ANDERSOM MILECH EINHARDT

NICKEL AND GLYPHOSATE ON THE POTENTIATION OF SOYBEAN RESISTANCE AGAINST INFECTION BY *Phakopsora pachyrhizi*

Thesis presented to the Universidade Federal de Viçosa, as part of the requeriments of the Graduate Program in Plant Physiology, to obtain the title of *Doctor Scientiae*.

Advisor: Fabrício de Ávila Rodrigues

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(Advisor)

To my parents Seldina and Rui Alberto,

my source of inspiration

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To God, for life and for showing me the way.

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BIOGRAPHY

ANDERSOM MILECH EINHARDT was born in São Lourenço do Sul-Rio Grande do Sul, Brazil, on November 07th, 1991. In 2014, he obtained his undergraduate degree in Agronomy at the Federal University of Pelotas, Pelotas-RS, Brazil. On July 2016, he obtained his *Master Scientiae* degree in Plant Physiology at the Federal University of Pelotas, Pelotas-RS, Brazil. In July 2016, he started his doctoral studies in Plant Physiology Program at Federal University of Viçosa, Viçosa-MG, Brazil.

"*Más vale buena esperanza que ruin posesión"*

(Miguel de Cervantes, 1605)

ABSTRACT

EINHARDT, Andersom Milech, D.Sc., Universidade Federal de Viçosa, March, 2020. **Nickel and glyphosate on the potentiation of the soybean resistance against infection by *Phakopsora pachyrhizi***. Adviser: Fabrício de Ávila Rodrigues.

In this study, the effects of nickel (Ni) (60 g ha^{-1} Ni) and glyphosate (Gl) (960 g ha^{-1} e.a. Gl) spray on the antioxidative, defense, and ethylene metabolisms of soybean plants inoculated with *P. pachyrhizi* were evaluated. In the first experiment, the severity of Asian soybean rust (ASR) decreased by 35% in plants of cv. TMG 135 treated with Ni (+Ni). The malondialdehyde (MDA) concentration was higher in plants not treated with Ni (-Ni) than in +Ni plants and was linked to ASR severity and extensive colonization of the palisade and spongy parenchyma cells by fungal hyphae. The lignin concentration, β-1,3-glucanase (GLU) activity, and expression of the *URE* gene and the defense-related genes *PAL1.1*, *PAL2.1*, *CHI1B1*, and *PR-1A* were up-regulated in +Ni infected plants. Taken together, the information provided in this study showed the great potential of Ni to increase the basal level of soybean resistance to ASR and to complement other control methods within the context of sustainable agriculture. In the second experiment, ASR severity in plants of cv. TMG 135 decreased by 34% due to Ni supply. In inoculated plants, the MDA concentration and superoxide (O_2^-) and hydrogen peroxide (H_2O_2) accumulation were lower for +Ni plants in comparison to -Ni plants. The antioxidant enzymes activities were inefficient to avoid the high reactive species of oxygen (ROS) accumulation on -Ni inoculated plants. The photosynthetic pigments, maximum photochemical efficiency of photosystem II (PSII), effective yield of PSII, electron transport rate, rate of net carbon assimilation, stomatal conductance to water vapor, and transpiration rate values were higher and the yield for other non-regulated losses and internal CO_2 concentration values were lower for +Ni inoculated plants in comparison to -Ni inoculated plants. High ROS production and the great damage to the photosynthetic apparatus damage caused by *P. pachyrhizi* infection on -Ni plants affected the synthesis of the sugars and increased the energetic consumption limiting therefore, the plant energetic reserves faster in contrast to +Ni plants. In conclusion, the cellular oxidative damage and the impairment on the photosynthetic apparatus of soybean plants caused by *P. pachyrhizi* infection were alleviated by supplying Ni foliarly. In the thirst experiment, ASR severity was lower by 37, 68, and 77% in plants of cv. TMG 132 supplied with Ni, Gl, and Ni and Gl (Ni+Gl) in comparison to plants supplied with water (control). The inoculation caused largest and fastest increase in the concentration of ROS and MDA in control plants in comparison to Ni and Gl plants. In inoculated plants, the Ni and Gl increased phenylalanine

ammonia lyase and GLU activities and phenolics concentration. Additionally, Ni-treated plants showed a fasted cell wall lignification than control plants. Polyphenoloxidase activity was increased by Gl at 5 days after inoculation, regardless of *P. pachyrhizi* infection. In conclusion, this study demonstrated that Ni and Gl regulate differently the activity of defense enzymes and did not affect the antioxidant enzymes in soybean plants infected by *P. pachyrhizi*. In the fourth experiment, the ASR severity was reduced on plants of cv. TMG 132 sprayed with Ni and Gl. Carotenoids and chlorophylls concentrations were preserved for Ni, Gl, and Ni+Gl inoculated plants in comparison to that in control plants. Parameters of chlorophyll *a* fluorescence revealed photosynthetic apparatus damage and lowest destination of energy to photochemistry process on inoculated plants from the control treatment. Limitations on the photosynthetic machinery capacity of inoculated plants to capture light and use the absorbed energy by PSII reflected on their capacity to reduce the CO_2 as indicated by the high values for internal CO_2 concentration and low values for rate of net carbon assimilation. Low sugars concentration on inoculated plants from the control treatment was linked to their reduced photosynthetic capacity due to the high ASR severity. For non-inoculated plants, ethylene concentration was not affected by Ni and Gl, but its concentration decreased for inoculated plants being more pronounced for plants from the control treatment. In conclusion, this study sheds light into the role played by both Ni and Gl on ASR control from a physiological point of view. Soybean plants exposed to Ni and Gl were able to maintain their photosynthetic capacity and the great ethylene concentration during the infection process of *P. pachyrhizi*.

Keywords: Plant nutrition. Herbicide. Host defense. Photosynthesis. Asian Soybean Rust.

RESUMO

EINHARDT, Andersom Milech, D.Sc., Universidade Federal de Viçosa, março de 2020. **Níquel e glifosato na potencialização da resistência da soja à infecção por *Phakopsora pachyrhizi***. Orientador: Fabrício de Ávila Rodrigues.

Neste estudo, foram avaliados os efeitos da aplicação foliar de níquel (Ni) (60 g ha^{-1} Ni) e glifosato (Gl) (960 g ha^{-1} e.a. Gl) nos metabolismos antioxidante, de defesa e do etileno de plantas de soja inoculadas com *P. pachyrhizi*. No primeiro experimento, a severidade da ferrugem asiática da soja (ASR) diminuiu em 35% nas plantas do cv. TMG 135 tratadas com Ni (+Ni). A concentração de malondialdeído (MDA) foi maior nas plantas não tratadas com Ni (-Ni) em comparação com as plantas +Ni, corroborando com os valores de severidade da ASR e com a elevada colonização das células dos tecidos paliçádico e esponjoso pelas hifas do fungo. A concentração de lignina, a atividade da enzima β-1,3-glucanase (GLU) e a expressão do gene *URE* e dos genes relacionados à resistência *PAL1.1*, *PAL2.1*, *CHI1B1* e *PR-1A* foram regulados positivamente nas plantas +Ni inoculadas. Em conjunto, a informação gerada a partir desse estudo mostrou o potencial do Ni para incrementar o nível basal de resistência da soja contra a ASR e complementar outros métodos de controle em sistemas agrícolas mais sustentáveis. No segundo experimento, a severidade da ASR em plantas de soja do cv. TMG 135 diminuiu em 34% devido ao suprimento de Ni. Em plantas inoculadas, a concentração de MDA e o acúmulo de superóxido (O^{2-}) e de peróxido de hidrogênio (H_2O_2) foram menores nas plantas +Ni em comparação com as plantas -Ni. As atividades das enzimas antioxidantes foram insuficientes para evitar o alto acúmulo de espécies reativas de oxigênio (ROS) nas plantas -Ni inoculadas. A concentração de pigmentos fotossintéticos e os valores da máxima eficiência fotoquímica do fotossistema II (PSII), produção efetiva do PSII, taxa de transporte de elétrons, taxa líquida de assimilação de carbono, condutância estomática de vapor de água e da taxa transpiratória foram maiores e os valores do rendimento por outras perdas não reguladas e da concentração interna de CO_2 foram menores nas plantas +Ni inoculadas em comparação com as plantas -Ni inoculadas. A elevada produção de ROS e o maior dano no aparato fotossintético causado pela infecção com *P. pachyrhizi* em plantas -Ni afetou a síntese de açúcares e incrementou o consumo energético, limitando, dessa forma, as reservas energéticas da planta mais rapidamente em comparação com as plantas +Ni. Como conclusão, os danos oxidativos celulares e os prejuízos no aparato fotossintético de plantas de soja causados pela infecção com *P. pachyrhizi* foram amenizados pelo suprimento foliar de Ni. No terceiro experimento, a severidade da ASR foi menor em 37, 68 e 77% em plantas do cv. RR TMG 132 supridas com Ni, glifosato (Gl) e

Ni e Gl (Ni+Gl) em comparação com plantas supridas com água (controle). A inoculação promoveu um maior e mais rápido incremento na concentração de ROS e MDA nas plantas controle em comparação com plantas Ni e Gl. Em plantas inoculadas, o Ni e o Gl incrementaram a atividade das enzimas fenilalanina amônia liase e GLU e a concentração de compostos fenólicos. Adicionalmente, plantas tratadas com Ni apresentaram uma lignificação celular mais rápida em comparação com plantas controle. A atividade da enzima polifenoloxidase foi aumentada pelo Gl aos 5 dias após a inoculação, independente da infecção por *P. pachyrhizi.* Em conclusão, este estudo demonstrou que o Ni e o Gl regulam diferencialmente a atividade de enzimas de defesa e não afetam a atividade de enzimas antioxidantes em plantas de soja infectadas com *P. pachyrhizi*. No quarto experimento, a severidade da ASR foi reduzida em plantas do cv. RR TMG 132 pulverizadas com Ni e Gl. As concentrações de carotenóides e clorofila foram preservadas em plantas inoculadas dos tratamentos Ni, Gl e Ni+Gl em comparação com plantas inoculadas do tratamento controle. Os parâmetros de fluorescência da clorofila *a* revelaram danos ao aparato fotossintético e menor destinação de energia para o processo fotoquímico em plantas inoculadas do tratamento controle em comparação com as plantas inoculadas dos demais tratamentos. As limitações da maquinaria fotossintética para capturar a luz e para usar a sua energia pelo PSII refletiram na sua capacidade em reduzir o CO_2, como demonstrado pelos elevados valores de concentração interna de CO_2 e baixos valores na taxa de assimilação líquida de carbono. A baixa concentração de açúcares em plantas do tratamento controle foi relacionada com sua reduzida capacidade fotossintética devido à elevada severidade da ASR. Para plantas não inoculadas, a concentração de etileno não foi afetada pelo Ni e Gl, contudo, a concentração desse hormônio foi diminuída em plantas inoculadas, sendo essa diminuição mais pronunciada para plantas do tratamento controle. Em conclusão, esse estudo esclarece o papel desempenhado pelo Ni e pelo Gl no controle da ASR de um ponto de vista fisiológico. Plantas de soja expostas ao Ni e ao Gl foram capazes de manter sua capacidade fotossintética e a alta concentração de etileno durante o processo infeccioso de *P. pachyrhizi*.

Palavras-chave: Nutrição vegetal. Herbicida. Defesa vegetal. Fotossíntese. Ferrugem asiática da soja.

SUMMARY

GENERAL INTRODUCTION

Soybean (*Glycine max* (L.) Merrill) is the main source of protein used worldwide (Ravindran 2013). The well-balanced essential amino acid profile of soybean enables it to balance most cereal-based diets (Beski *et al.* 2015). Asian soybean rust (ASR), caused by the fungus *Phakopsora pachyrhizi* H. Sydow & P. Sydow, is considered one of the most important diseases affecting soybean production worldwide, with the potential to cause yield losses greater than 90% (Hartman *et al.* 2015). As a biotrophic pathogen, *P. pachyrhizi* depends on living plant tissues to complete its life cycle. The urediniospores are dispersed by the wind and begin to germinate under favorable environmental conditions as soon as they are deposited on leaf blades (Langenbach *et al.* 2016). ASR symptoms first appear as small chlorotic lesions delimited by the veins and evolve into larger necrotic lesions mainly on the abaxial leaf surface. After five to eight days, uredinia are detectable on the leaf blades and release many urediniospores during the infectious period (Goellner *et al.* 2010). Due to its biotrophic lifestyle, small cellular damage is observed at the beginning of the fungal infection process. However, under favorable environmental conditions and using susceptible cultivars, the efficient colonization of soybean leaf tissues by *P. pachyrhizi* is associated with high consumption of plant nutrients and disturb on the plant metabolism mainly due to oxidative stress (Waszczak *et al.* 2018).

