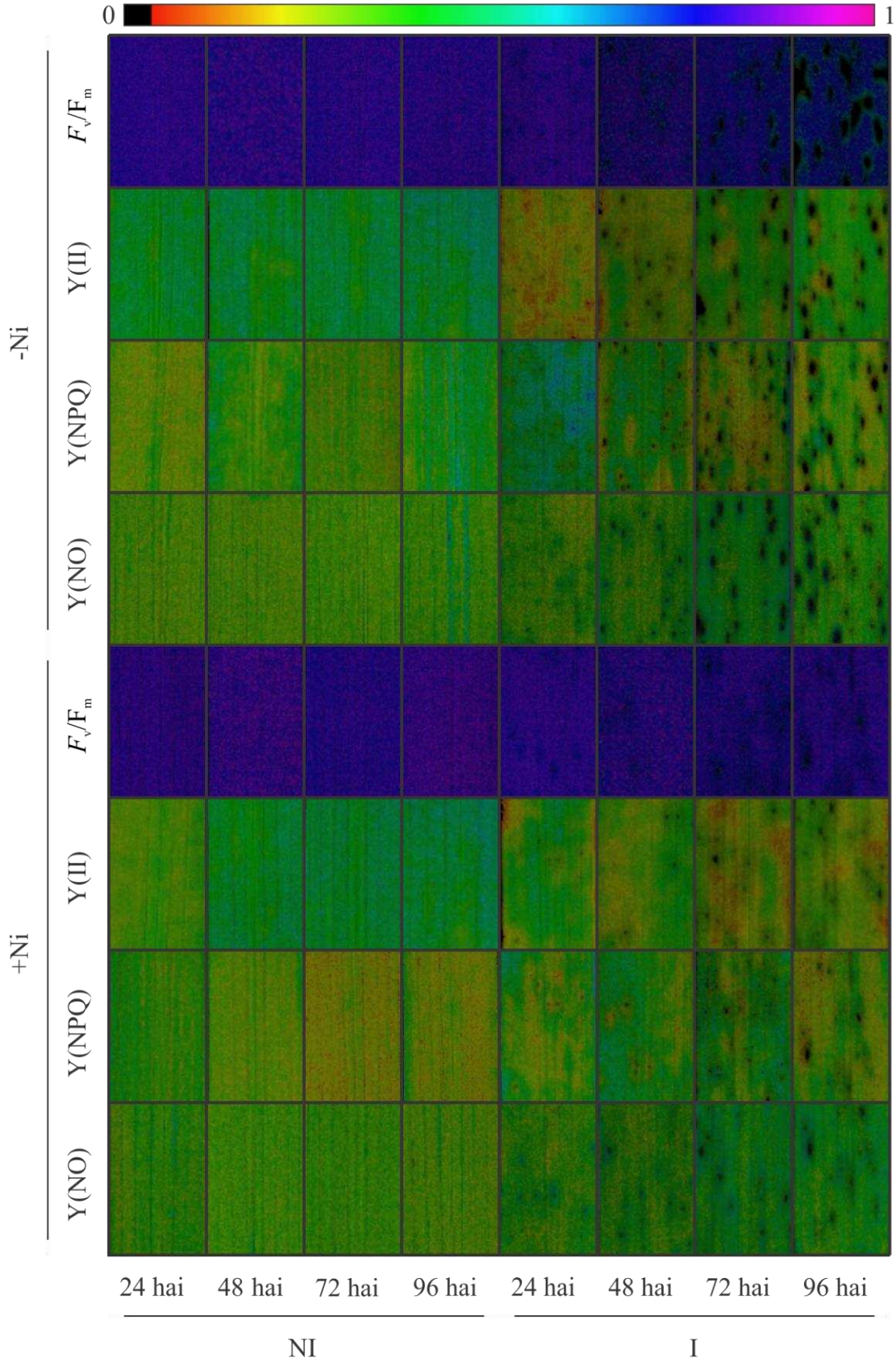


Figure 4. Light (A-F) and fluorescence (G-J) micrographs of transverse sections of leaf tissue from rice plants non-supplied (-Ni) (A, C, E, G, and I) or supplied (+Ni) (B, D, F, H, and J) with nickel (Ni) at 96 hours after inoculation with *Bipolaris oryzae*. The hatched squares (A and B) indicate the necrotic leaf tissue that are highlighted in C and D. Fungal hyphae (arrowheads) colonized the bulliform, epidermal, and mesophyll cells as well as the bundle sheath and vascular bundle of both -Ni and +Ni plants. Mesophyll cells presented extensive disorganization, especially in -Ni plants as a result of *B.*

oryzae colonization. Fluorescence images showed the green fluorescence of *B. oryzae* hyphae that were stained with WGA-AF 488 at the necrotic leaf tissue areas (asterisks). Fungal colonization was restricted in +Ni plants. Bulliform cell (bc), bundle sheath (bs), epidermis (e), intercellular space (ic), mesophyll cells (m), phloem (p), sclerenchyma (s), stomata (st), trichome (t), vascular bundle (vb), and xylem vessels (xv). Scale bars: 20 µm (A-F) and 50 µm (G-J).



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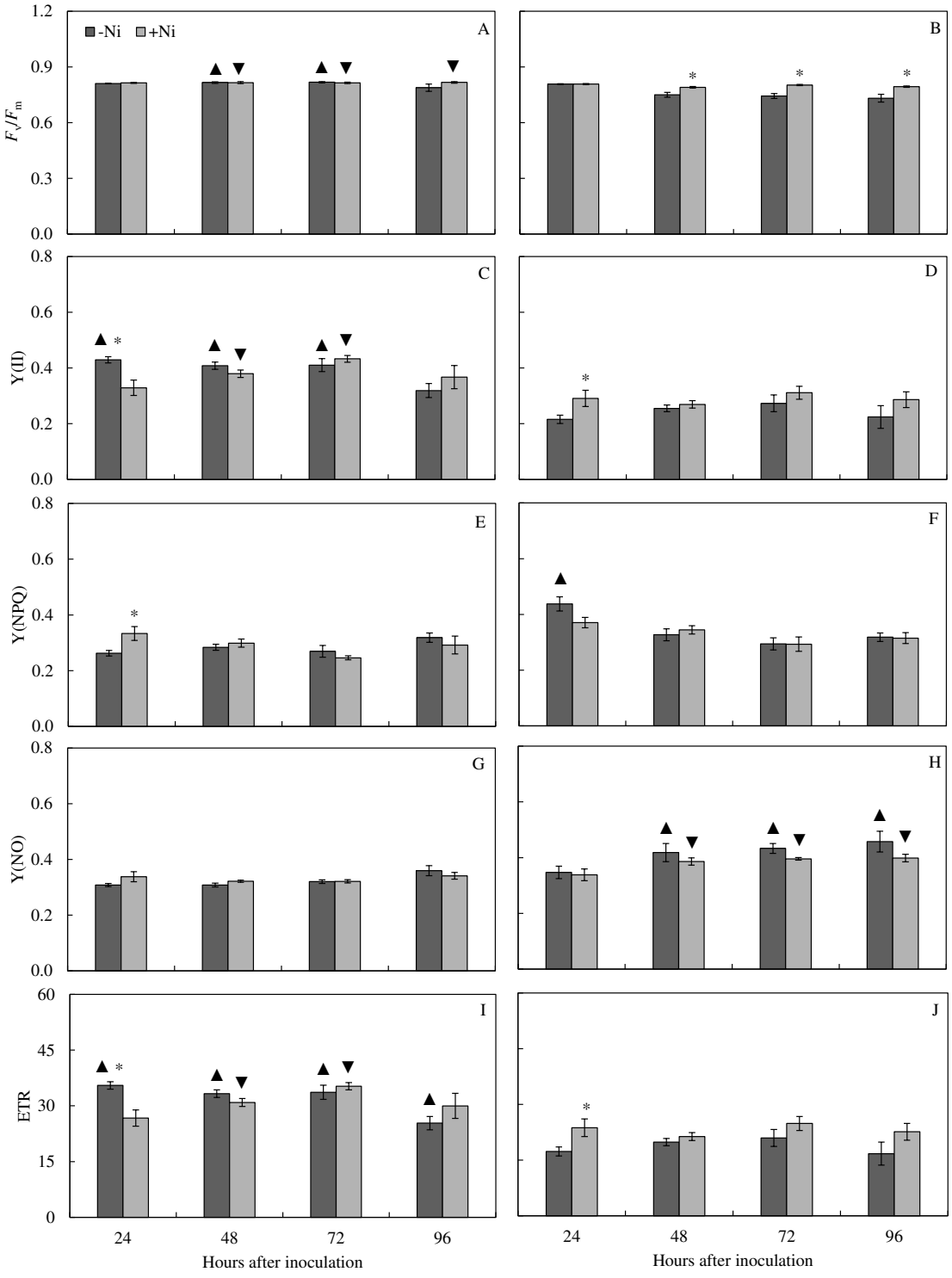