The oxidative damage on cellular constituents of plants is caused by reactive species of oxygen (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and other types of ROS (Demidchik 2015). The action of ROS in cellular membranes generates an increase in malondialdehyde concentration that is used as an indicator of cellular damage (Schmid-Siegert *et al.* 2016). Moreover, ROS causes disturbs on the photosynthetic apparatus of plants with a consequent decrease in the synthesis of ATP, NADPH, and trioses phosphates (Foyer 2018). Limitations on photosynthesis and nutrients consumption by *P. pachyrhizi* causes energetic lose to soybean plants and can be the major factor associated with the great yield losses (Barón *et al.* 2012, Rios *et al.* 2018). Another important energetic cost imposed by ASR on soybean plants is related to the activation of host defense mechanisms against fungal infection and ROS detoxification (Neilson *et al.* 2013). In the last case, the enzymes superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) deserve to be highlighted in this process (Demidchik 2015).

Another important group of enzymes is involved more directly on the plant defense to pathogens, acting on the degradation of fungal cell wall (chitinases, CHI; and β-1,3-glucanase,

GLU) (Malik 2019), synthesis of quinones with antimicrobial action (polyphenoloxidase, PPO) (Taranto *et al.* 2017), oxidation of phenolics (POX) (Almagro *et al.* 2009), synthesis of compounds with signaling functions (lipoxygenases, LOX) (Wasternack and Feussner 2018), and in the phenylpropanoids pathway (phenylalanine ammonia lyase, PAL) (Hossain *et al.* 2018).

Mineral nutrients play a vital role in the metabolism of cultivated plants by influencing their growth, development and potentiating (increasing resistance from its basal level) or reducing their resistance to pathogen infection (Mur *et al.* 2016). Nickel (Ni) is an micronutrient involved in nitrogen metabolism, the formation of several biomolecules, and binding to approximately 500 proteins and peptides (Brown *et al.* 1987). Several enzymes require Ni for their catalytic process, such as urease (URE), SOD, CAT, NiFe hydrogenases, methyl coenzyme-M reductase, carbon monoxide dehydrogenase, acetyl coenzyme-A synthase, and RNAse-A (Harasim and Filipek 2015). Moreover, the degradation of purine bases in plants (*e.g.*, adenine and guanine) occurs via ureides, and Ni deficiency alters the metabolism of ureides and amino acids (Bai *et al.* 2006).

Deficiency of Ni results in the foliar accumulation of urea due to depression of urease activity, generating a lower conversion of urea to ammonia (Dixon *et al.* 1975). In pecan plants, Ni-deficiency developed a symptom characterized as ‘mouse-ear' (Brown *et al.* 1987). However, the occurrence of Ni-deficiency symptoms under field conditions is rarely reported due to its low requirement by plants and to its content (low as 0.2 mg kg^{-1} or as high as 450 mg kg^{-1}) in the soil solution (Harasim and Filipek 2015). Despite the low Ni-requirement by plants, the considerable Ni-content in the soil, and the high Ni-mobility in the plant tissues, suboptimal supply of Ni to plants is noticed when the mostly Ni-accumulation in the roots than in the other parts of the plant occur through roots uptake (Antonkiewicz *et al.* 2016). Thus, the foliar supply of Ni would be an interesting alternative to provide this micronutrient to the plants.

Few studies about the Ni effect on plant-pathogen interactions have been reported in the literature. The beneficial effects of this micronutrient on diseases control have been reported for the sugarcane-*Puccinia melanocephala* (Singh and Muthaujan 1968), lettuce-*Fusarium oxysporum* f. sp. *lactucae*, tomato-*F. oxysporum* f. sp. *lycopersici* (Ahmed *et al.* 2016), and soybean-*Microsphaera diffusa* (Barcelos *et al.* 2018) interactions.

The mechanism by which Ni potentiates host resistance to pathogen infection requires further elucidation. In adequate doses, Ni has a positive effect on plant growth and favors the activities of enzymes involved in antioxidative metabolism (Gomes-Junior *et al.* 2006), the biosynthesis of some phytoalexins (Ahmad and Ashraf 2011), and the activation of urease (Bai

et al. 2006). Despite the role of Ni in plant metabolism, its adequate supply is impaired in economically valuable crops growing in Ni-deficient soils. Considering that urease has a pivotal role on the resistance of soybean plant against ASR, the effect of the Ni on the plant-pathogen interaction soybean-*P. pachyrhizi* is an interesting case to be studied (Wiebke-Strohm *et al.* 2012).

Glyphosate (Gl) is the most successful herbicide in crop production areas (Duke 2018). As the Ni, the Gl affects the plant protection to pathogens, such alfalfa-*Colletotrichum trifolii* and -*Phoma medicaginis* (Samac and Foster-Hartnett 2012), wheat-*Puccinia triticina* and *P. striiformis* f. sp. *tritici* (Feng *et al.* 2005), eucalyptus-*Austropuccinia psidii* (Santos *et al.* 2019), and soybean-*P.pachyrhizi* (Feng *et al.* 2005, 2008). It is known that Gl have ability to interact with divalent cations and to form poorly soluble compounds (Duke *et al.* 2012), alter the hormonal balance in sublethal doses (Baylis 2000), increase the PAL activity, and promote alterations on the synthesis of amino acids (Hoagland *et al.* 1979). However, the mechanism that Gl act in the soybean-*P. pachyrhizi* interaction requires further elucidation. Non-lethal doses of Gl increased the production of ethylene by Gl non-resistant plants of common beans (Abu-irmaileh *et al.* 1979) and paper birch (Stasiak *et al.* 1992). On cotton plants, Gl acts disrupting auxin transport leading, therefore, to an increase on ethylene synthesis (Beyer and Morgan 1969). Distinctly, Pennazio and Roggero (1992) reported that Ni inhibited ethylene production in soybean plants by depressing ethylene-forming enzyme activity although it stimulated the production of free ACC (1-Aminocyclopropane-1-carboxylic acid), precursor of ethylene. It is commonly accepted that ethylene/jasmonic acid-dependent defenses are generally active against necrotrophic pathogens whereas salicylic acid-inducible defenses predominantly effective against the biotrophics (Zhang *et al.* 2009, Sun *et al.* 2017, Van Der Ent and Pieterse 2012). However, the effect of both Ni and Gl on the regulation of ethylene biosynthesis in soybean plants infected by *P. pachyrhizi* becomes one interesting topic to be investigated.

To gain further insights into the role of Ni and Gl in host-pathogen interactions, this study aimed to evaluate the effect of Ni, Gl, and their combination, in antioxidative, defense, sugars, and ethylene metabolisms, and in alleviating the cellular damage and the impairment of photosynthesis as a result of *P. pachyrhizi* infection on soybean plants.

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1. CHAPTER 1: NICKEL POTENTIATES SOYBEAN RESISTANCE AGAINST INFECTION BY *Phakopsora pachyrhizi*

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Abstract: Asian soybean rust (ASR), caused by the fungus *Phakopsora pachyrhizi*, causes significant yield losses worldwide. Nickel (Ni) plays a key role in the metabolism of some profitable crops, such as soybeans, because it is a constituent of several biomolecules and is required for the catalytic process of several enzymes. This study investigated the effect of foliar Ni spray (60 g ha^{-1}) on the potentiation of soybean (cultivar 'TMG 135') resistance to *P. pachyrhizi* infection at the microscopic, biochemical, and molecular levels. The severity of ASR decreased by 35% in plants treated with Ni. The malondialdehyde concentration, an indicator of cellular oxidative damage, was high in the leaves of plants that were not treated with Ni and was linked to ASR severity and the extensive colonization of the palisade and spongy parenchyma cells by fungal hyphae. The lignin concentration, β-1,3-glucanase activity, and expression of the *URE* gene and the defense-related genes *PAL1.1*, *PAL2.1*, *CHI1B1*, and *PR-1A* were up-regulated in Ni-treated plants infected with *P. pachyrhizi*. Taken together, the information provided by this study showed the great potential of Ni to increase the basal level of soybean resistance to ASR and to complement other control methods within the context of sustainable agriculture.

Keywords: *Glycine max*. Biotrophic pathogen. Host defense. Plant nutrition. Rust.

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1.1. INTRODUCTION

Asian soybean rust (ASR), caused by the fungus *Phakopsora pachyrhizi* H. Sydow & P. Sydow, is considered one of the most important diseases affecting soybean production worldwide, with the potential to cause yield losses greater than 90% (Hartman *et al.* 2015). As a biotrophic pathogen, *P. pachyrhizi* depends on living plant tissues to complete its life cycle. The urediniospores are dispersed by the wind and begin to germinate under favorable environmental conditions as soon as they are deposited on leaf blades (Langenbach *et al.* 2016). ASR symptoms first appear as small chlorotic lesions delimited by the veins and evolve into larger necrotic lesions mainly on the abaxial leaf surface. After five to eight days, uredinia are detectable on the leaf blades and release many urediniospores during the infectious period (Goellner *et al.* 2010).

Mineral nutrients play a vital role in the metabolism of cultivated plants by influencing their growth and development and potentiating (increasing resistance from its basal level) or reducing their resistance to pathogen infection (Mur *et al.* 2016). Nickel (Ni) is an essential micronutrient in higher plants, as it is involved in nitrogen metabolism, the formation of several biomolecules, and binding to approximately 500 proteins and peptides (Brown *et al.* 1987). Several enzymes require Ni for their catalytic process, such as urease (URE), superoxide dismutase, catalase, NiFe hydrogenases, methyl coenzyme-M reductase, carbon monoxide dehydrogenase, acetyl coenzyme-A synthase, and RNAse-A (Harasim and Filipek 2015). Moreover, the degradation of purine bases in plants (*e.g.*, adenine and guanine) occurs via ureides, and Ni deficiency alters the metabolism of ureides and amino acids (Bai *et al.* 2006).

The effect of Ni on the control of rusts caused by *Puccinia triticina* and *P. recondita* on wheat and rye, respectively, has been reported since the 1930s and before its essentiality to plants was established (Sempio 1936, Keil *et al.* 1958). Other host-pathogen interactions, such as sugarcane-*P. melanocephala* (Singh and Muthaujan 1968), lettuce-*Fusarium oxysporum* f. sp. *lactucae*, tomato-*F. oxysporum* f. sp. *lycopersici* (Ahmed *et al.* 2016), and soybean-*Microsphaera diffusa* (Barcelos *et al.* 2018), are also negatively affected by Ni.

The mechanism by which Ni potentiates host resistance to pathogen infection requires further elucidation. In adequate doses, Ni has a positive effect on plant growth and favors the activities of enzymes involved in antioxidative metabolism (Gomes-Junior *et al.* 2006), the biosynthesis of some phytoalexins (Ahmad and Ashraf 2011), and the activation of urease (Bai *et al.* 2006). Despite the role of Ni in plant metabolism, its adequate supply is impaired in economically valuable crops growing in Ni-deficient soils.

To gain further insights into the role of Ni in host-pathogen interactions, this study aimed to elucidate whether this micronutrient could potentiate defense mechanisms in soybean plants in response to *P. pachyrhizi* infection by analyses at the microscopic, biochemical, and molecular levels.

1.2. MATERIAL AND METHODS

1.2.1. Experimental design

A 2 × 2 factorial experiment, consisting of plants non-sprayed or sprayed with Ni (refereed as -Ni and +Ni plants thereafter) and non-inoculated or inoculated plants with *P. pachyrhizi*, was arranged in a completely randomized design with sixteen replications. Each experimental unit consisted of a plastic pot containing five plants. The experiment was carried out twice. Data from the variables evaluated from the two experiments were analyzed using the MIXED procedure of the SAS software (Release8.02Level02M0for Windows, SAS Institute) to determine if data from the experiments could be combined (Moore and Dixon 2015).

1.2.2. Plant growth

Soybean seeds from cultivar 'TMG 135' were sown in sand previously washed with HCl 1 N. This cultivar was selected for this study for having good growth architecture and with great susceptibility to ASR. Five seedlings were transferred to each 5 L plastic pots and cultivated in hydroponic system containing nutrient solution from Hoagland and Arnon (1950) with pH adjusted to 6.0. The nutrient solution was kept aerated, changed every four days, and with pH adjusted to 6.0 daily. Plants were kept in greenhouse (25 ± 3°C temperature, 80 ± 5% relative humidity) during the experiments.

1.2.3. Application of Ni and plant inoculation

Based on preliminary experiments realized (unpublished data) and on data provided by Barcelos *et al.* (2017, 2018), the dose of 60 g ha^{-1} Ni was used in the present study. Plants at the V4 growth stage (three fully expanded leaves) were sprayed with a solution (7.2 mL per plant) of 0.19 g L^{-1} $NiSO_4.6H_2O$ (equivalent to 60 g ha^{-1} Ni). At three days after Ni spray, plants were inoculated with a suspension of 1 × 10^5 urediniospores of *P. pachyrhizi* mL^{-1}. The urediniospores were collected from soybean plants (cv. 'TMG 135') previously inoculated with the monouredinial isolate UFV-DFP *Pp*25. The urediniospores were collected, preserved at -80ºC (Furtado *et al.* 2008), and their viability determined before plant inoculation. Vials

containing urediniospores with viability greater 50% were used to prepare the suspension. Gelatin (0.5% w/v) was added to the suspension to aid urediniospores adhesion to the leaf blades. The suspension was applied as a fine mist to the abaxial leaf surface of each plant using a VL Airbrush atomizer (Paasche Airbrush Co., IL, USA). Inoculated plants were kept in a plastic mist growth chamber (temperature of 25 ± 3°C and relative humidity of 90 ± 5%) inside a greenhouse. At 16 hours after inoculation, plants were transferred to a greenhouse with the same conditions previously described.

1.2.4. Evaluation of ASR severity

The first, second, and the third trifoliate leaves, from base to top, of five plants from replication (four replications and 20 plants total) of -Ni and +Ni treatments were collected at 15 days after inoculation (dai). The abaxial surface of the leaves was scanned at 1200 dpi resolution and the obtained images were processed using the software QUANT (Vale *et al.* 2003) to quantify ASR severity.

1.2.5. Determination of foliar Ni concentration

The second and third trifoliate leaves from plants used to evaluated ASR severity (15 dai) and also from the non-inoculated plants both non-supplied or supplied with Ni were collected (20 plants and 40 leaves total per treatment), washed in deionized water, and dried in a drying oven with forced ventilation. The foliar Ni was extracted by nitric-perchloric digestion method and determined by inductively coupled plasma-optical emission spectrometry (ICP-OES).

1.2.6. Biochemical analysis

The second and third trifoliate leaves, from base to top, of two plants from replication of each treatment (four replications and 8 plants per each sampling time) were collected at 1, 2, 3, 5, 10, and 15 dai. Leaf samples were kept in liquid nitrogen during sampling and stored at -80°C until further analysis. Part of these leaf samples were saved to be used for the molecular analysis. Plants that had their leaves collected were discarded after each sampling.

1.2.7. Concentration of malondialdehyde (MDA)

Cellular oxidative damage was estimated based on the production of total 2-thiobarbituric acid reactive substances and expressed as equivalents of MDA according to Heath and Packer (1968) with a few modifications. A total of 100 mg of leaf tissue collected at 10 and 15 dai was ground into a fine powder using a vibration ball mill (Retsch, Haan, Germany) with liquid

nitrogen and homogenized in 2 mL of 0.1% (w/v) trichloroacetic acid solution. The homogenate was centrifuged at 12,000 *g* for 15 min at 4°C. After centrifugation, 0.25 mL of the supernatant was added to 0.75 mL of thiobarbituric acid solution (0.5% in 20% trichloroacetic acid) and held for 60 min at 99°C in a thermomixer (Eppendorf, Hamburg, Germany). The reaction was stopped in an ice bath. After the solution reached room temperature, the absorbance was read at 532 nm and discounting the non-specific absorbance at 600 nm. The molar extinction coefficient of 155 mM^{-1} cm^{-1} was used to calculate the MDA concentration.

1.2.8. Concentrations of total soluble phenolics (TSP) and lignin-thioglycolic acid (LTGA) derivatives

A total of 100 mg of leaf tissue collected at 5, 10, and 15 dai was ground into a fine powder with liquid nitrogen using a vibration ball mill 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 following the procedures of Fortunato *et al.* (2015).

1.2.9. Activities of chitinase (CHI) (EC 3.2.1.14), *β*-1,3-glucanase (GLU) (EC 3.2.1.39), polyphenoloxidase (PPO) (EC 1.10.3.1), peroxidase (POX), and lipoxygenase (LOX) (EC 1.13.11.12)

A total of 200 mg of leaf tissue collected at 1, 2, 3, 5, and 10 dai was ground into a fine powder using a vibration ball mill with liquid nitrogen. The fine powder was homogenized in 2 mL of a solution containing 50 mM potassium phosphate buffer (pH 6.8), 0.1 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, and 2% (m/v) polyvinylpyrrolidone. The homogenate was centrifuged at 13,000 *g* for 15 min at 4°C and the supernatant was collected to be used to determine CHI, GLU, PPO, POX, and LOX activities. The activities of these enzymes were determined as previously described by Fortunato *et al.* (2015) and expressed on the basis of protein.

1.2.10. Genes expression using quantitative real-time PCR

The second and third trifoliate leaves, from base to top, of two plants from replication of each treatment (four replications and 8 plants per each sampling time) were collected at 1, 2, 3, 5, and 10 dai. Leaf samples were kept in liquid nitrogen during sampling and stored at -80°C until further analysis. A total of 75 mg of leaf tissue was ground into a fine powder using a

vibration ball mill with liquid nitrogen with four replications. RNA was extracted with Trizol (Invitrogen®). DNA contaminations were removed with RQ1 RNase-Free DNase (Promega). The amount of RNA was measured in a Qubit fluorometer using Qubit RNA HS Assay Kit (Invitrogen, São Paulo, Brazil) and the quality and integrity of the RNA were verified by 1% agarose gel electrophoresis. Single-stranded cDNAs were synthesized by reverse transcription using 3 μg of total RNA with oligo(dT) primers in a final volume of 20 μL using the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen®). The qRT-PCR was performed on a Bio-Rad CFX Real Time Thermal Cycler using SYBR Green PCR Master Mix according to the manufacturer's recommendations. All reactions were performed in triplicate and the relative expression values for each gene studied were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Expression analysis of genes coding for phenylalanine ammonia-lyase (*PAL1.1*, *PAL1.3*, *PAL2.1*, and *PAL3.1*), chitinase (*CHIA1*), chalcone isomerase (*CHI1B1*), lipoxygenase (*LOX7*), metalloproteinase (*MMP2*), isochorismate synthase (*ICS1* and *ICS2*), urease (*URE*), resistance-related protein (*PR-1A*), and an AP2/ERF type transcription factor (*ERF3*) was performed using specific primers sequence (Table S1). The *TEF1* gene expression that corresponded to the 18S ribosomal portion of *P. pachyrhizi* was also quantified as an indicator of fungal presence in the plant tissue. The Ubiquitin-3 gene was used as a reference for normalization as previously described by Mortel *et al.* (2007).

1.2.11. Light microscopy

The second trifoliate leaf, from base to top, of one plant per replication of each treatment (four replications and 4 plants per each sampling time) were collected at 6 and 12 dai. From each leaf, a total of 15 fragments (≈ 25 mm^2) were collected and fixed in a solution of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C. Afterwards, leaf fragments were dehydrated in an ethyl series and included in Historesin methacrylate (Leica Instruments, Heidelberg, Germany). Four blocks were prepared for each treatment. Each block contained two leaf fragments. Cross sections of 4 μm thick, obtained in an automatic advance rotary microtome model RM 2255 (Leica Microsystems Inc., Deerfield, IL, USA), were stained with 0.1% toluidine blue solution (pH 4.7). Twelve semi-thin sections, obtained per each block, were placed in glass slides. The images of the infected leaf tissues were acquired digitally (Axio Cam HR; Carl Zeiss) using a Carl Zeiss Axio Imager A1 microscope (Carl Zeiss) and further processed using the AXION VISION v. 4.8.1 software.

1.2.12. Data analysis

Data from the variables evaluated were checked for normality and homogeneity of variance and subjected to analysis of variance (ANOVA) thereafter. Treatments means were compared by F test ($P \leq 0.05$). For defense enzymes activities, gene expression, MDA, TSP, LTGA derivatives, and foliar Ni concentrations, ANOVA was considered to be a 2×2 factorial experiment consisting of non-inoculated and inoculated plants and plants non-sprayed or sprayed with Ni with four replications. Principal components analysis (PCA) using data from genes expression was performed aiming to determine the relationship between the variables evaluated. Data were analyzed using the Minitab software (version 18, Minitab Corporation).

1.3. RESULTS

1.3.1. Foliar Ni concentration

For -Ni plants, the foliar Ni concentrations were 4.6 and 4.5 mg kg^{-1} dry weight for non-inoculated and inoculated plants, respectively. The foliar Ni concentrations were 40.4 and 18.5 mg kg^{-1} dry weight for non-inoculated and inoculated +Ni plants, respectively.

1.3.2. Symptoms of ASR and ASR severity

On the leaves of -Ni plants, there were many necrotic lesions containing several uredinia, while on the leaves of +Ni plants, the lesions were much less developed (Fig. 1A-B). The ASR severity in +Ni plants was significantly reduced by 35% in comparison to that in -Ni plants (Fig. 1C).

1.3.3. Histopathology of the *P. pachyrhizi* infection and MDA concentration

Fungal hyphae colonized the palisade and spongy parenchyma cells more abundantly on the leaf tissues of -Ni plants (Fig. 2A and C) in comparison to +Ni plants (Fig. 2B and D). The palisade and spongy parenchyma cells were more degraded in the leaf tissues of -Ni plants (Fig. 2A and C) in comparison to +Ni plants (Fig. 2B and D), due to the extensive colonization of fungal hyphae. At 6 dai, uredinia formation was first observed on the leaves of -Ni plants (Fig. 2A) in comparison to +Ni plants (Fig. 2B) and was more abundant and developed on the leaves of -Ni plants (Fig. 2C) than on the leaves of +Ni plants at 12 dai (Fig. 2D). The MDA concentration was significantly higher by 37% at 15 dai in -Ni inoculated plants in comparison to +Ni inoculated plants. In -Ni inoculated plants, the MDA concentration at 15 dai increased by 33% in comparison to that at 10 dai (Fig. 3A-B).

1.3.4. Concentrations of TSP and LTGA derivatives

There was no significant difference in the TSP concentration between the -Ni and +Ni plants regardless of inoculation with *P. pachyrhizi* (Fig. 4A-B). Comparing non-inoculated and inoculated plants, the increase in the LTGA derivatives concentration was significantly higher by 49 and 31% in -Ni and +Ni plants at 10 dai, respectively, and by 40 and 65% in -Ni and +Ni plants at 15 dai, respectively (Fig. 4C-D). For inoculated plants at 15 dai, the concentration of LTGA derivatives in +Ni plants significantly increased by 34% in comparison to that in -Ni plants (Fig. 4D).

1.3.5. Defense-related enzyme activities

There was no significant difference between -Ni non-inoculated plants and +Ni non-inoculated plants regardless of the enzymes studied and the evaluation time (Fig. 5A, C, E, and I), except for POX, which had significantly higher activity by 31, 39, and 56% at 1, 2, and 3 dai, respectively, in +Ni plants in comparison to -Ni plants (Fig. 5G). For inoculated plants, CHI activity increased by 48 and 49% in -Ni plants and by 37 and 66% in +Ni plants at 2 and 10 dai, respectively (Fig. 5A-B). GLU activity was significantly higher by 35 and 23% at 5 and 10 dai, respectively, in +Ni inoculated plants in comparison to -Ni inoculated plants (Fig. 5D). Comparing non-inoculated and inoculated plants at each evaluation time and Ni supply, GLU activity was significantly higher by 73 and 84% at 5 and 10 dai, respectively, in +Ni inoculated plants in comparison to +Ni non-inoculated plants. GLU activity was also significantly higher by 38% at 10 dai in -Ni inoculated plants than in -Ni non-inoculated plants (Fig. 5C-D). PPO activity increased by 33 and 24% at 1 dai in inoculated -Ni and +Ni plants, respectively, in comparison to non-inoculated plants (Fig. 5E-F). POX activity was higher in -Ni inoculated plants than in -Ni non-inoculated plants at each evaluation time. For the +Ni inoculated plants, POX activity increased by 24 and 63% at 1 and 10 dai, respectively (Fig. 5G-H). There was no effect of Ni and plant inoculation on LOX activity (Fig. 5I-J).

1.3.6. Expression of defense-related genes

Considering that there were no significant differences among the evaluated times for -Ni and +Ni non-inoculated plants (Fig. 6A-B), the fold change was based on the medium values of $2^{-\Delta CT}$ in -Ni non-inoculated plants at 1 dai. The expression of the genes evaluated in the +Ni non-inoculated plants did not differ from that in the -Ni non-inoculated plants (Fig. 6A-B). The expression of defense-related genes (*PAL3.1*, *CHIA1*, *CHI1B1*, *PR-1A*, and *MMP2*) increased 1 dai for both the inoculated -Ni and +Ni plants (Fig. 6A-D). Additionally, *PAL1.1*, *PAL2.1*, and *URE* gene expression in +Ni inoculated plants increased at 1 dai in comparison to that in

+Ni non-inoculated plants (Fig. 6B and D). At 2 dai, *PAL1.1* gene expression in +Ni inoculated plants was 0.6 times higher than that in +Ni non-inoculated plants. The expression of the *CHI1B1* and *MMP2* genes was high at 2 dai in -Ni and +Ni inoculated plants. The expression of the *MMP2* gene was higher 3 dai in inoculated than in non-inoculated plants regardless of Ni treatment (Fig. 6A-D). At 3 dai, *CHI1B1* gene expression was high only in +Ni inoculated plants in comparison to +Ni non-inoculated plants (Fig. 6B and D). No significant difference in the expression of the majority of the defense-related genes occurred at 5 dai between non-inoculated and inoculated plants, except for the *ICS2* and *ICS2* genes, which showed reduced expression in inoculated compared to non-inoculated plants regardless of Ni supply. The expression of the majority of defense-related genes (*PAL1.3*, *PAL2.1*, *PAL3.1*, *CHIA1*, *PR-1A*, and *MMP2*) at 10 dai increased more in inoculated plants than in non-inoculated plants regardless of Ni supply. There was no significant change in *ERF3* gene expression regardless of Ni supply, plant inoculation, or evaluation time (Fig. 6A-D). Considering that the *TEF1* gene was not expressed in non-inoculated plants, its fold change was based on the medium values of $2^{-\Delta CT}$ for -Ni inoculated plants at 1 dai. At each evaluation time, *TEF1* gene expression was significantly higher by 0.6, 0.8, and 2.2 times at 3, 5, and 10 dai, respectively, in -Ni inoculated plants in comparison to +Ni inoculated plants. Following this same comparison, there was a significant increase in the expression of the genes *PAL1.1* at 2 dai (0.6-fold increase), *PAL2.1* at 1 dai (one-fold increase), *CHI1B1* at 1 (two-fold increase), 2 (0.5-fold increase), and 3 dai (0.6-fold increase), and *PR-1A* at 1 (0.7-fold increase) and 10 dai (0.9-fold increase) in +Ni plants (Fig. 6C-D).