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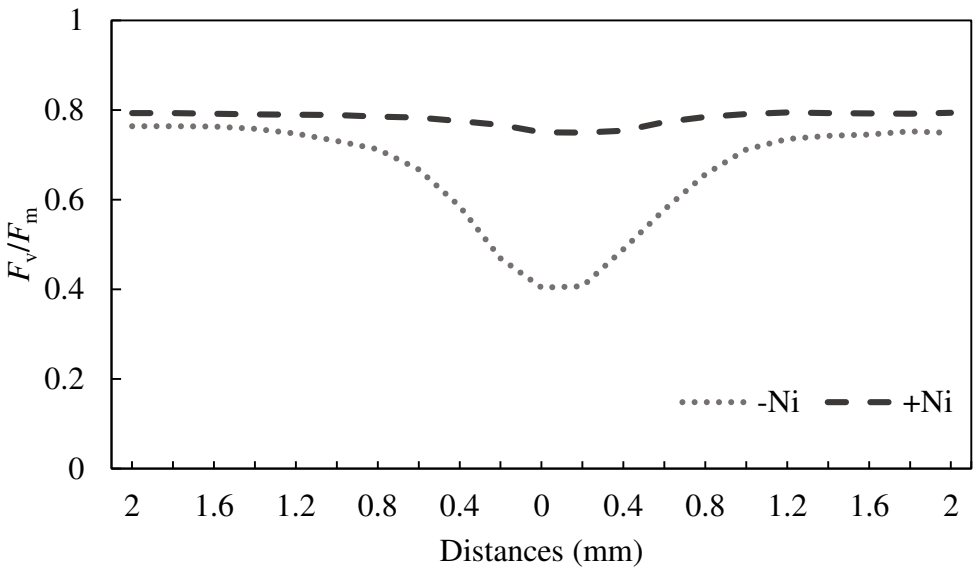
inoculated (I) (B, D, F, H, and J) with *Bipolaris oryzae*. Means for -Ni and +Ni treatments, at each evaluation time, followed by an asterisk (*) are significantly different ($P \leq 0.05$) according to *F* test. The symbols filled triangle (▲) and filled inverted triangle (▼) indicate differences between NI and I treatments, respectively, for -Ni and +Ni treatments, at each evaluation time, according to *F* test. Bars represent the standard errors of the means. $n = 6$.



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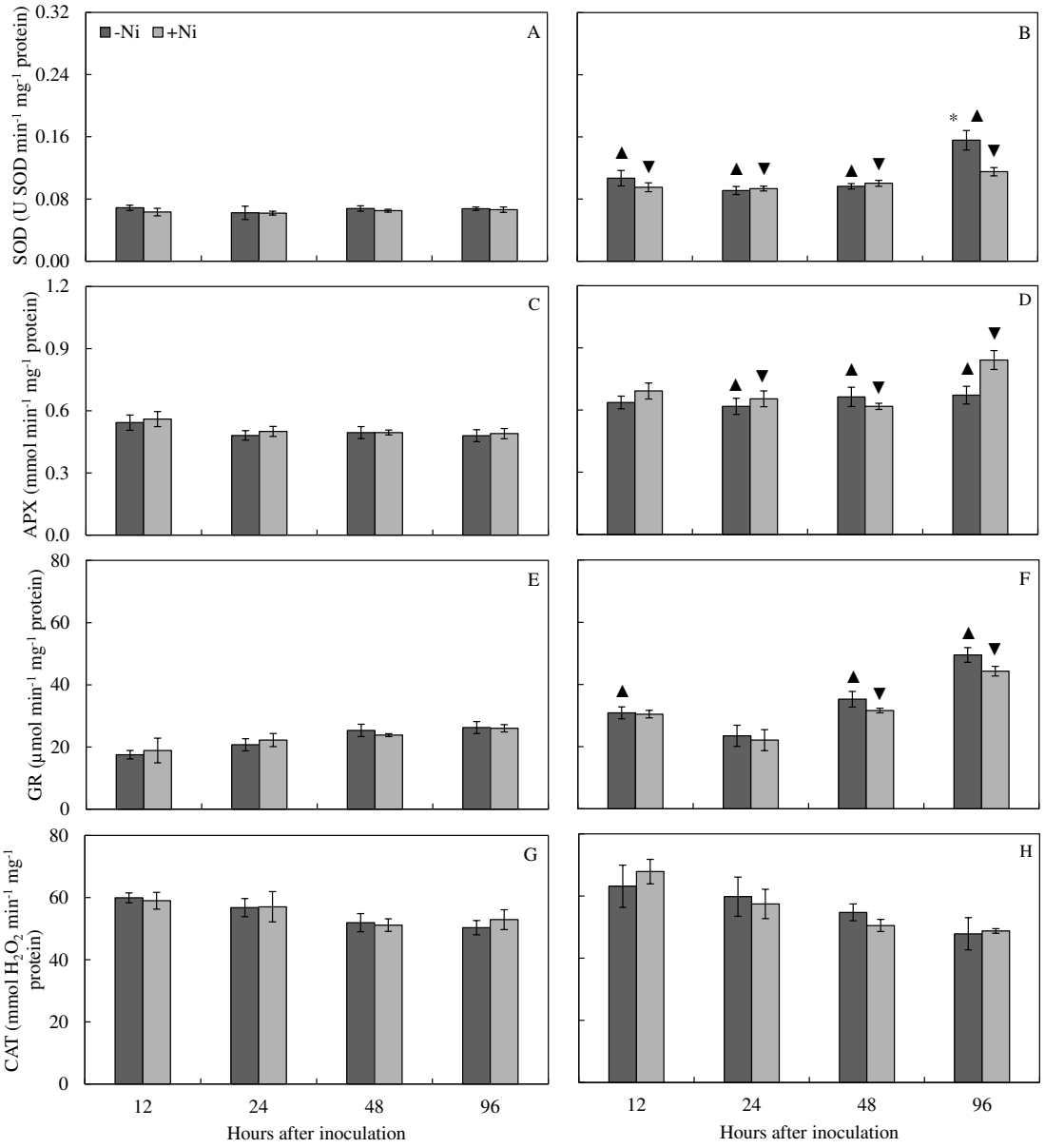

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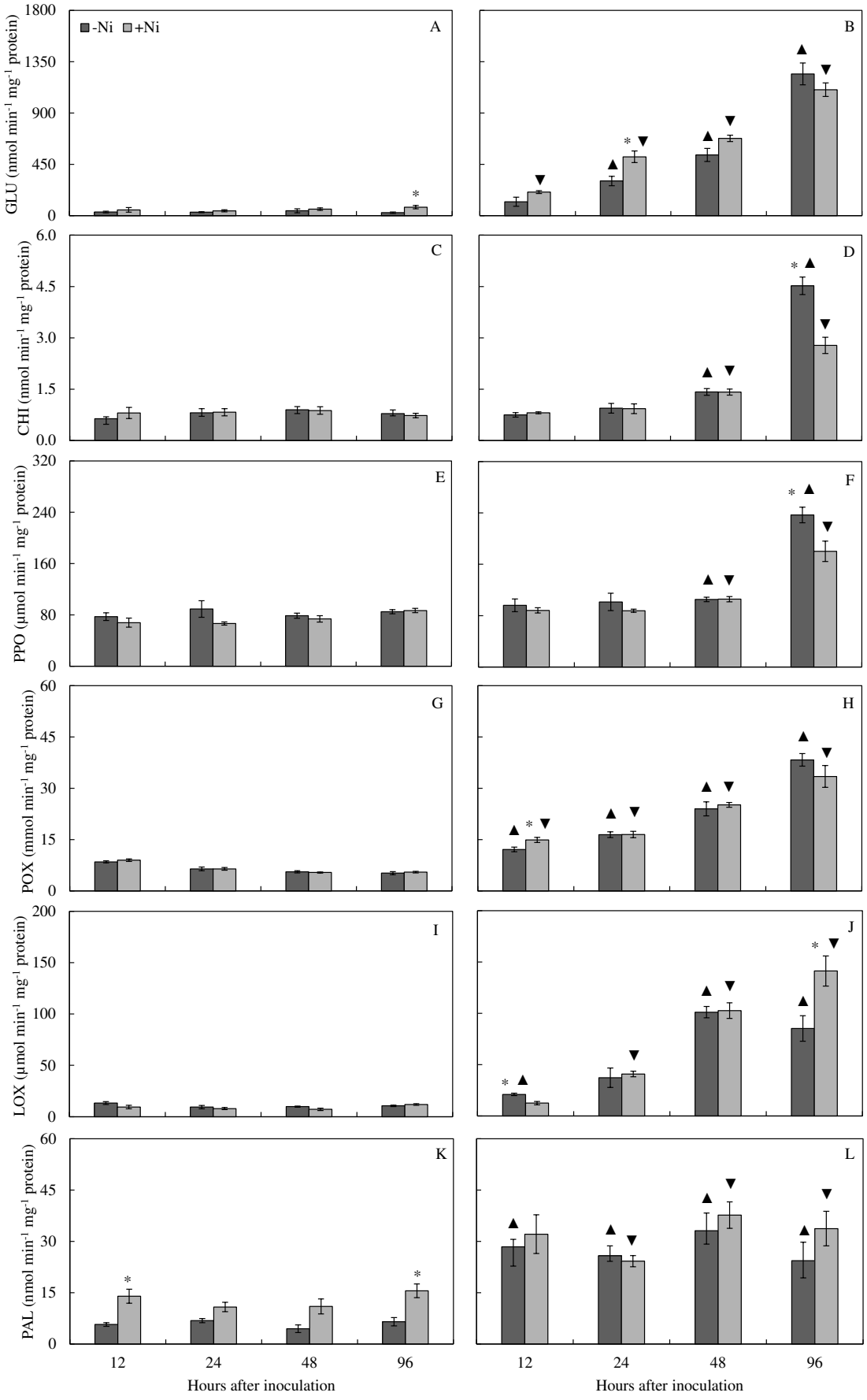