The first two PCs covered most of the variation in the dataset; PC 1 and PC 2 explained 53 and 25% of the total variance, respectively (Fig. 7A-B). Based on cluster analysis with complete linkage and Pearson distance, four clusters (C) were generated: C1 (+Ni inoculated plants at 1 dai), C2 (+Ni inoculated plants and -Ni inoculated plants at 10 dai), C3 (+Ni inoculated plants at 2 dai and -Ni inoculated plants at 1 dai), and C4 (other treatments and evaluation times) (Fig. 7A). PC 1 showed positive scores for *URE*, *PAL1.1*, *PAL1.3*, *PAL2.1*, *PAL3.1*, *CHI1B1*, *LOX7*, *PR-1A*, *CHIA1*, *MMP2*, *ERF3*, and *TEF1* and negative scores for *ICS1* and *ICS2* (Fig. 7B).

1.4. DISCUSSION

The present study provides, to the best of the authors' knowledge, novel evidence of the effect of foliar Ni application on the potentiation of the resistance of the specific soybean cultivar 'TMG 135' to *P. pachyrhizi* infection. The high foliar Ni concentration in plants

supplied with this micronutrient, regardless of inoculation with *P. pachyrhizi*, confirmed its absorption. On the other hand, the lowest foliar Ni concentration in inoculated Ni-supplied plants in comparison to non-inoculated Ni-supplied plants may be explained by Ni mobilization to other parts of the plant as a result of fungal infection. The high expression of the *TEF1* gene corroborated the increased ARS severity, indicating that Ni played an important role in affecting the colonization of leaf tissues by fungal hyphae. The great ASR symptoms on the leaves of plants that were not treated with Ni were accompanied by high MDA concentration at advanced stages of fungal infection, indicating intense damage to cellular membranes caused by the extensive colonization of leaf tissues by fungal hyphae.

In the present study, the high concentration of LTGA derivatives in the leaves of +Ni inoculated plants may have contributed to impeding further leaf tissue colonization by *P. pachyrhizi,* corroborating the least severe ASR symptoms in +Ni plants. Lygin *et al.* (2009) reported that the cell walls of the leaves of soybean plants from cultivars resistant to ASR showed higher lignin deposition than those from susceptible ones.

PAL catalyzes the first step in the phenylpropanoid pathway, producing cinnamic acid and ammonia (Hossain *et al.* 2018). In soybean, PAL is encoded by a small gene family consisting of only three members with different subgroups (Frank and Vodkin 1991). In the present study, *PAL* transcripts increased earlier (1 dai) and at the advanced (10 dai) stage of *P. pachyrhizi* infection. In the presence of Ni and *P. pachyrhizi* infection, the transcript levels of *PAL2.1* at 1 dai and *PAL1.1* at 1 and 2 dai were high, indicating their importance in soybean resistance to ASR. According to Zhang *et al.* (2017), high *PAL2.1* gene expression was associated with the resistance of soybean plants to *Phytophthora sojae* infection. Soybean plants infected with *Pseudomonas syringae* and *P. sojae* also showed an increase in the expression of the *PAL1.1*, *PAL1.3*, and *PAL3.1* genes (Shine *et al.* 2016). Mortel *et al.* (2007) compared two soybean genotypes contrasting in their resistance to ASR and noticed an earlier increase in the expression of these genes in plants from the resistant genotype (PI230970) than in the susceptible genotype (Embrapa-48). Moreover, genes related to the phenylpropanoid pathway were upregulated in soybean plants from a cultivar carrying R-genes for resistance to ASR (Hossain *et al.* 2018).

Cinnamic acid, the first phenolic compound produced due to PAL activity in the phenylpropanoid pathway, can be directed to the synthesis of flavonoids, salicylic acid, or other phenolics (Dixon *et al.* 2002). Thus, high PAL activity should result in an increase in the concentration of phenolics. No changes in the TSP concentration occurred for soybean plants regardless of *P. pachyrhizi* inoculation and Ni application. The phenolics produced may have

been allocated to the synthesis of lignin, a polymer formed by the enzymatic oxidation of phenolic monomers (Sangha *et al.* 2014), mainly in +Ni inoculated plants.

The expression of the *CHI1B1* gene, which encodes chalcone isomerase, was high in +Ni inoculated plants at early stages of *P. pachyrhizi* infection and may be linked to driving the products of the phenylpropanoid pathway into the production of flavonoids. The isoflavonoids glyceollin, daidzein, and genistein are involved in the resistance of soybean plants to ASR (Lygin *et al.* 2009, Ahuja *et al.* 2012).

Soybean plants transport substantial amounts of nitrogen as ureides rather than as amides or amino acids. Ureides can be metabolized to urea and subsequently converted to NH_4^+ and therefore play an important role as key N compounds (McClure and Israel 1979). Urease plays a role in ureide catabolism in pecan (Bai *et al.* 2006) but not in soybean (Stebbins and Polacco 1995). Urea can also be generated from the conversion of arginine to ornithine (Witte 2011). Considering that ureases are Ni-dependent metalloenzymes that catalyze the conversion of urea to ammonia and carbon dioxide (Bai *et al.* 2006), the high expression of the *URE* gene in +Ni inoculated plants may have contributed to modifying the NH_4^+ pool in the infected leaf tissues and increasing its availability for the production of compounds containing nitrogen. Reduced urease activity in Ni-deficient soybean plants resulted in urea accumulation in leaf tissues (Eskew *et al.* 1983). Wiebke-Strohm *et al.* (2012) reported a differential expression of the *GmEu4* gene, which encodes urease, between resistant and susceptible soybean genotypes to ASR mainly at 24 h after fungal infection. These authors reinforced the importance of urease in the resistance of soybean to *P. pachyrhizi* infection with the generation of mutants deficient in the synthesis of this enzyme.

Plants adapt to changes in metabolism by altering their physiological status and coordinating different biochemical reactions. Changes in protein metabolism that lead to a high and rapid production of PR proteins are a common response of plants to counteract infection by pathogens (Jain and Khurana 2018). The expression of the *PR-1A* gene in +Ni plants infected with *P. pachyrhizi* can be linked to increased resistance to ASR. The matrix metalloproteinase *MMP2* gene that produces antimicrobial peptides is activated in soybean in response to pathogen infection (Liu *et al.* 2001). However, in the present study, no change in the expression of the *MMP2* gene was detected in +Ni plants infected with *P. pachyrhizi*.

In contrast to the reported results of soybean mosaic virus infection in soybean, hormone application, and exposure to abiotic stress (Zhang *et al.* 2009, Hernandez-Garcia and Finer 2016), the expression of the *ERF3* gene was unchanged in +Ni inoculated plants until 10 dai. The *ICS1* and *ICS2* genes were downregulated in infected soybean plants at 5 dai in contrast to

non-inoculated plants regardless of Ni supply. In contrast, in Arabidopsis, in which *ICS1*-derived biosynthesis contributes to approximately 98% of pathogen-induced salicylic acid production (Wildermuth *et al.* 2001), the PAL and ICS (isochorismate synthase) pathways in soybean are equally important for pathogen-induced salicylic acid biosynthesis (Shine *et al.* 2016).

Chitinases hydrolyze internal β-1,4-N-acetyl-D-glucosamine linkages of chitin polymers, a major component of fungal cell walls (Hamid *et al.* 2013). The class I chitinases are localized to vacuoles and are among the major PR-related proteins reported to be important in many host-pathogen interactions, such as potato-*Phytophthora infestans* (Beerhues and Kombrink 1994), wheat-*Puccinia graminis* (Liao *et al.* 1994), and soybean-*P. sojae* (Gijzen *et al.* 2001). The infection of soybean plants with *P. pachyrhizi* resulted in the upregulation of the *CHIA1* gene at 1 and 10 dai and was associated with an increase in CHI activity at 2 and 10 dai. Cruz *et al.* (2013) reported high CHI activity in soybean plants infected with *P. pachyrhizi* at 3 dai. No changes in *CHIA1* gene expression and CHI activity were detected between -Ni and +Ni plants. β-1-3 glucan is an important constituent of many fungal cell wall, and an increase in the activity of the hydrolase GLU has been linked to the resistance of plants in response to fungal infection (Huang 2001). In the present study, a substantial and rapid increase in GLU activity in inoculated +Ni plants could explain the reduction in ASR symptoms. PPO catalyzes the oxidation of polyphenols into quinones, which exhibit antimicrobial action against several plant pathogens (Taranto *et al.* 2017). PPO activity was high only in infected soybean plants regardless of Ni supply. Kováčik *et al.* (2009) did not detect an effect of Ni on PPO activity in the leaves of chamomile plants. Interestingly, Ni played a regulatory effect on POX activity in non-inoculated plants. POX is important in eliminating excess hydrogen peroxide and for the oxidation of phenolics (Rossi *et al.* 2017). In general, POX activity increased in infected soybean plants regardless of Ni supply. POX activity also increased in wheat plants supplied with Ni (Gajewska and Sklodowska 2007). LOX promotes the oxidation of polyunsaturated fatty acids, initiating the synthesis of oxylipins, which have different cellular functions (Wasternack and Feussner 2018). In plants infected with pathogens, LOX is linked to the synthesis of different compounds with signaling functions (Porta and Rocha-Sosa 2002). In the present study, in addition to Ni, plant inoculation had no effect on *LOX* gene expression or LOX activity, probably due to the biotrophic lifestyle of *P. pachyrhizi*, which does not need to release hydrolytic enzymes or even nonhost selective toxins to successfully infect soybean leaves (Mendgen and Hahn 2002). Based on the PCA analysis of the gene expression data, there was

a high and rapid expression of defense genes in response to *P. pachyrhizi* infection potentiated by Ni.

Based on the results of the present study, Ni had a beneficial effect on the potentiation of the defense responses of a susceptible soybean genotype against infection by *P. pachyrhizi* mainly by stimulating the expression of the *CHI1B1*, *PAL*, *PR-1A*, and *URE* genes and *β*-1,3-glucanase activity in addition to increasing lignin production. The energetic cost of the activation of plant defense mechanisms can result in a negative effect on crop yield (Neilson *et al.* 2013). Thus, the cost of increasing soybean resistance to ASR, potentiated by Ni, needs to be better elucidated. Moreover, additional studies with other soybean genotypes and other isolates of *P. pachyrhizi* are required to gain additional insights into the mechanisms underlying the increase in soybean resistance by Ni application.

1.5. TABLES AND FIGURES

Genes	GenBank Id.	Primer sense 5'-3'	Primer antisense 5'-3'
PAL1.1	Glyma19g182300	GCAAGTGCAACCATAATCATTT	AACCAAAGCTCCGGCAAA
PAL1.3	Glyma03g181600	TTTGTACCTATGCAAGAAAAACCA	TGAAGGAACATTGAAATTAGGCT
PAL2.1	Glyma10g058200	ATCTCCCTCCACTCACCATA	GTTCAAGGGGTCATTAGCAC
PAL3.1	Glyma02g309300	TGCTCTTCAGAAGGAAATGGT	GTTGCTGATTTAGGCAGTGT
CHIA1	Glyma02g0425001	TTCTTGGCTCAAACTTCTCATGAA	CCCACGCATATGGACCATCT
CHI1B1	Glyma20g2416001	GTTTCCCCTGCTTTGAAAGAGA	GGATTGGCCTCTAACTCTTTGAAG
LOX7	Glyma13g347800	ACAAGCTAGGCACAACAAAA	TTGTTTCCTCCGATGATTCCAA
PR-1A	AF136636.1	GCACTACACACAGGTCGTTTGG	CCTCCGTTATCACATGTCACTTTG
MMP2	Glyma01g036900.1	TGGGCTCTTCCCAGTGAAA	TTGCCGCACTCTCCAAGTC
TEF1	EF560586.1	ATTCGAAGCCGGTATTTCTAAAG	CCACTTGGTTGTGTCCATCTTAT
URE	Glyma11g248700	AGTTAGTCACCATTCATGACCT	CAAGTGAAGGTACTGGAAGAAAA
ERF3	Glyma03g263700	AACTCGCTCAAGCATCTCTT	CAACAATCGAGATTTCCCCAA
ICS1	Glyma01g104100	GAAACAGTACAGTCCCTGCT	TGTGGCTGGGAAAAGAAAAC
ICS2	Glyma03g070600	GCAACATCCTCGTACCTCTT	CTCTCTGCAACCGTTCATTG
UBIQ	Glyma20g141600	TGTAATGTTGGATGTGTTCCC	GGGACACAATTGAGTTCAACA

Table S1. Genes and their primer sequences analyzed in the leaves of soybean plants non-sprayed or sprayed with nickel and non-inoculated or inoculated with *Phakopsora pachyrhizi* by using quantitative reverse transcription PCR.