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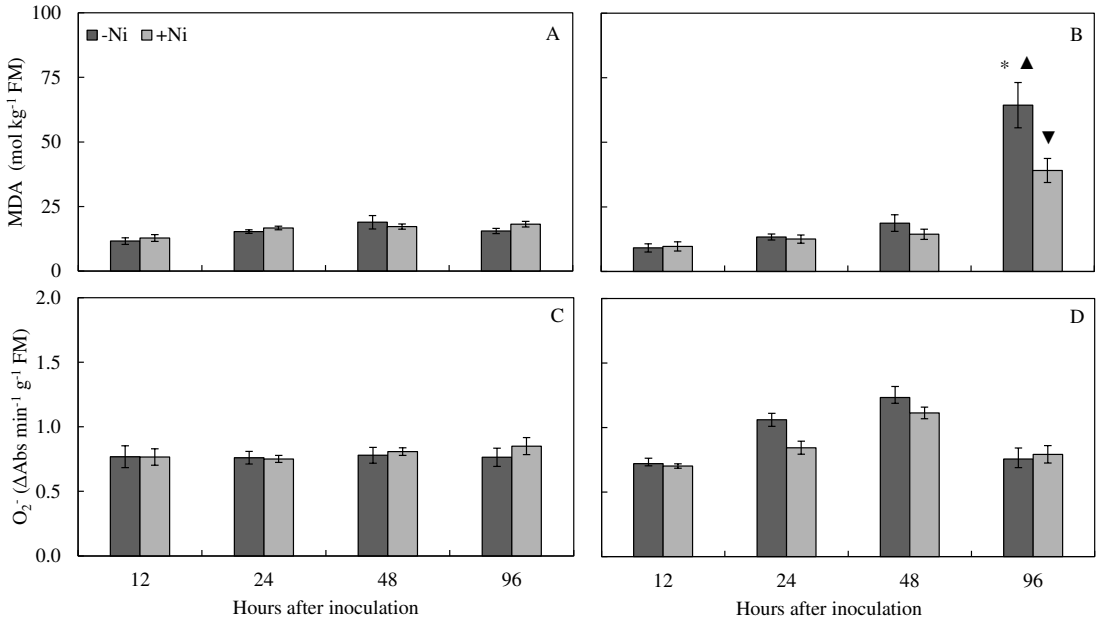

Figure 10. Concentrations of malondialdehyde (MDA) (A and B) and superoxide (O_2^-) (C and D) in the leaves of rice plants supplied with 0 (-Ni) or with 0.1 mM (+Ni) nickel (Ni) and non-inoculated (A and C) or inoculated (B and D) with *Bipolaris oryzae*. Means for -Ni and +Ni treatments, at each evaluation time, followed by an asterisk (*) are significantly different ($P \leq 0.05$) according to *F* test. The symbols filled triangle (▲) and filled inverted triangle (▼) indicate differences between NI and I treatments, respectively, for -Ni and +Ni treatments, at each evaluation time, according to *F* test. Bars represent the standard errors of the means. FM = fresh matter. $n = 4$.

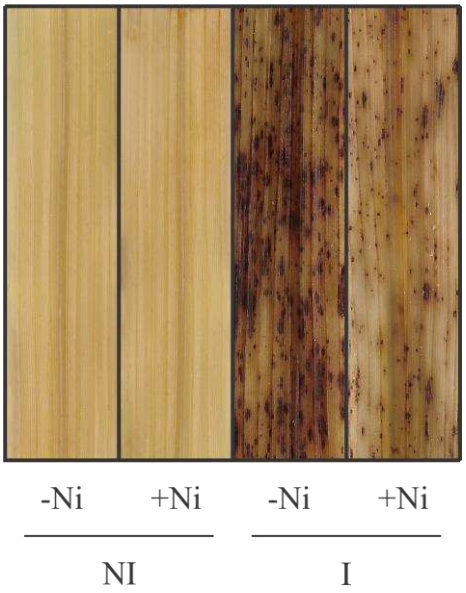

Figure 11. Histochemical detection of hydrogen peroxide (H_2O_2) on the leaves of rice plants supplied with 0 (-Ni) or with 0.1 mM (+Ni) nickel (Ni) and non-inoculated (NI) or at 96 hours after inoculation (I) with *Bipolaris oryzae*.

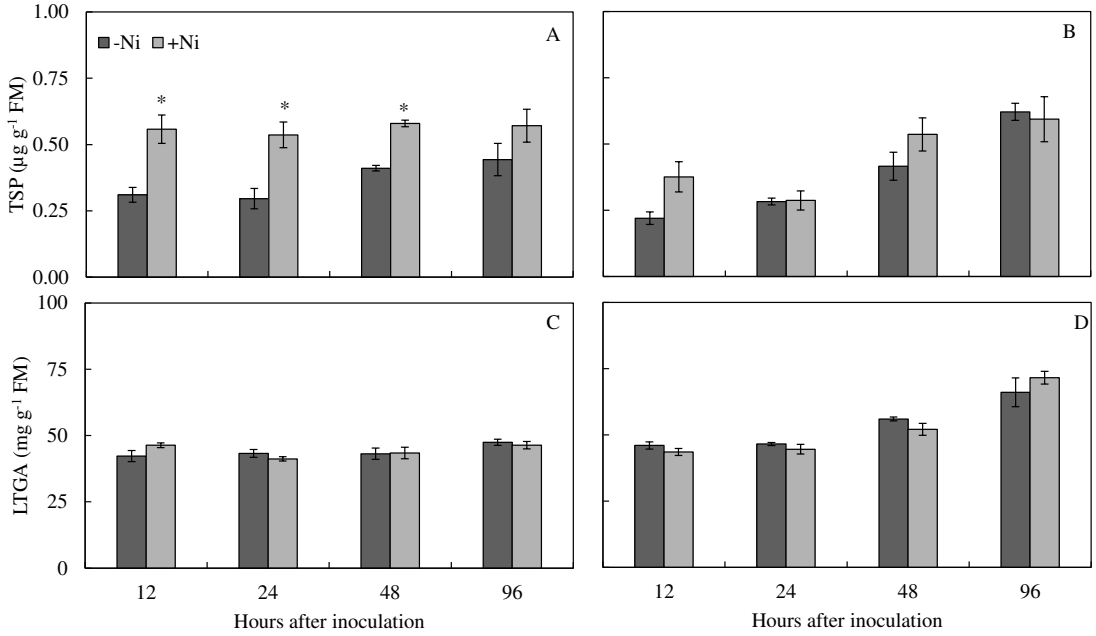

Figure 12. Concentrations of total soluble phenolics (TSP) (A and B) and lignin-thioglycolic acid (LTGA) derivatives (C and D) in the leaves of rice plants supplied with 0 (-Ni) or with 0.1 mM (+Ni) nickel (Ni) and non-inoculated (A and C) or inoculated (B and D) with *Bipolaris oryzae*. Means for -Ni and +Ni treatments, at each evaluation time, followed by an asterisk (*) are significantly different ($P \leq 0.05$) according to *F* test. The symbols filled triangle (▲) and filled inverted triangle (▼) indicate differences between NI and I treatments, respectively, for -Ni and +Ni treatments, at each evaluation time, according to *F* test. Bars represent the standard errors of the means. FM = fresh matter. $n = 4$.

CHAPTER 2

DUAL EFFECT OF PICOLINIC ACID IN THE PHOTOSYNTHETIC PERFORMANCE OF RICE PLANTS IN RESPONSE TO *Bipolaris oryzae* INFECTION

ABSTRACT

Brown spot, caused by *Bipolaris oryzae*, is one of the most important diseases of rice. The non-host toxin α-picolinic acid (PA) has great potential to be used to enhance plant resistance against pathogens infection. The present study aimed to investigate the effect of PA spray in rice plants (cultivar Metica-1) at the concentrations of 0 (PA 0), 1 (PA 1), 3 (PA 3), and 5 mg mL^{-1} (PA 5) on the photosynthetic performance of infected rice plants. Moreover, it was also evaluated whether the PA concentrations, especially at the highest concentration, could affect brown spot development. The chlorophyll *a* fluorescence parameters variable-to-maximum chlorophyll *a* fluorescence ratio (F_v/F_m), photochemical yield [Y(II)], yield for dissipation by down-regulation [Y(NPQ)], yield for non-regulated dissipation [Y(NO)], and electron transport rate (ETR) as well as the concentration of photosynthetic pigments were determined. Based on the *in vitro* assays, PA inhibited fungal mycelial growth in a dose-dependent manner, conidia germination was not affected, conidial germ tube length only decreased for PA 5. Necrotic lesions caused by PA were observed for non-inoculated plants at PA 3 and PA 5. Symptoms of brown spot were reduced on plants sprayed with PA 1 and PA 3 in comparison to non-sprayed plants. Lesions originated from *B. oryzae* infection and PA toxicity overlapped for inoculated plants sprayed with PA 3 and PA 5. The photochemical performance of non-inoculated plants was hampered by PA 3 and PA 5. The high concentration of photosynthetic pigments and less impairment on the photosynthetic performance of inoculated plants sprayed with PA 1 were noticed based on the values of F_v/F_m, Y(II), Y(NPQ), Y(NO), and ETR in comparison to inoculated plants non-sprayed with PA. In conclusion, the use of a low PA concentration showed a potential to decrease brown spot severity while preserving the photosynthetic capacity of the infected plants. The cellular damage generated by the highest PA concentration did not seem to favor *B. oryzae* infection.

Keywords: brown spot, Chlorophyll *a* fluorescence, foliar disease, necrotrophic pathogen, non-host selective toxin, photosynthesis

INTRODUCTION

Brown spot, caused by the necrotrophic fungus *Bipolaris oryzae* (Breda de Haan) Shoemaker, is one of the most important diseases affecting rice worldwide production for dramatically decreasing yield and impacting grains' quality (Barnwal et al., 2013; Ou, 1985). Rice plants are infected by *B. oryzae* from seedlings to the milk growth stage (Sunder et al., 2014). Epidemics of brown spot are often reported in rice production regions where water supply is scarce and plants suffer from nutritional imbalance (Barnwal et al., 2013). Brown spot management is dependent on correct soil fertilization along well with fungicides spray (Barnwal et al., 2013). On leaves, disease symptoms are noticed as small and circular dark brown spots that become oval with gray centers often surrounded by a yellow halo (Mew & Gonzales, 2002). Lesions coalescence and extensive chlorosis around them cause the leaves to dry, impairing photosynthesis (Dallagnol et al., 2009, 2011).

Several ophiobolins, a family of sesterterpenoid non-host selective toxins, are produced during the infection process of *B. oryzae* on rice leaf tissues, which have great importance for disease development (Au et al., 2000; Xiao et al., 1991). On infected rice leaves, degeneration of chloroplasts and the browning following the death of the parenchyma cells are reported to be caused by the action of ophiobolins A and B that are produced since conidia germinate over the leaf surface (Horino & Akai, 1970; Tullis, 1935; Xiao et al., 1991). Toxins are compounds of low molecular weight that originated from the secondary metabolism of fungi and are involved in disease development (Möbius & Hertweck, 2009). Rice genotypes have been screened for resistance to blast with the use of α-picolinic acid (PA), one of the non-host selective toxins produced by *Pyricularia oryzae* when infecting rice (Iwasaky et al., 1999; Prabhu & Rush, 1997). The resistance of rice plants to blast increased when they were sprayed with low PA concentrations through the induction of cell death associated with the production of reactive oxygen species (ROS) and defense-related genes transcripts (Zhang et al., 2004b). Blast symptoms on the leaves of wheat plants were reduced upon PA application due to a more robust antioxidative metabolism that rapidly scavenged the ROS generate during *P. oryzae* infection associated with a better photosynthetic performance of the infected plants (Aucique-Pérez et al., 2019). However, at high concentrations, PA was able to induce cell death (small lesion) in the leaf tissues of rice

and wheat plants (Aucique-Pérez et al., 2019; Zhang et al., 2004b). Bouizgarne et al. (2006) reported that the concentration of fusaric acid, a non-selective toxin produced by *Fusarium* spp., required to induce programmed cell death in *Arabidopsis thaliana* was 100-fold higher than the concentration used to trigger defense-associated responses such as the production of the phytoalexin camalexin. According to Jayaraj et al. (2010), the exogenous application of oxalic acid, a non-host selective toxin produced by *Sclerotinia sclerotiorum*, reduced the severity of sheath blight, caused by *Rhizoctonia solani*, on rice plants due to a great production of phenolics and high peroxidase, chitinase, β-1,3-glucanase activities.

Considering the potential of using the PA to increase the resistance of plants against diseases, this study was carried out to test the following hypothesis: (*i*) the photosynthetic performance of plants infected by *B. oryzae* could be less impaired by PA spray ensuring more capacity for defense and (*ii*) the highest PA concentration could affect both fungal infection process and photosynthesis.

MATERIAL AND METHODS

Plant growth

Rice seeds (cultivar Metica-1, susceptible to brown spot) (Moreira et al., 2013), were surface disinfected in 0.5% (v/v) NaOCl for 3 min, rinsed in tap water, and germinated on a sand substrate. The 7-day old seedlings were transferred to plastic pots (five plants per plastic pot) containing 5 liters of one-half strength of a nutrient solution prepared according to Hoagland & Arnon (1950) with some modification as follow: 1.0 mM KNO_3, 0.25 mM $NH_4H_2PO_4$, 0.1 mM NH_4Cl, 0.5 mM $MgSO_4$.7H2O, 1.0 mM $Ca(NO_3)_2$, 0.3 µM $CuSO_4.5H_2O$, 0.33 µM $ZnSO_4.7H_2O$, 11.5 µM H_3BO_3, 3.5 µM $MnCl_2.4H_2O$, 0.1 µM $(NH_4)_6Mo_7O_{24}.4H_2O$, 25µM $FeSO_4.7H_2O$, and 25 µM EDTA disodium. The nutrient solution strength was increased over time and changed every four days. The pH was checked daily and maintained at approximately 5.5 by adding NaOH or HCl (1 M) solutions as needed.

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

Plants were inoculated with the isolate UFV/DFP *Bo*-01 of *B. oryzae* following the procedures of Dallagnol et al. (2011). The isolate was preserved on filter paper in glass vials with silica gel at 4°C. Pieces of filter paper containing the fungus were transferred to Petri dishes with potato-dextrose-agar (PDA) medium. After 3 days, PDA plugs containing fungal mycelia were transferred to new Petri dishes containing oat-agar (OA) medium. The Petri dishes were kept in a growth chamber at 25°C with 12 h photoperiod for 10 days. After this period, conidia were carefully removed from the dishes with a soft bristle brush using water containing gelatin (1% w/v). The conidial suspension was calibrated using a hemacytometer to obtain a concentration of 5×10^3 conidia mL^{-1}.

At 24 h before inoculation, rice plants (40 days after emergence; V_5 growth stage) (Counce et al., 2000) were sprayed with PA solutions at the concentrations of 0, 1, 3, and 5 mg mL^{-1}. The conidial suspension was sprayed on the adaxial surface of the leaves until runoff using a VL Airbrush atomizer (Paasche Airbrush Co.). Immediately after inoculation, plants were transferred to a mist chamber (temperature of 25 ± 2°C and relative humidity of 90 ± 5%) for an initial 24 h dark period. After this period, plants were transferred to a growth chamber with 12 h photoperiod of photon irradiance of 350 µmol m^{-2} s^{-1} provided by cool-white fluorescent lamps. Plants were kept inside

the mist chamber for the duration of the experiments. The non-inoculated plants were kept in a separate growth chamber, but exposed to the same environmental conditions.