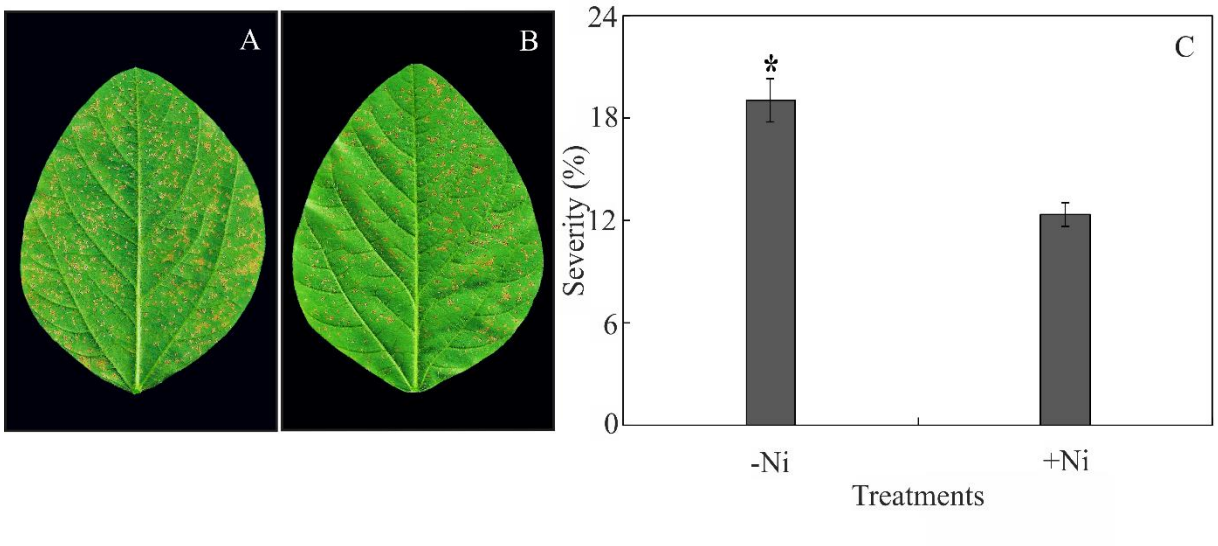

Figure 1. Symptoms of Asian soybean rust (A and B) and severity (C) in the leaves of soybean plants non-sprayed (-Ni) or sprayed (+Ni) with nickel (Ni). Means for each treatment followed by an asterisk (*) are significantly different ($P \leq 0.05$) according to F test. Bars represent the standard error of the means. $n = 8$.

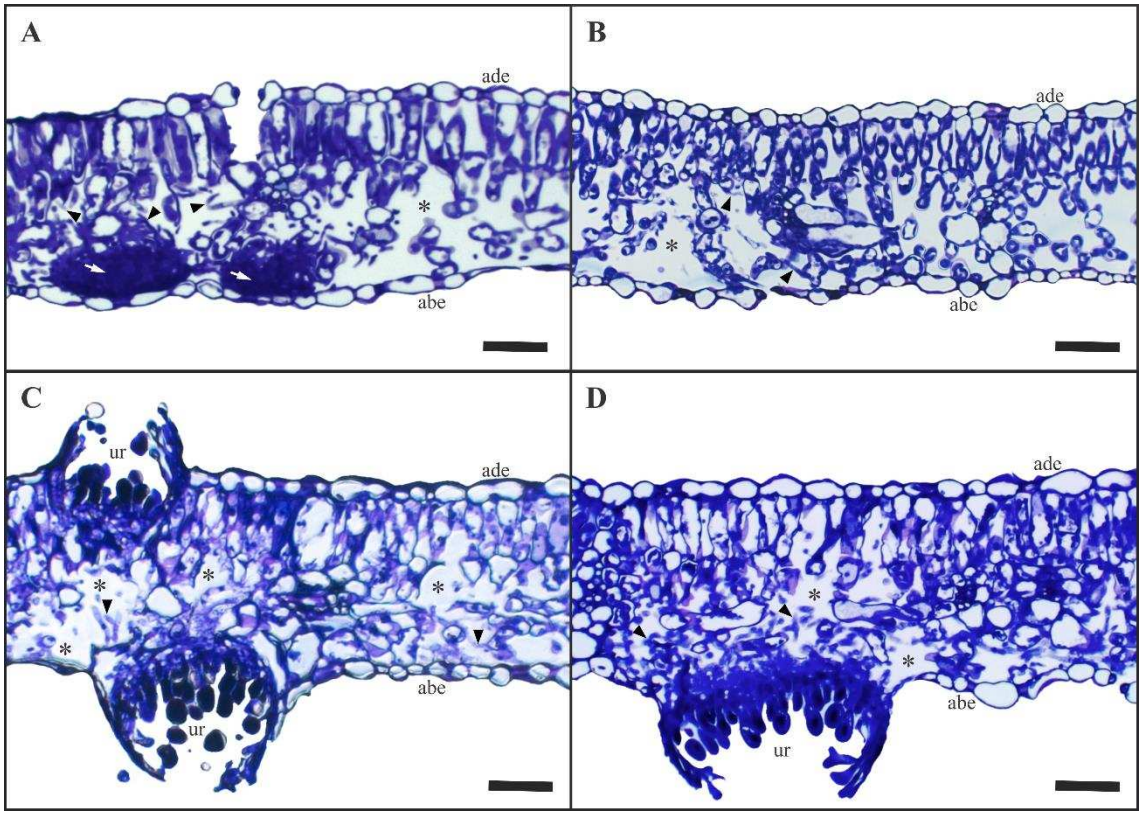

Figure 2. Light micrographs of transverse sections of leaf tissues of soybean plants non-sprayed (-Ni) (A and C) or sprayed (B and D) (+Ni) with nickel (Ni) at 6 (A and B) and 12 (C and D) days after inoculation (dai) with *Phakopsora pachyrhizi*. Fungal hyphae (arrowheads) colonized more abundantly the palisade and the spongy parenchyma cells on the leaf tissues of -Ni plants (A and C) in comparison to +Ni plants (B and D). The palisade and spongy parenchyma cells were more degraded (asterisks) in the leaf tissues of -Ni plants (A and C) due to the massive fungal hyphae colonization in comparison to +Ni plants (B and D). Uredinia formation were first noticed on the leaves of -Ni plants at 6 dai (A, arrows) and were more abundant and developed (C) in comparison to the leaves of +Ni plants (D). Adaxial epidermis (ade), abaxial epidermis (abe), and ur = uredinia. Scale bars = 20 µm.

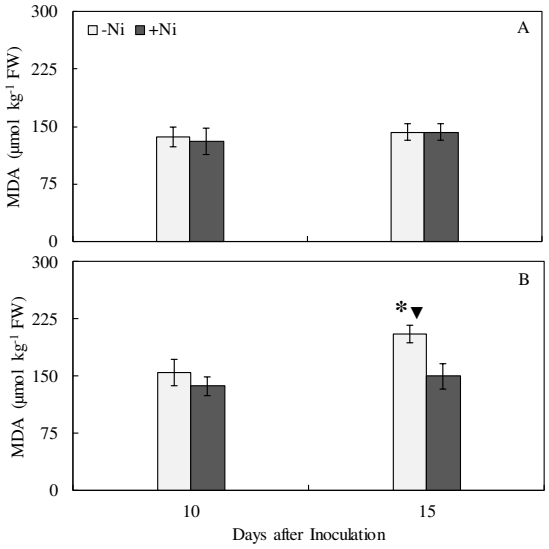

Figure 3. Concentration of malondialdehyde (MDA) in the leaves of soybean plants non-sprayed (-Ni) or sprayed (+Ni) with nickel (Ni) and non-inoculated (NI) (A) or inoculated (I) (B) with *Phakopsora pachyrhizi*. For each evaluation time, means for the -Ni and +Ni treatments followed by an asterisk (*) and for the NI and I treatments followed by an inverted triangle (▼) are significantly different ($P \leq 0.05$) according to Tukey's test. FW = fresh weight. Bars represent the standard error of the means. $n = 8$.

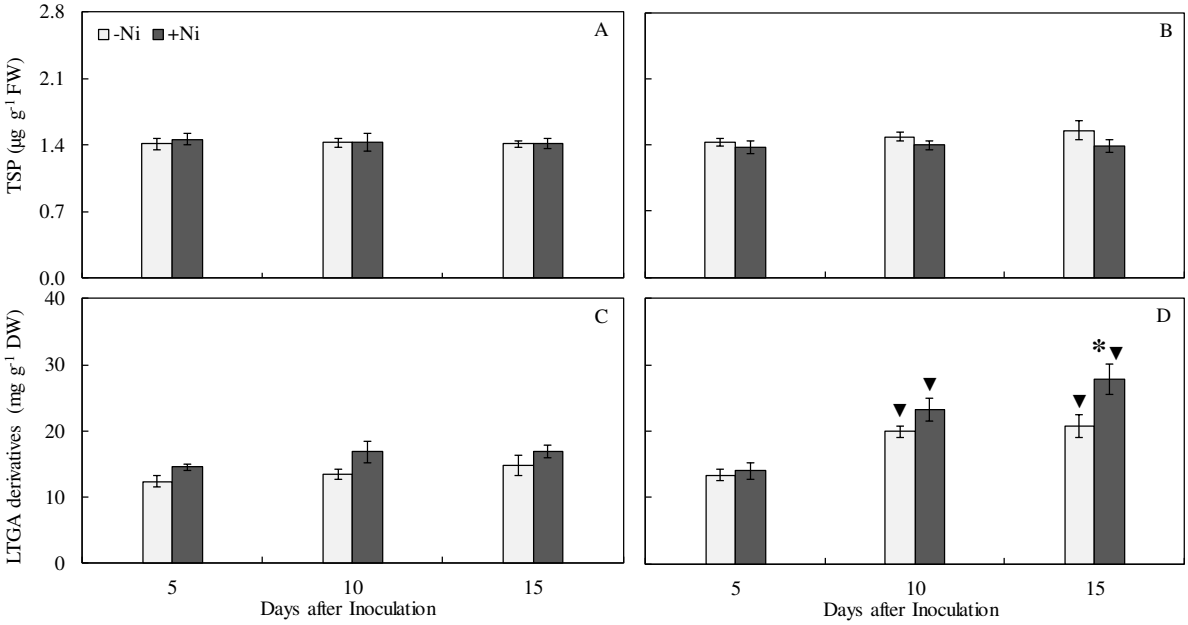

Figure 4. Concentrations of total soluble phenolics (TSP) (A and B) and lignin-thioglycolic acid (LTGA) derivatives (C and D) in the leaves of soybean plants non-sprayed (-Ni) or sprayed (+Ni) with nickel (Ni) and non-inoculated (NI) (A and C) or inoculated (I) (B and D) with *Phakopsora pachyrhizi*. For each evaluation time, means for the -Ni and +Ni treatments followed by an asterisk (*) and for the NI and I treatments followed by an inverted triangle (▼) are significantly different ($P \leq 0.05$) according to Tukey's test. FW = fresh weight, DW = dry weight. Bars represent the standard error of the means. $n = 8$.