***In vitro* assays**

The sensitivity of *B. oryzae* to PA solutions prepared at the concentrations of 1, 3, and 5 mg PA mL^{-1} of OA medium was evaluated *in vitro*. Melted OA medium was amended with the PA solutions above mentioned and then poured into Petri plates (20 mL per plate). Melted OA medium non-mended with PA served as the control treatment. An OA plug (9 mm in diameter), containing fungal mycelial obtained from the edge of a 10-day-old *B. oryzae* colony, was placed in the center of each Petri dish. The Petri dishes were kept in a growth chamber (25°C and 12 h photoperiod). Fungal colony in each Petri dish was measured daily in two orthogonal directions (to obtain the diameter) for 6 days by using a digital caliper. Conidia obtained from 10-day-old colonies of *B. oryzae* were used to obtain a conidial suspension of 5×10^3 conidia mL^{-1}. To determine the PA effect on conidia germination and germ tube length, a total of 1 mL of conidial suspension prepared with the PA solutions at the concentrations mentioned above (including the control treatment) was transferred to each Petri dish (total of five Petri dishes) containing agar-agar medium. The suspension was homogeneously distributed over the medium in each Petri dish using a Drigalski glass. Petri dishes were transferred to a growth chamber at 25°C. After 4 h, lactophenol was added to the dishes to stop conidia germination. One hundred conidia were randomly examined from each Petri dish under a microscope Carl Zeiss Axio Imager A1 microscope (Carl Zeiss, Germany) at 40 × magnification. A conidium was considered germinated when the germ tube was longer than its diameter. The percentage of germination was calculated. The germ tube length of each conidium was measured using the AXION VISION v.4.8.1 software.

Determination of chlorophyll (Chl) *a* fluorescence

The third and fourth leaf of each plant per replication of each treatment were collected at 48, 72, and 96 hours after inoculation (hai) to obtain the images of the parameters of Chl *a* fluorescence using the MAXI version of the Imaging-PAM chlorophyll fluorometer and the Imaging Win software (Heinz Walz GmbH, Effeltrich, Germany) according to Tatagiba et al. (2016). Leaves from non-inoculated plants were sampled at these evaluation times to serve as the control treatment. Leaves were individually fixed

in the holder at a distance of 18.5 cm from the recording camera CCD ('charge-coupled device') coupled to the fluorescence device and then exposed to a weak, modulated measuring beam (0.5 μmolm^{-2} s^{-1}, 100 μs, 1 Hz) to determine the initial fluorescence (F_0) when all the PS II reaction centers were 'open'. Next, a saturating white light pulse of 2400 μmol m^{-2} s^{-1} (10 Hz) was applied for 0.8 s to ensure the maximum fluorescence emission (F_m) when all the PS II reaction centers were 'closed'. Based on these initial measurements, the maximum PS II photochemical efficiency of the dark-adapted leaves was estimated through the variable-to-maximum Chl fluorescence ratio, $F_v/F_m = [(F_m - F_0)/F_m)]$. The leaves were subsequently exposed to actinic photon irradiance (185 μmol m^{-2} s^{-1}) for 300 s to obtain the steady-state fluorescence yield (F_s), after which a saturating white light pulse (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 & Baker (1997). Following the calculations of Kramer et al. (2004), the energy absorbed by PS II for the following three yield components for dissipative processes was determined: the yield of photochemistry [Y(II) = $(F_m' - F_s)/F_m')$], the yield for dissipation by down-regulation [Y(NPQ) = $(F_s/F_m') - (F_s/F_m)$], and the yield for other non-photochemical (non-regulated) losses [Y(NO) = F_s/F_m]. The apparent electron transport rate was calculated as ETR = Y(II) × PPFD × f × α according to Baker (2008). The rectangle option on the Imaging Win software was used to determine the parameters of Chl *a* fluorescence.

Determination of the concentration of photosynthetic pigments

Four squared leaf areas (1 cm^2) were punched from the fourth leaf of each plant per replication of each treatment at 48 and 96 hai to determine the concentrations of Chl *a*, Chl *b*, and carotenoids. Leaf samples were immersed in glass tubes containing 4 mL of dimethyl sulfoxide (DMSO) solution (saturated with calcium carbonate ($CaCO_3$), 5 g L^{-1}) and kept in the dark at room temperature for 24 h. The absorbance of the extracts was read at 480, 649, and 665 nm in a spectrophotometer using $CaCO_3$ saturated solution of DMSO as a blank. Concentrations of Chl *a*, Chl *b*, and carotenoids were calculated according to Wellburn (1994).

Experimental design and statistical analysis

A 4 × 2 factorial experiment, consisting of four PA concentrations (0, 1, 3, and 5 mg mL^{-1}, hereafter referred to as PA 0, PA 1, PA 3, and PA 5, respectively) and plant inoculation (non-inoculated or inoculated plants), was arranged in a completely randomized design with three replications. Each replication consisted of a plastic pot with five plants. The experiment was repeated once. Data from all the variables and parameters evaluated were subjected to analysis of variance (ANOVA) and regression models were fitted to relate the dependent variables to the picolinic acid concentrations. Models were chosen whose regression coefficients were significant up to the 5% level of probability and which exhibited the greatest adjusted coefficient of determination.

RESULTS

Effect of PA alone and in combination with infection by *B. oryzae* on lesions development on rice leaves

The leaves of plants sprayed with PA solutions of 3 and 5 mg mL^{-1} displayed visible necrotic lesions starting at 72 and 48 hai, respectively (Fig. 1A4 and C3). There were no visible necrotic lesions on the leaves of plants sprayed with PA at 1 mg mL^{-1} (Fig. 1A2, C2, and E2) in comparison to the leaves of non-sprayed plants (Fig. 1A1, C1, and E1) regardless of the sampling time. Many necrotic lesions surrounded by intense chlorosis were noticed on leaves of plants non-sprayed with PA and infected by *B. oryzae* (Fig. 1B1, D1, and F1). By contrast, the number and size of the necrotic lesions were reduced, the quantity of coalescing lesions was less, and chlorosis was reduced on leaves of plants sprayed with PA at 1 (Fig. 1B2, D2, and F2) and 3 mg mL^{-1} (Fig. 1B3, D3, and F3). On the infected leaves of plants sprayed with PA at 3 and 5 mg mL^{-1}, the lesions originated from fungal infection and also caused by PA itself were somehow overlapped making it challenging to separate their effect (Fig. 1B3-B4, D3-D4, and F3-F4). Brown spot lesions may have their development impaired on the leaves of plants sprayed with PA at 5 mg mL^{-1} likely due to its immediate and more toxic effect (Fig. 1B4, D4, and F4).

In vitro effect of PA on fungal mycelial growth, conidia germination, and conidial germ tube length

Fungal mycelial growth was inhibited entirely on the OA medium as the PA concentration increased from 1 to 5 mg mL^{-1} in comparison to PA 0 mg mL^{-1} (Fig. 2A). The diameter of the fungal colony was significantly reduced by 35, 68, and 82% on OA medium containing 1, 3, and 5 mg PA mL^{-1}, respectively, in comparison to PA 0 mg mL^{-1} (Fig. 2B). Compared to PA 0 mg mL^{-1}, conidial germ tube length was significantly reduced by 30% on the OA medium containing 5 mg PA mL^{-1} (Fig. 2C). Conidia germination was not affected by PA regardless of the concentration tested (data not shown).

Effect of PA alone and in combination with infection by *B. oryzae* on Chl *a* fluorescence parameters

Images of Chl *a* fluorescence evidenced changes only for Y(NPQ) at 96 hai for non-inoculated plants sprayed with PA at 1 and 3 mg mL^{-1}. For non-inoculated leaves of plants sprayed with PA at 5 mg mL^{-1}, there was a progressive loss of their photosynthetic capacity from 48 to 96 hai as indicated by the dark areas in the images (Fig. 3). Changes in all parameters on the leaves of inoculated plants started on 48 hai regardless of PA concentration used (Fig. 3). The imaging of the parameters F_v/F_m, Y(II), Y(NPQ), and Y(NO) became more pronounced (seen as many necrotic lesions caused by both fungal infection and PA action) as the PA concentrations increased from 1 to 5 g mL^{-1} in association with fungal infection (Fig. 3).

A linear model best described the relationship of PA concentrations and the parameter F_v/F_m for inoculated plants at 48 hai, meanwhile a quadratic model best fitted for non-inoculated plants (Fig. 4A). At 72 and 96 hai, second order regression curves best described the relationship of PA concentrations and the parameter F_v/F_m without significant difference ($P \leq 0.05$) between non-inoculated and inoculated plants (Fig. 4B and C). The relationship between the parameters Y(II) and ETR and PA rates were quadratic for both non-inoculated and inoculated plants at 48 hai, and linear and quadratic, respectively, at 72 and 96 hai (Fig. 4C-E and M-N). There is no relationship between PA concentrations and Y(NPQ) at 48 hai (Fig. 4G). However, second order regression curves best described the relationship of PA concentrations and the parameter Y (NPQ) without significant difference ($P \leq 0.05$) between non-inoculated and inoculated plants at 72 and 96 hai (Fig. 4H and I). The relationship between Y(NO) and PA concentrations were quadratic for non-inoculated and inoculated plants at 48 hai and 72 and 96 hai without significant difference ($P \leq 0.05$) between non-inoculated and inoculated plants (Fig. 4J-L).