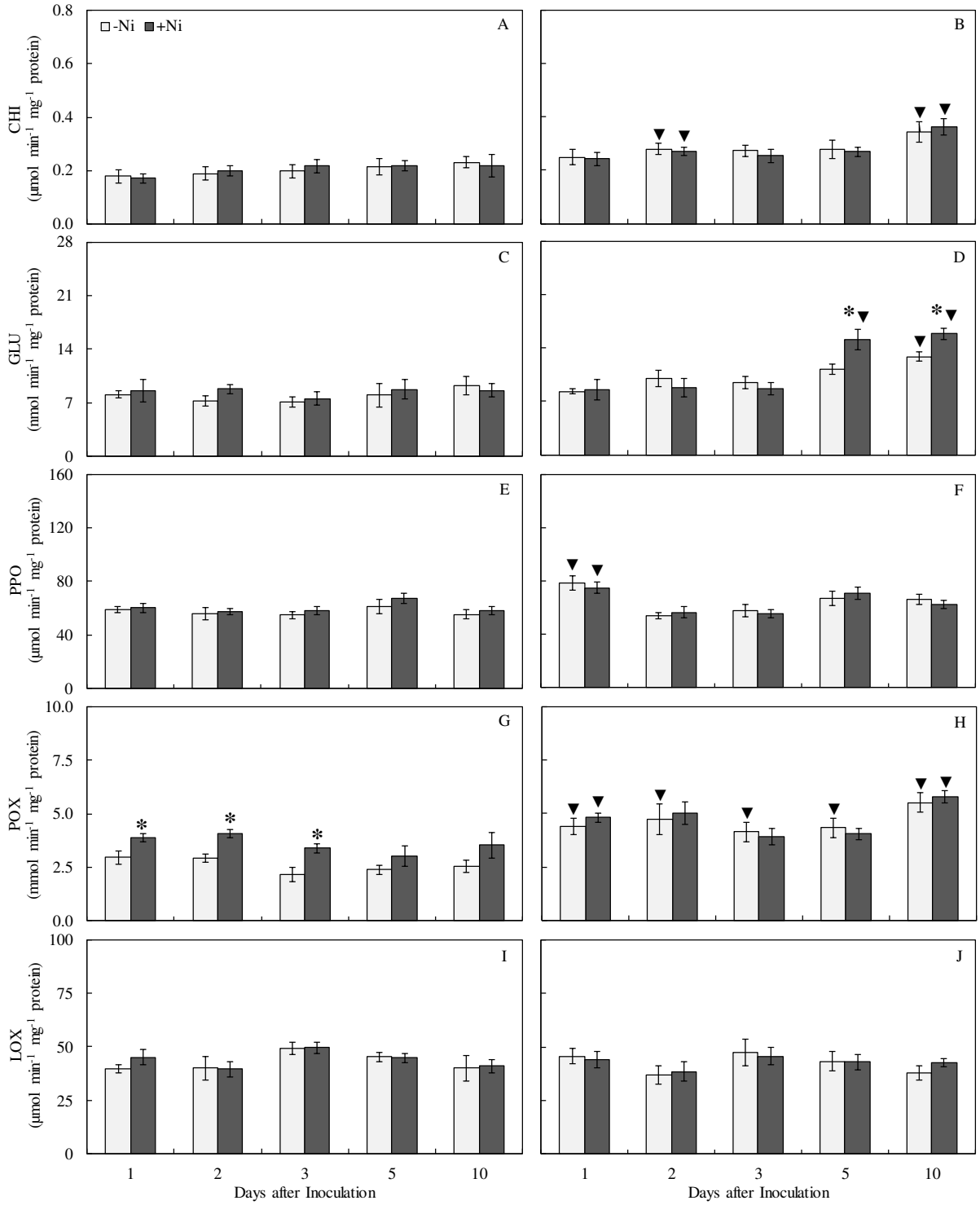

Figure 5. Activities of chitinase (CHI) (A and B), β-1,3-glucanase (GLU) (C and D), polyphenoloxidase (PPO) (E and F), peroxidase (POX) (G and H), and lipoxygenase (LOX) (I and J) in the leaves of soybean plants non-sprayed (-Ni) or sprayed (+Ni) with nickel (Ni) and non-inoculated (NI) (A, C, E, G, and I) or inoculated (I) (B, D, F, H, and J) with *Phakopsora pachyrhizi*. For each evaluation time, means for the -Ni and +Ni treatments followed by an asterisk (*) and for the NI and I treatments followed by an inverted triangle (▼) are significantly different ($P \leq 0.05$) according to Tukey's test. Bars represent the standard error of the means. $n = 8$.

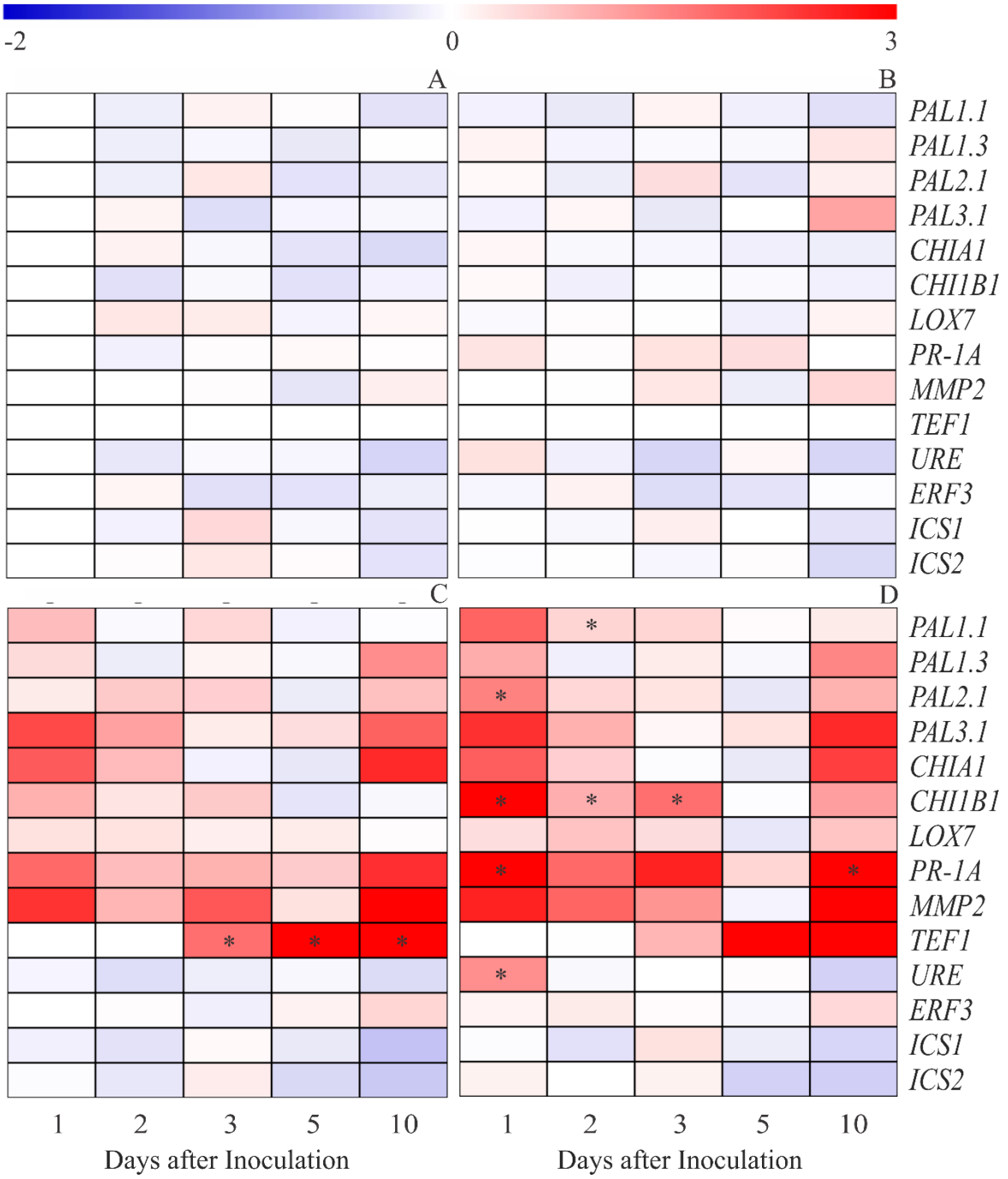

Figure 6. Expression profile of defense genes determined in the leaves of soybean plants from the following treatments: (A) non-sprayed with nickel (-Ni) and non-inoculated (NI) with *Phakopsora pachyrhizi*, (B) sprayed with nickel (+Ni) and NI with *P. pachyrhizi*, (C) -Ni and inoculated (I) with *P. pachyrhizi*, and (D) +Ni and I with *P. pachyrhizi* (D). Color cells represents the relative transcript levels ranging from blue (-2) to red (4). The amplification of the Ubiquitin-3 (UBIQ) gene from soybean was used as an internal control for data normalization. Fold changes were calculated based on the transcript level for the -Ni NI treatment at 1 day after inoculation (dai), except for *TEF-1* gene where the transcript level for the -Ni I treatment at 1 dai was used in the calculation. For each leaf sample, four biological replications were used with their respective three technical replicates. For each evaluation time, means for the -Ni and +Ni treatments followed by an asterisk (*) are significantly different ($P \leq 0.05$) according to F test. $n = 8$.

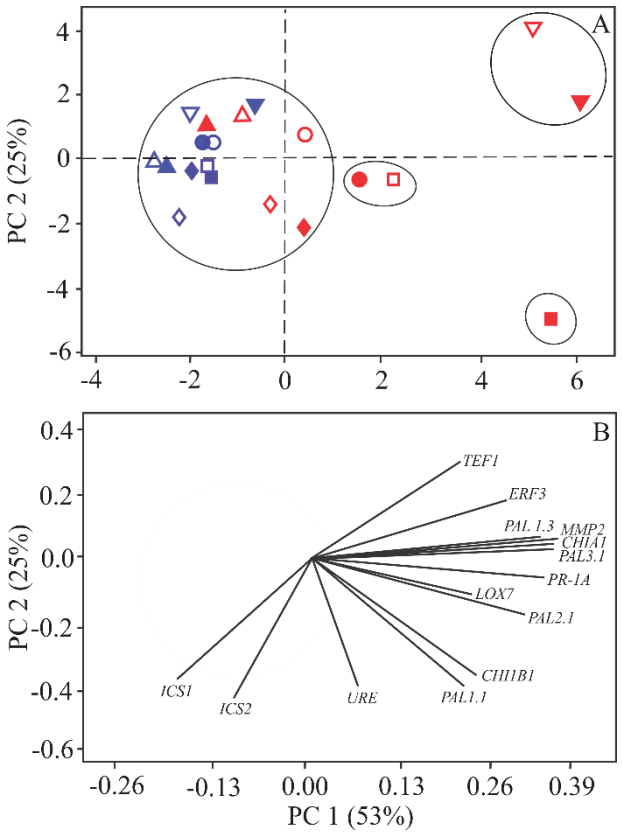

Figure 7. Score (A) and loading (B) plots of principal component analysis comparing the expression of genes coding for phenylalanine ammonia-lyase (*PAL1.1, PAL1.3, PAL2.1,* and *PAL3.1*), chitinase (*CHIA1*), chalcone isomerase (*CHI1B1*), lipoxygenase (*LOX7*), metalloproteinase (*MMP2*), isochorismate synthase (*ICS1* and *ICS2*), urease (*URE*), resistance-related protein (*PR-1A*), AP2/ERF type transcription factor (*ERF3*), and 18S ribosomal portion of *Phakopsora pachyrhizi* (*TEF1*) in the leaves of soybean plants. Empty forms: plants non-sprayed with nickel; completed forms: plants sprayed with nickel; blue forms: non-inoculated plants; red forms: plants inoculated with *P. pachyrhizi;* square: 1 day after inoculation (dai); circle: 2 dai; rhombus: 3 dai; triangle: 5 dai; and inverted triangle: 10 dai. Groups were generated from cluster analysis with complete linkage and Pearson distance.

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2. CHAPTER 2: CELLULAR OXIDATIVE DAMAGE AND IMPAIRMENT ON THE PHOTOSYNTHETIC APPARATUS CAUSED BY ASIAN SOYBEAN RUST ON SOYBEANS ARE ALLEVIATED BY NICKEL

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Abstract: Asian Soybean Rust (ASR), caused by *Phakopsora pachyrhizi* H. Sydow & P. Sydow, is one of the most severe diseases of soybean. The oxidative damage in cellular constituents and the disruption in the photosynthetic apparatus are the major negative effects of ASR on soybean plants. Considering the importance of nickel (Ni) on plant metabolism, this study evaluated the effect of foliar Ni-supply on ASR control, reactive oxygen species (ROS) accumulation, activities of antioxidant enzymes, parameters related to photosynthesis, and sugars concentration on soybean plants. ASR severity decreased by 34% due to Ni supply. Infected and Ni-supplied (+Ni) plants showed lower malondialdehyde concentration and superoxide (O_2^-) and hydrogen peroxide (H_2O_2) accumulation in contrast to infected leaf tissues of non-Ni-supplied (-Ni) plants. The antioxidant enzymes activities were inefficient to avoid the high ROS accumulation for -Ni inoculated plants. The photosynthetic pigments, maximum photochemical efficiency of photosystem II (PSII), effective yield of PSII, electron transport rate, rate of net carbon assimilation, stomatal conductance to water vapor, and transpiration rate values were higher and the yield for other non-regulated losses and internal CO_2 concentration values were lower for +Ni inoculated plants in comparison to -Ni inoculated plants. High ROS production and the greater damage to the photosynthetic apparatus caused by *P. pachyrhizi* infection on -Ni plants affected the synthesis of the sugars. The infection also decreased the plant's energetic reserves faster in -Ni plants in comparison to +Ni plants. In conclusion, the cellular oxidative damage and the impairment on the photosynthetic apparatus of soybean plants caused by *P. pachyrhizi* infection were alleviated by supplying Ni foliarly.