Concentrations of photosynthetic pigments

For non-inoculated plants, the concentration of Chl *a+b* was negatively affected by PA concentration, while for inoculated plants, the concentration of Chl *a+b* was affected by PA regardless of its concentration (Fig. 5A and B). The concentration of Chl *a+b* was 45 and 23% higher for PA 0 mg mL^{-1}, respectively, at 48 and 96 hai compared to PA 5 mg mL^{-1}. For inoculated plants, the concentration of Chl *a+b* was higher by 27% for PA

0 mg mL^{-1} in comparison to PA 3 mg mL^{-1} at 48 hai (Fig. 5A). At 96 hai, the concentration of Chl *a+b* was lower by 41 and 30% for PA 0 and 3 mg mL^{-1}, respectively, in comparison to PA 5 mg mL^{-1} and by 45 and 35% for PA 0 and 3 mg mL^{-1}, respectively, in comparison to PA 1 mg mL^{-1} (Fig. 5B). The concentration of carotenoids was impaired by PA concentration at both 48 and 96 hai (Fig. 5 C and D). For non-inoculated plants, the concentration of carotenoids was higher by 41 and 19% for PA 0 mg mL^{-1} in comparison to PA 5 mg mL^{-1}, respectively, at 48 and 96 hai (Fig. 5C and D). For inoculated plants, the concentration of carotenoids was higher by 59% for PA 0 mg mL^{-1} at 48 hai and by 59% for PA 1 mg mL^{-1} at 96 hai in comparison to PA 3 (Fig. 5C and D).

DISCUSSION

This study brings new insights, to the best of authors' knowledge, into the effect of PA on rice resistance to brown spot through a detailed physiological analysis. It is known that non-host selective toxins, such as the PA, present a great potential to be used in decreasing diseases symptoms development through the potentiation of host defense responses (Aucique-Pérez et al., 2019; Zhang et al., 2004ab). In the present study, the PA was able to reduce brown spot symptoms on rice leaves in a concentration-dependent matter. The spray of rice plants with a low PA concentration (1 mg mL^{-1}), that resulted in the lowest brown spot severity, was associated with a high concentration of photosynthetic pigments in comparison to non-sprayed plants and, therefore, a better photosynthetic performance. According to Dallagnol et al. (2011), the infection of rice leaves by *B. oryzae* dramatically decreased the concentration of photosynthetic pigments due to the extensive damage caused by the fungus producing non-host selective toxins on the leaf tissues. Mycelial growth of *B. oryzae in vitro* was inhibited as the concentration of PA increased from 1 to 5 mg mL^{-1}, suggesting its effect on fungal physiology. Interestingly, PA did not affect conidia germination regardless of the concentration used while the germ tube length was inhibited at the highest PA concentration. Aucique-Pérez et al. (2019) reported a reduction in conidia production of *P. oryzae* and their germination at PA concentration of 0.3 mg mL^{-1} and complete inhibition of fungal mycelial growth with PA at concentrations above 0.5 mg mL^{-1}. Considering the fact that the pre-penetration stage of *B. oryzae* into the leaf surface was slightly affected by PA, reduction on brown spot symptoms on plants sprayed with the lowest PA concentration could be partially accounted for by the direct action of this toxin on *B. oryzae* based on the delay for the appearance of brown spot symptoms. Several non-host selective toxins released by fungi during their infection process may act as elicitors to stimulate defense responses on their hosts preceded mostly by an increase in ROS production (Averyanov, 2008). Rice cells exposed to PA were elicited to produce ROS, cell death in a hypersensitive-like response, and enhanced resistance against *P. oryzae* infection (Zhang et al., 2004b). In *A. thaliana*, PA-induced oxidative burst on leaf cells and their death through the activation of the salicylic acid-dependent and jasmonic acid/ethylene-dependent pathways suggesting that PA acted as a non-specific elicitor in the activation of host defense responses (Zhang et al., 2004a). For the

wheat-*P. oryzae* interaction, reduction on blast severity mediated by PA was linked with an increase on the activities of antioxidant enzymes (*e.g.*, ascorbate peroxidase, catalase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase, peroxidase, and superoxide dismutase) and lower ROS production and malondialdehyde concentration (Aucique-Pérez et al., 2019). The tenuazonic acid, another non-host selective toxin produced by *P. oryzae*, increased rice resistance to *P. oryzae* mediated by high ROS production (Aver'yanov et al., 2007).

The functionality of the photosynthetic apparatus was negatively impaired on the leaves of rice plants sprayed with the highest PA concentration. The F_v/F_m values were below 0.83 for non-inoculated and inoculated plants sprayed with PA concentration of 5 mg mL^{-1}, indicating the photoinhibition of the photosynthesis. The Y(NPQ) values did not suffer alterations regardless of plant inoculation at none of the PA concentrations tested, while the Y(NO) values were high as the PA concentration increased from 1 to 5 mg mL^{-1}. Similar results were reported by Aucique-Peréz et al. (2019) for the wheat-*P. oryzae* interaction plants. According to these authors, F_v/F_m values decreased by 11% while the Y(NO) increased by 34% for wheat plants sprayed with PA at the concentration of 5 mg mL^{-1} in comparison to non-sprayed plants. The Y(II) parameter, a relative measure of photochemical energy conversion in PSII, was inhibited on the leaves of non-inoculated plants sprayed with PA concentrations of 3 and 5 mg mL^{-1} indicating that light energy absorbed by the photosynthetic pigments was not allocated to the photochemical process probably due to photooxidative damage. On the other hand, for inoculated plants, the values for Y(II) and ETR (a measure of the electron transportation on the thylakoid membranes) were simultaneously harmed by the cellular damage caused both by *B. oryzae* infection and PA. Rice plants sprayed with PA at the lowest concentration (1 mg mL^{-1}), for which no visible damage to the leaf tissues was noticed and the brown spot symptoms were less developed, presented a better photosynthetic performance based on the values of Y(II) and ETR parameters suggesting that at lower PA concentration the rice plants could counteract against *B. oryzae* infection more efficiently. In comparison to rice plants suffering only from *B. oryzae* infection without PA interference, those inoculated plants sprayed with PA concentrations of 3 and 5 mg mL^{-1} were more impacted by the dual effect of PA and *B. oryzae* infection especially for the former. The photosynthesis of rice plants infected by *B. oryzae* decreased due to photochemical and biochemical limitations on the leaf tissues massively colonized by fungal hyphae (Dallagnol et al., 2011; Debona et al.,

2016). Necrotrophic pathogens, such as *B. oryzae*, are very efficient in killing the tissues of their hosts due to a massive production of non-host selective toxins that work together with hydrolytic enzymes to destroy the tissues to obtain the necessary nutrients toward a successfully infection process (Kan, 2006; Dallagnol et al., 2011; Debona et al., 2016). Therefore, the spray of PA at a specific concentration capable of causing severe cellular damage could boost the colonization on rice leaf tissues by *B. oryzae*. The PA was able to enhance rice resistance to blast, owing to the generation of ROS and inducing cell death in a hypersensitive-like response (Zhang et al., 2004ab). The necrotrophic fungus *Botrytis cinerea* had its infection process maximized on the leaves of *A. thaliana* plants due to a hypersensitive response and ROS generation (Govrin & Levine, 2000). By contrast, in the present study, the lowest PA concentration was able to reduce brown spot symptoms in a scenario where the cellular damage caused by this toxin was not able to favor *B. oryzae* infection.

The results from the current study demonstrated the potential of using PA at a low and non-phytotoxic concentration to decrease brown spot symptoms associated with partial preservation of their photosynthetic capacity. Moreover, lesions resulted from a high PA concentration impaired photosynthesis and was not able to stimulate the infection process of *B. oryzae*.