Keywords *Glycine max* • *Phakopsora pachyrhizi*. Micronutrient. Antioxidant enzymes. Leaf gas exchange. Chlorophyll *a* fluorescence

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2.1. INTRODUCTION

Nickel (Ni) has been recognized as an essential nutrient for plant growth since 1987 (Brown *et al.* 1987). The importance of Ni in the plant metabolism is related to its participation in the constitution of several biomolecules, binding to high number of proteins and peptides, and also for being required for the catalytic process of several enzymes such carbon monoxide dehydrogenase, catalase, NiFe hydrogenases, superoxide dismutase, and urease (Harasim and Filipek 2015).

Deficiency of Ni results in the foliar accumulation of urea due to depression of urease activity, generating a lower conversion of urea to ammonia (Dixon *et al.* 1975). In pecan plants, Ni-deficiency developed a symptom characterized as 'mouse-ear' (Brown *et al.* 1987). However, the occurrence of Ni-deficiency symptoms under field conditions is rarely reported due to its low requirement by plants and to its content (low as 0.2 mg kg^{-1} or as high as 450 mg kg^{-1}) in the soil solution (Harasim and Filipek 2015). Despite the low Ni-requirement by plants, the considerable Ni-content in the soil, and the high Ni-mobility in the plant tissues, suboptimal supply of Ni to plants is noticed when the mostly Ni-accumulation in the roots than in the other parts of the plant occur through roots uptake (Antonkiewicz *et al.* 2016). Thus, the foliar supply of Ni would be an interesting alternative to provide this micronutrient to the plants.

Few studies about the Ni effect on plant-pathogen interactions have been reported in the literature. The beneficial effects of this micronutrient for disease control have been reported for the sugarcane-*Puccinia melanocephala* (Singh and Muthaujan 1968), lettuce-*Fusarium oxysporum* f. sp. *lactucae*, tomato-*F. oxysporum* f. sp. *lycopersici* (Ahmed *et al.* 2016), and soybean-*Microsphaera diffusa* (Barcelos *et al.* 2018) interactions. The effect of Ni on soybean-*Phakopsora pachyrhizi* interaction is a compelling case to be investigated since urease plays a pivotal role in soybean resistance against this pathogen and for Ni being a cofactor for this enzyme (Dixon *et al.* 1975, Wiebke-Strohm *et al.* 2012). Moreover, a positive effect on plant growth and the activities of enzymes involved in the antioxidative metabolism was reported for plants supplied with adequate doses of Ni (Gomes-Junior *et al.* 2006).

Asian Soybean Rust (ASR), caused by *P. pachyrhizi* H. Sydow & P. Sydow, is one of the most severe diseases of soybean (Langenbach *et al.* 2016). Due to its biotrophic lifestyle, small cellular damage is observed at the beginning of the fungal infection process. However, under favorable environmental conditions and using susceptible cultivars, the efficient colonization of soybean leaf tissues by *P. pachyrhizi* is associated with the high consumption of plant nutrients and disrupts plant metabolism via oxidative stress (Waszczak *et al.* 2018).

The oxidative damage on cellular constituents of plants is caused by reactive species of oxygen (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and other types of ROS (Demidchik 2015). The action of ROS in cellular membranes generates an increase in malondialdehyde concentration that is used as an indicator of cellular damage (Schmid-Siegert *et al.* 2016). Moreover, ROS disrupts the photosynthetic apparatus of plants with a consequent decrease in the synthesis of ATP, NADPH, and trioses phosphates (Foyer 2018). Limitations on photosynthesis and nutrients consumption by *P. pachyrhizi* causes energetic lose to soybean plants and can be the major factor associated with the great yield losses (Barón *et al.* 2012, Rios *et al.* 2018). Another important energetic loss to soybean plants is related to the activation of host defense mechanisms against fungal infection and ROS detoxification (Neilson *et al.* 2013). In the last case, the enzymes superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) deserve to be highlighted in this process (Demidchik 2015).

Considering the great Ni-mobility in plant tissues and its potential to be used for ASR control, this study aimed to evaluate the effect of foliar Ni supply in alleviating the cellular damage and the impairment of photosynthesis as a result of *P. pachyrhizi* infection of soybean plants.

2.2. MATERIAL AND METHODS

2.2.1. Plant growth conditions and experimental design

Soybean seeds from the cultivar 'TMG 135' were sown in sand previously washed with HCl 1 N. This cultivar was selected for this study for having good growth architecture and highly susceptible to ASR. Five seedlings were transferred to a 5 L plastic pots and cultivated in a hydroponic system containing nutrient solution from Hoagland and Arnon (1950) with pH adjusted to 6.0. The nutrient solution was kept aerated, changed every four days, and had the pH adjusted to 6.0 daily. Plants were kept in a greenhouse located at the geographic coordinates of 20° 44' 52.6" S and 42° 51' 06.2" W, under natural daylight conditions, 25 ± 3°C temperature, and 80 ± 5% relative humidity. A 2 × 2 factorial experiment, consisting of plants non-sprayed or sprayed with Ni (refereed to as -Ni and +Ni plants thereafter) and non-inoculated or inoculated plants with *P. pachyrhizi*, was arranged in a completely randomized design with sixteen replications. Each experimental unit consisted of a plastic pot containing five plants. The experiment was conducted twice. Data from the variables evaluated from the two experiments were analyzed using the MIXED procedure of the SAS software

(Release8.02Level02M0for Windows, SAS Institute) to determine if data from the experiments could be combined (Moore and Dixon 2015).

2.2.2. Spray of Ni and plant inoculation with *P. pachyrhizi*

Plants at the V4 growth stage (three fully expanded leaves) were sprayed with a solution (7.2 mL per plant) of 0.19 g L^{-1} $NiSO_4.6H_2O$ (equivalent to 60 g ha^{-1} Ni) and 0.01% (v/v) Tween-20 as a surfactant. This dose of Ni was based on preliminary experiments realized (unpublished data) and on data provided by Barcelos *et al.* (2017, 2018). Plants sprayed with water served as the control treatment. At three days after Ni spray, plants were inoculated with a suspension of 1×10^5 urediniospores of *P. pachyrhizi* mL^{-1}. The urediniospores were collected from soybean plants (cv. 'TMG 135') previously inoculated with the monouredinial isolate UFV-DFP *Pp*25. The urediniospores were collected, preserved at -80ºC (Furtado *et al.* 2008), and their viability determined before plant inoculation. Vials containing urediniospores with viability greater 50% were used to prepare the suspension. Gelatin (0.5% w/v) was added to the suspension to aid urediniospores adhesion to the leaf blades. The suspension was applied as a fine mist to the abaxial leaf surface of each plant using a VL Airbrush atomizer (Paasche Airbrush Co., IL, USA). Inoculated plants were kept in a plastic mist growth chamber (temperature of 25 ± 3°C and relative humidity of 90 ± 5%) inside a greenhouse. At 16 hours after inoculation, plants were transferred to a greenhouse with the same conditions previously described.

2.2.3. Determination of ASR severity

The second and third trifoliate leaves, from base to top, of five plants from each replication (four replications and 20 plants total) of -Ni and +Ni treatments were collected at 16 days after inoculation (dai). The abaxial surface of the leaves was scanned at 1200 dpi resolution, and the obtained images were processed using the software QUANT (Vale *et al.* 2003) to quantify ASR severity.

2.2.4. Determination of foliar Ni concentration

The second and third trifoliate leaves from plants used to evaluated ASR severity (16 dai) and also from the non-inoculated plants both non-supplied or supplied with Ni were collected (20 plants and 40 leaves total per treatment), washed in deionized water, and dried in a drying oven with forced ventilation. The foliar Ni was extracted by nitric-perchloric digestion method and determined by inductively coupled plasma-optical emission spectrometry (ICP-OES).

2.2.5. Determination of chlorophyll (Chl) *a* fluorescence

The Imaging-PAM fluorometer and the Imaging Win software MAXI version (Heinz Walz GmbH, Effeltrich, Germany) were used to obtain the images and parameters of Chl *a* fluorescence. The second and third trifoliate leaves, from base to top, of one plant from a replication of each treatment (four replications and four plants per each sampling time) were collected at 8 and 13 dai. The methodology used was described by Fagundes-Nacarath *et al.* (2018) changing only the time of actinic photon irradiance to obtain the steady-state fluorescence yield that was fixed in 5 min.

2.2.6. Determination of photosynthetic pigments

The second and third trifoliate leaves from plants used to evaluated Chl *a* fluorescence parameters (8 and 13 dai) were used to perform the pigments quantification (four replications and four plants per each sampling time). The photosynthetic pigments of six-leaf discs (0.8 cm^3 each) were extracted with dimethyl sulfoxide (DMSO) saturated with calcium carbonate (Santos *et al.* 2008). The concentrations of chlorophyll (Chl) *a*, Chl *b*, Chl *a*+*b*, and carotenoids were determined based on the absorbance at 480, 649, and 665 nm (Sumanta *et al.* 2014).

2.2.7. Gas-exchange parameters

Leaf gas exchange parameters were determined in the lateral leaflet of the third leaf, from base to top, of one plant from the replication of each treatment (four replications and four plants per each sampling time) at 8 and 13 dai. The rate of net carbon assimilation (*A*), stomatal conductance to water vapor (g_s), internal CO_2 concentration (C_i), and transpiration rate (*E*) were estimated from 09:00 to 12:00 h under artificial and saturating photon irradiance (1200 μmol m^{-2} s^{-1}) and an external CO_2 concentration of 400 μmol mol^{-1} using a portable open-system infrared gas analyzer (LI-6400, LI-COR Inc., Lincoln, NE, USA). All measurements were performed by setting the block temperature at 25°C.

2.2.8. Histochemical detection of O_2^- and H_2O_2

In situ localization of O_2^- and H_2O_2 on the second trifoliate leaf, from base to top, of one plant from the replication of each treatment (four replications) was performed at 16 dai using 3,3′-diaminobenzidine and nitro-blue tetrazolium chloride; respectively, according to the methodology described by Aucique-Pérez *et al.* (2019). The stained leaflets were scanned at 1200 dpi resolution to obtain the images showing the histochemical localization of O_2^- and H_2O_2.

2.2.9. Biochemical analysis

The second and third trifoliate leaves, from base to top, of two plants from the replication of each treatment (four replications and 8 plants per each sampling time) were collected at 4, 8, 12, and 16 dai. Leaf samples were kept in liquid nitrogen during sampling and stored at -80°C until further analysis. Plants that had their leaves collected were discarded after each sampling.

2.2.10. Determination of sugars

Approximately 50 mg of fresh ground material was used for sugars analyses. Samples were subjected to ethanol extraction according to Medeiros *et al.* (2017). The ethanol-soluble phase was used for the quantification of sugars and the pellet was used for the quantification of starch (Fernie *et al.* 2001). The soluble sugars (glucose, fructose, and sucrose) and starch were analyzed as described by Daloso *et al.* (2015).

2.2.11. Determination of malondialdehyde (MDA)

Cellular oxidative damage was estimated based on the production of total 2-thiobarbituric acid (TBA) reactive substances and expressed as equivalents of MDA according to Hodges *et al.* (1999) with a few modifications. A total of 100 mg of leaf tissue was ground into a fine powder using a vibration ball mill (Retsch, Haan, Germany) with liquid nitrogen and homogenized in 2 mL of 0.1% (w/v) trichloroacetic acid (TCA) solution. The homogenate was centrifuged at 12,000 *g* for 15 min at 4°C. After centrifugation, 0.25 mL of the supernatant was added to 0.75 mL of TBA solution (0.5% in 20% TCA) and held for 60 min at 99°C in a thermomixer (Eppendorf, Hamburg, Germany). The reaction was stopped in an ice bath. After the solution reached room temperature, the absorbance was read at 532 and 600 nm. The non-specific absorbance value (at 600 nm) was discounted of the absorbance value at 532 nm. A stand curve of sucrose was used to correct the interference of soluble sugars in the samples. The molar extinction coefficient of 155 mM^{-1} cm^{-1} was used to calculate the MDA concentration.

2.2.12. Determination of antioxidant enzyme activities

A total of 200 mg of leaf tissue was ground into a fine powder using a vibration ball mill with liquid nitrogen. The fine powder was homogenized in 2 mL of a solution containing 100 mM potassium phosphate buffer (pH 7.8), 0.1 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, and 0.5% (m/v) polyvinylpyrrolidone. The homogenate was centrifuged at 13,000 *g* for 15 min at 4°C and the supernatant was collected and used to determine SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), and APX (EC 1.11.1.1) activities. The

activities of the antioxidant enzymes were determined as previously described by Debona *et al.* (2012).

2.2.13. Statistical analysis

The Minitab software was used for statistical analysis. Data from the variables evaluated were checked for normality and homogeneity of variance and subjected to analysis of variance (ANOVA) thereafter. Treatments means were compared by F test ($P \leq 0.05$). For ASR severity, ANOVA considered plants non-sprayed or sprayed with Ni with four replications. For biochemical analysis, pigments and foliar Ni concentrations, Chl *a* fluorescence, and leaf gas exchange parameters, ANOVA was considered to be a 2 × 2 factorial experiment consisting of non-inoculated and inoculated plants and plants non-sprayed or sprayed with Ni with four replications.