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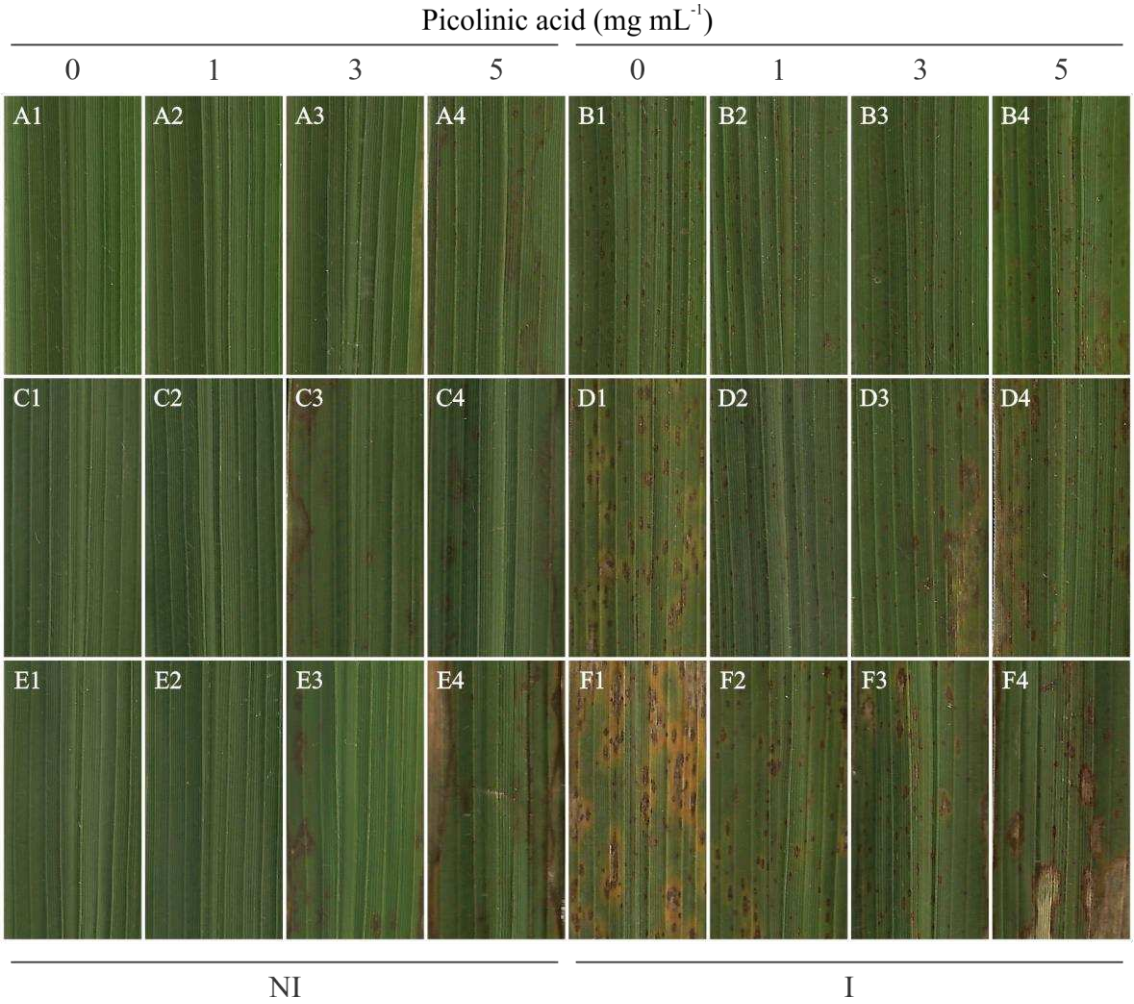

Figure 1. Leaves of rice plants sprayed with picolinic acid at the concentrations of 0, 1, 3, and 5 mg mL^{-1} and non-inoculated (NI) or inoculated (I) with *Bipolaris oryzae*. Leaves were sampled at 48 (A1-A4 and B1-B4), 72 (C1-C4 and D1-D4), and 96 (E1-E4 and F1-F4) hours after inoculation for both NI and I plants.

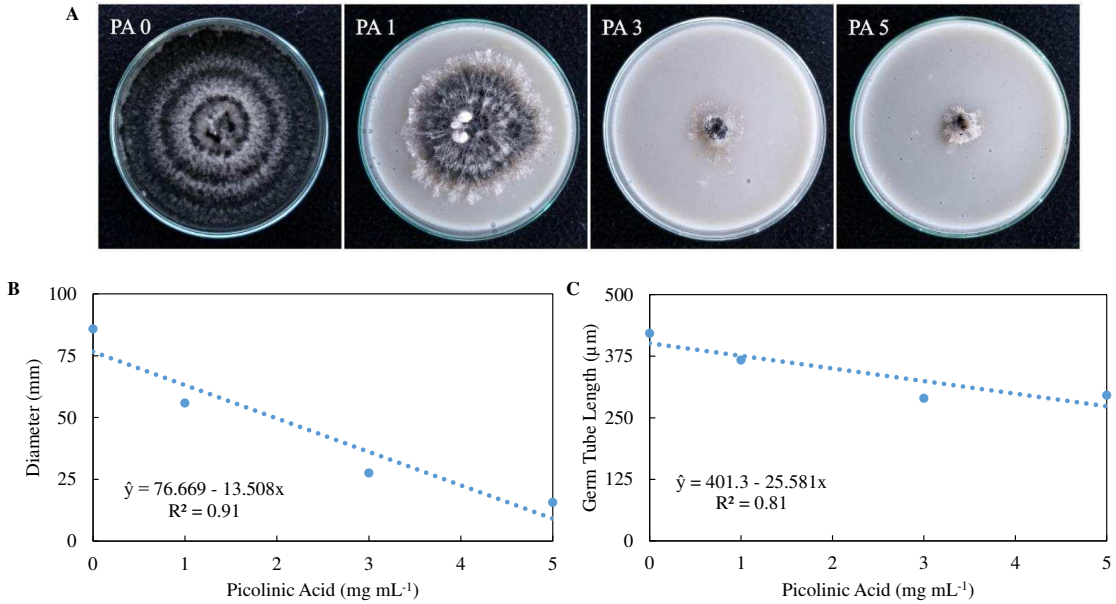

Figure 2. *In vitro* effect of picolinic acid (PA), at the concentrations of 0 (PA 0), 1 (PA 1), 3 (PA 3), and 5 mg mL^{-1} (PA 5), on fungal mycelial growth (A), diameter of fungal colony (B), and conidial germ tube length (B) of *Bipolaris oryzae*. * and **: significant at 1 and 5%, respectively.

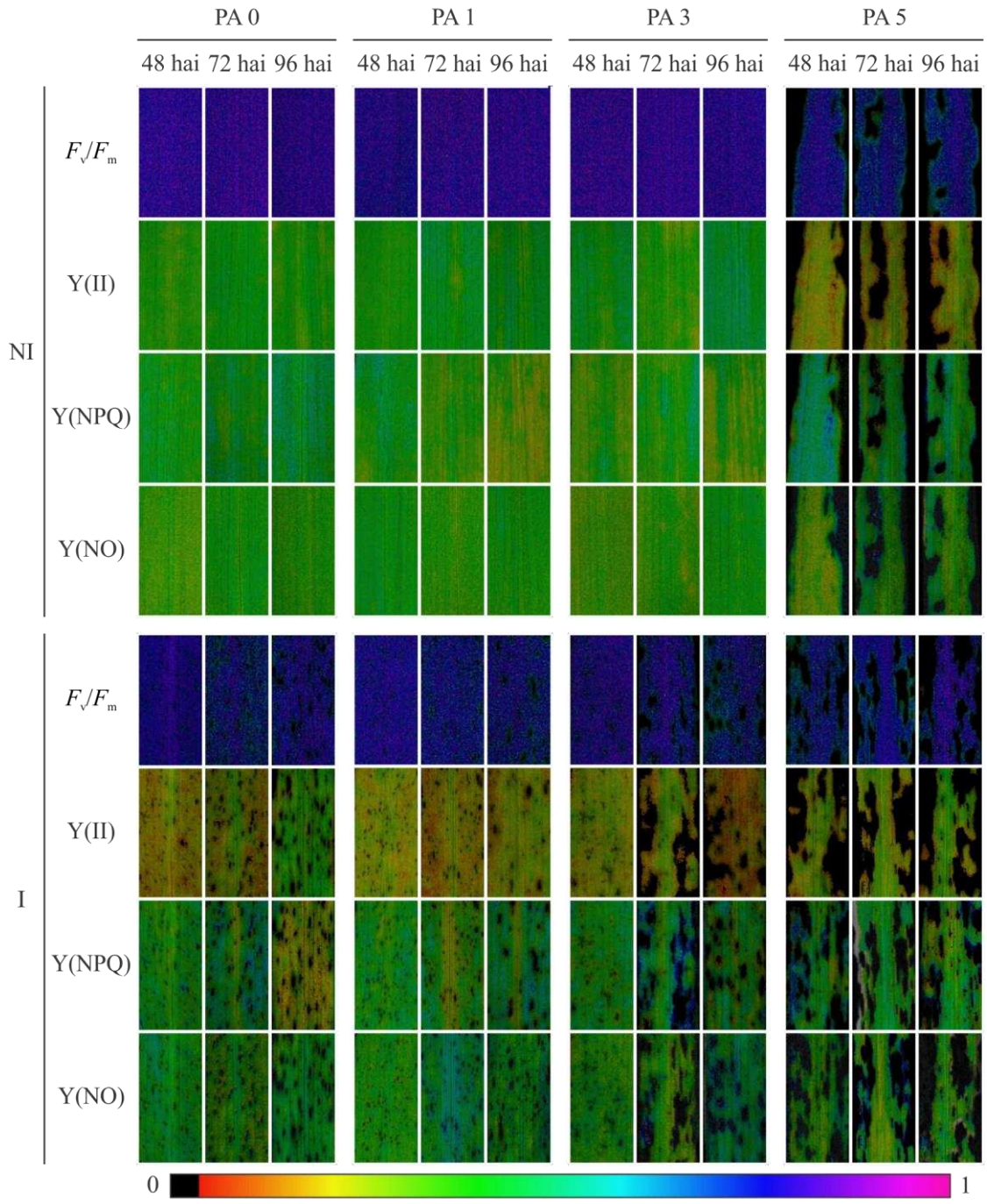

Figure 3. Images of the chlorophyll *a* fluorescence parameters: maximum PSII quantum efficiency (F_v/F_m), effective PSII quantum yield (Y(II)), quantum yield of regulated energy dissipation (Y(NPQ)), and quantum yield of non-regulated energy dissipation (Y(NO)) determined on the leaves of rice plants sprayed with picolinic acid (PA) at the concentrations of 0 (PA 0), 1 (PA 1), 3 (PA 3), and 5 mg mL^{-1} (PA 5) and non-inoculated (NI) or inoculated (I) with *Bipolaris oryzae*. hai = hours after inoculation.

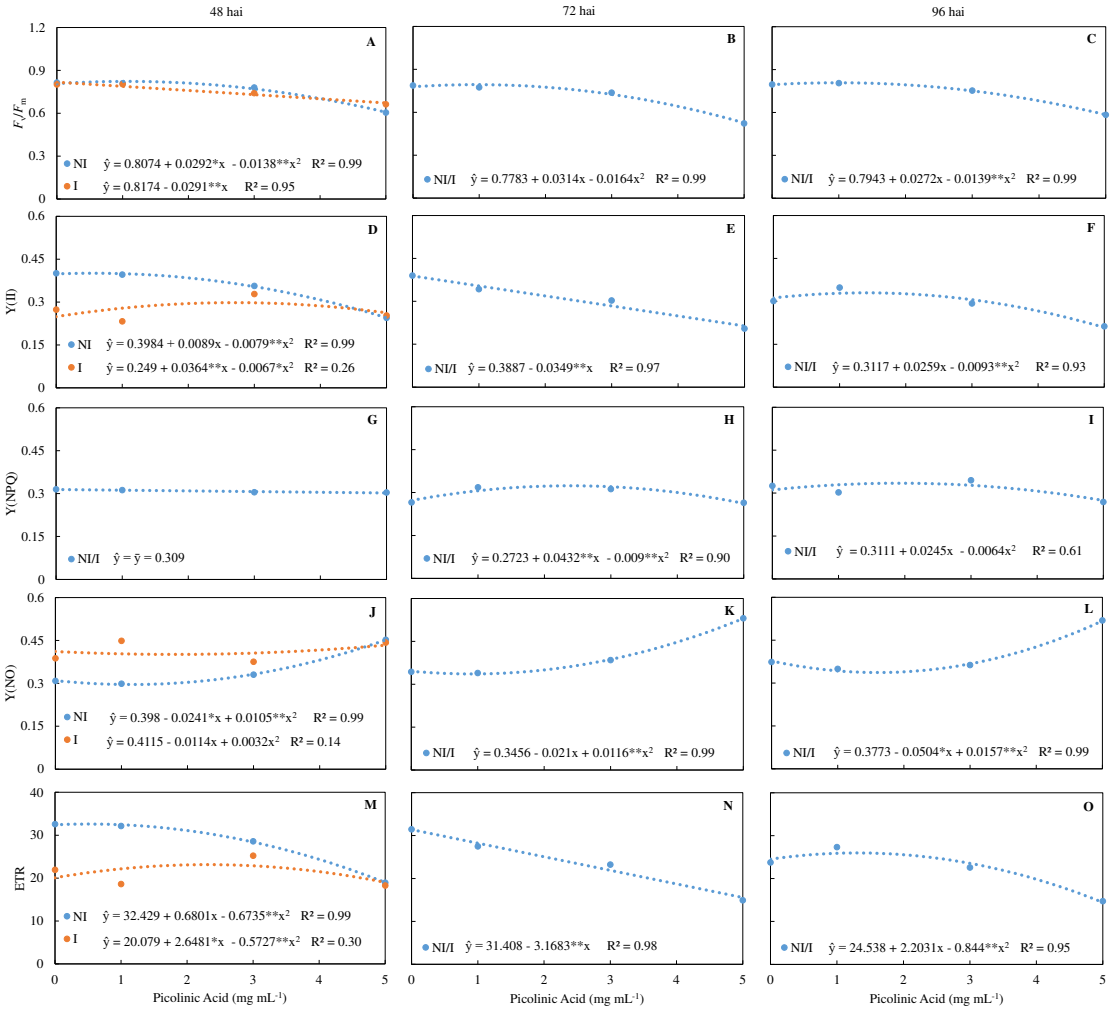

Figure 4. Chlorophyll (Chl) *a* fluorescence parameters: maximum PSII quantum efficiency (F_v/F_m) (A-C), effective PSII quantum yield (Y(II)) (D-F), quantum yield of regulated energy dissipation (Y(NPQ)) (G-I), quantum yield of non-regulated energy dissipation (Y(NO)) (J-L), and apparent electron transport rate (ETR) (M-O) determined on the leaves of rice plants sprayed with picolinic acid at the concentrations of 0, 1, 3, and 5 mg mL^{-1} and non-inoculated (NI) (blue dots) or inoculated (I) (orange dots) with *Bipolaris oryzae* at 48, 72, and 96 hours after inoculation (hai). * and **: significant at 1 and 5%, respectively.

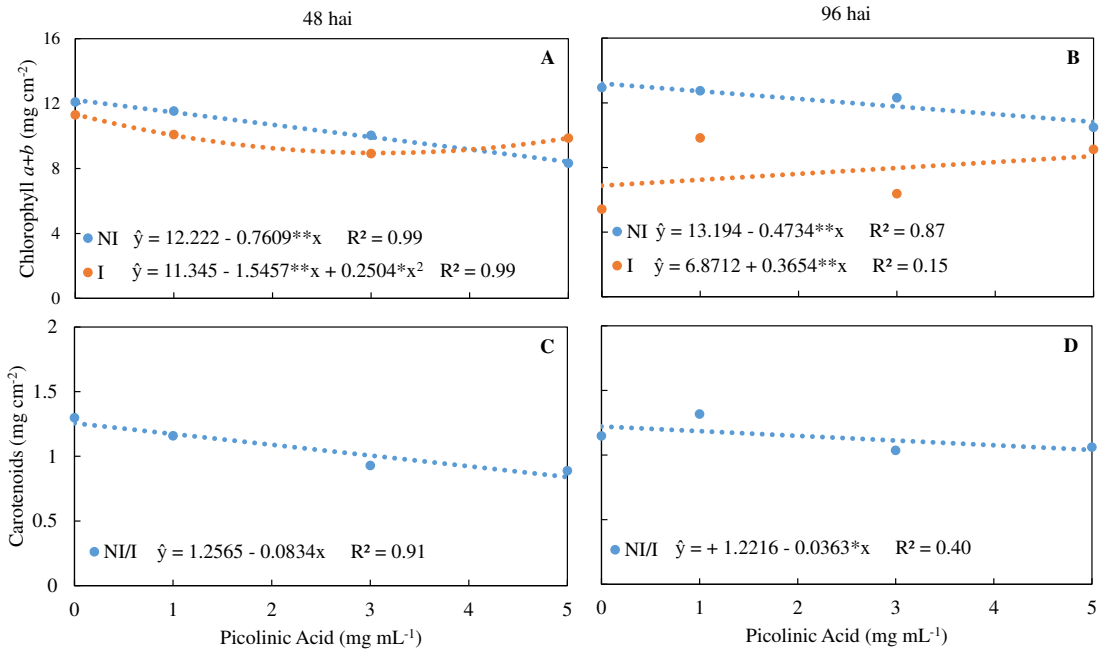

Figure 5. Concentrations of chlorophyll *a+b* (A and B) and carotenoids (C and D) determined on the leaves of rice plants sprayed with picolinic acid (PA) at the concentrations of 0, 1, 3, and 5 mg mL^{-1} and non-inoculated (NI) (blue dots) or inoculated (I) (orange dots) with *Bipolaris oryzae*. * and **: significant at 1 and 5%, respectively.