2.3. RESULTS

2.3.1. Foliar Ni concentration

The foliar Ni concentration for -Ni non-inoculated and -Ni inoculated plants were 5.6 and 5.8 mg kg^{-1}, respectively. By contrast, foliar Ni concentration was increased by 445 and 645% for +Ni non-inoculated plants and +Ni inoculated plants, respectively, in comparison to -Ni plants (Fig. 1). For the +Ni plants, foliar Ni concentration was 42% higher for inoculated plants than for non-inoculated plants.

2.3.2. Symptoms of ASR and ASR severity

For leaves treated with Ni, both the chlorotic and necrotic tissue was reduced in comparison to -Ni plants (Fig. 2A-D). ASR severity for +Ni plants was significantly reduced by 34% in comparison to -Ni plants (Fig. 3).

2.3.3. Concentration of MDA

The MDA concentration for -Ni inoculated plants increased at 12 and 16 dai by 17 and 28%, respectively, and for +Ni inoculated plants at 16 dai by 15% in comparison to non-inoculated plants (Fig. 4A-B). The MDA concentration for -Ni inoculated plants was significantly increased by 18% at 16 dai in comparison to +Ni inoculated plants (Fig. 4B).

2.3.4. Histochemical detection of O_2^- and H_2O_2

From the analysis of the color of the tissues, is possible detect a greater accumulation of O_2^- (blue-colored tissues) and H_2O_2 (brown-colored tissues) for inoculated leaves of both -Ni and +Ni plants in comparison to non-inoculated leaves (Fig. 5A-H). In inoculated plants, the accumulation of O_2^- and H_2O_2 was less pronounced for +Ni plants in comparison to -Ni plants (Fig. 5C, D, G, and H).

2.3.5. Antioxidant enzyme activities

For non-inoculated plants, there was no significant difference between -Ni and +Ni plants regardless of the enzymes studied and the evaluation time (Fig. 6A-C). At each evaluation time, SOD activity was significantly increased at 8, 12, and 16 dai for -Ni inoculated plants and at 12 and 16 dai for +Ni inoculated plants in comparison to those non-inoculated. SOD activity was significantly higher for -Ni inoculated plants by 22 and 38% at 12 and 16 dai, respectively, in comparison to +Ni inoculated plants (Fig. 6A). CAT activity was significantly higher by 56 and 29% at 16 dai for -Ni and +Ni inoculated plants, respectively, in comparison to those non-inoculated (Fig. 6B). APX activity was increased for inoculated plants at 8, 12, and 16 dai in comparison to non-inoculated plants regardless of Ni supply (Fig. 6C).

2.3.6. Photosynthetic pigments

For non-inoculated plants, there was no significant difference between -Ni and +Ni plants regardless of the photosynthetic pigment studied and the evaluation time (Fig. 7A-D). At each evaluation time, the concentrations of Chl *a*, Chl *b*, Chl *a*+*b*, and carotenoids were significantly lower at 8 and 13 dai for inoculated plants in comparison to non-inoculated plants regardless of Ni supply. For inoculated plants, the concentrations of Chl *a*, Chl *b*, and Chl *a*+*b* were higher by 43, 54, and 45%, respectively, at 13 dai for +Ni plants in comparison to -Ni plants (Fig. 7A-C).

2.3.7. Chl *a* fluorescence parameters

Irrespective of Ni supply, the changes in color intensity following a color scale of the Chl *a* fluorescence images evidenced changes caused by fungal inoculation in all parameters examined (Fig. 8). For non-inoculated plants, there was no significant difference between -Ni and +Ni plants regardless of the Chl *a* fluorescence parameter examined and the evaluation time (Fig. 9A-E). Significant decreases on the maximum photochemical efficiency of photosystem II (PSII) (F_v/F_m), effective yield of PSII (Y(II)), and apparent electron transport rate (ETR)

were observed for infected leaves of both -Ni and +Ni plants regardless of the evaluation time (Fig. 9A, B, and E).

The yield for dissipation by down-regulation energy (Y(NPQ)) decreased by 15 and 49% at 8 and 13 dai, respectively, for -Ni inoculated plants in comparison to -Ni non-inoculated plants (Fig. 9C). The yield for other non-photochemical (non-regulated) losses (Y(NO)) was higher by 30 and 51% at 8 and 13 dai, respectively for -Ni inoculated plants in comparison to -Ni non-inoculated plants and by 11% at 13 dai for +Ni inoculated plants in comparison to +Ni non-inoculated plants.

At each evaluation time, F_v/F_m, Y(II), and ETR values were higher and Y(NO) values were lower for +Ni inoculated plants in comparison to -Ni inoculated plants (Fig. 9A, B, D, and E). Y(NPQ) values were higher by 51% at 13 dai for +Ni inoculated plants in comparison to -Ni inoculated plants.

2.3.8. Leaf gas-exchange parameters

There was decrease for A, g_s, and E values for -Ni and +Ni inoculated plants in comparison to non-inoculated plants regardless of the evaluation time, except for g_s at 13 dai, which had no significantly difference between +Ni inoculated and +Ni non-inoculated plants (Fig. 10A, B, and D). C_i values were higher by 6% at 8 dai for -Ni inoculated plants and by 20 and 14% at 13 dai for -Ni and +Ni inoculated plants, respectively, in comparison to non-inoculated plants (Fig. 10C). At each evaluation time, A values were higher by 82 and 416% at 8 and 13 dai, respectively, for +Ni inoculated plants in comparison to -Ni inoculated plants (Fig. 10A). Following this same comparison, g_s and E values for +Ni inoculated plants were increased by 111 and 52% at 13 dai, respectively, in comparison to -Ni inoculated plants (Fig. 10B and D). C_i values for +Ni inoculated plants were decreased by 4 and 10% at 8 and 13 dai, respectively, in comparison to -Ni plants (Fig. 10C).

2.3.9. Sugars

There was no significant difference between -Ni non-inoculated plants and +Ni non-inoculated plants regardless of the concentrations of glucose, fructose, sucrose, and starch and the evaluation time (Fig. 11A-D). Glucose, fructose, and sucrose concentrations were significantly lower at 16 dai for inoculated plants in comparison to non-inoculated plants regardless of Ni supply (Fig. 11A-C). Fructose and sucrose concentrations were lower at 12 dai for -Ni inoculated plants in comparison to -Ni non-inoculated plants (Fig. 11B and C). Moreover, there was a decrease in starch concentration for -Ni inoculated plants and +Ni

inoculated plants in comparison to non-inoculated plants regardless of the evaluation time (Fig. 11D). Comparing -Ni and +Ni plants at each evaluation time, starch concentration was higher by 31, 139, and 82% at 8, 12, and 16 dai, respectively, for +Ni inoculated plants in comparison to -Ni inoculated plants. Sucrose concentration was higher by 55 and 104%, respectively, at 12 and 16 dai for +Ni inoculated plants in comparison to -Ni inoculated plants. Glucose and fructose concentrations were higher by 100 and 72% at 16 dai, respectively, for +Ni inoculated plants in comparison to -Ni inoculated plants (Fig. 11 A-D).

2.4. DISCUSSION

In the present study, the high foliar Ni concentration for Ni-supplied plants reduced the ASR symptoms as a result of the less cellular damage (lower MDA concentration and reduced accumulation of O_2^- and H_2O_2) caused by fungal infection as well as for keeping the photosynthesis at a high level of functionality. The higher foliar Ni concentration for +Ni inoculated plants in comparison to +Ni non-inoculated plants could be related to the great mobility of this micronutrient in the plant tissues (Chen *et al*. 2009) and a possible nutrient accumulation at the infection sites as observed for assimilates in French bean (*Phaseolus vulgaris*) infected by *Uromyces appendiculatus* (Livne and Daly 1966). However, further studies are needed to support this hypothesis.

Plants developed an efficient system for cell detoxification composed of non-enzymatic and enzymatic mechanisms during their evolution. Considering the enzymatic mechanism, SOD plays a crucial role in O_2^- detoxification (Demidchik 2015). There was an early and high SOD activity for -Ni inoculated plants in comparison to +Ni inoculated plants. Despite the greater SOD activity, O_2^- accumulation in leaf tissues of -Ni plants at 16 dai was greater than in +Ni plants. Considering that H_2O_2 is the product formed by SOD activity, the increase in CAT and APX activities may not have been sufficient to detoxify the high H_2O_2 generated by SOD in the leaf tissues of -Ni inoculated plants in comparison to +Ni inoculated plants. This finding is corroborated by the histochemical analysis performed and by the no significative difference for CAT and APX activities between -Ni and +Ni plants. Distinctly, Barcelos *et al*. (2018) reported an increase of CAT and SOD activities in soybean plants cultivated in field conditions and infected by *M. diffusa* at 10 days after treatment with Ni doses between 10 and 60 g ha^{-1}.

Despite its biotrophic lifestyle, *P. pachyrhizi* infection caused several impairments on photosynthesis starting at 8 dai. Rios *et al*. (2018) reported a negative effect of ASR on

photosynthesis starting at 13 dai. The least severe ASR symptoms for +Ni plants in comparison to -Ni ones resulted in the maintenance of the photosynthesis at its higher level of functionality.

Considering that the photosynthetic pigments play a pivotal role on the light-harvesting complexes (LHCs) (Akhtar *et al*. 2015), a great decrease in their concentrations due to *P. pachyrhizi* infection may have limited the correct LHCs operation resulting in less energy transfer to PSII, and ultimately limiting the following photosynthetic processes. The Chl biosynthesis is affected by nitrogen (N) because it is a constituent of tetrapyrroles and is an essential component of amino acids, enzymes, hormones, phenolics, phytoalexins, and proteins that are involved on biochemical processes (Tanaka and Tanaka 2007). The N is demanded in a higher quantity for plant defense against pathogens infection (Elmer and Datnoff 2014) and this increase of Ni demand may have limited the N availability for Chl synthesis faster for Ni-non-supplied plants than for Ni-supplied plants. The beneficial effect of Ni on N metabolism via ureide catabolism, amino acid metabolism, and ornithine cycle intermediates (Bai *et al*. 2006) could explain the higher N availability and ultimately the higher Chl concentration for +Ni infected plants in comparison to -Ni infected plants.

Comparing the three yield components for dissipative processes between non-inoculated and inoculated plants, a decrease was observed on the energy destined to photochemical process (Y(II)) and dissipation by down-regulation energy (Y(NPQ)) and an increase on the energy destined to other non-regulated losses (Y(NO)) for inoculated -Ni plants. In contrast to -Ni plants, Y(II) values were higher and Y(NO) values were lower for +Ni inoculated plants. This variation of energy between the different dissipative processes reflects the higher damage on the photosynthetic apparatus for -Ni plants in comparison to +Ni plants as a result of *P. pachyrhizi* infection. Rios *et al*. (2018) reported for this same plant-pathogen interaction a similar destination of electronic excitation energy between the dissipative processes. The energy absorbed by the photosystem II was directed from the photochemical and regulated dissipation processes to the non-regulated dissipation process as a result of the great ASR symptoms for -Ni plants. This excess of energy dissipated by the non-regulated process can be used for the production of singlet oxygen in PSII and O_2^- in the acceptor side of photosystem I (PSI) (Huang *et al*. 2018). Singlet oxygen and the O_2^- cause photoinhibition of PSII being the former degrading the D1 protein (Vass 2011, Krieger-Liszkay *et al*. 2011). Progressive decrease on the F_v/F_m, ETR, and Y(NPQ) values and increase on Y(NO) values for -Ni inoculated plants provided evidence that the photoprotection mechanisms became ineffective and that the thermal dissipation of the energy was not sufficient to prevent photooxidative damages in these plants. Greater F_v/F_m and ETR values and a lower Y(NO) values for +Ni inoculated plants in

comparison to -Ni inoculated plants provided proof that Ni helped to maintain the integrity of the photosynthetic apparatus of plants infected by *P. pachyrhizi*.

The capacity of the photosynthetic machinery to capture light and to use the absorbed energy by PSII determine firstly its capacity to process CO_2 (Urban *et al*. 2017). In fact, *A* values were lower for inoculated plants in comparison to non-inoculated plants regardless of Ni supply. Colonization by *Phakopsora euvitis* also caused a significant reduction in CO_2 assimilation rate in leaves of fox grapes plants (Nogueira Júnior *et al*. 2017). Beyond limitations caused by photosynthetic apparatus damage, diffusive (lower g_s values) and biochemical (high C_i values) photosynthetic limitations were also responsible for decreasing *A* during the time-course evaluated. Bonde *et al*. (1976) reported that *P. pachyrhizi* infection promoted reduction of intercellular spaces and hypertrophy of spongy parenchyma cells of soybean plants that acted as a barrier to CO_2 diffusion. This finding corroborates with the lower g_s and *E* values observed in the present study for soybean infected plants in comparison to non-infected plants. The C_i values were maintained lower and the g_s and *E* values were kept higher for +Ni inoculated plants in comparison to -Ni inoculated plants, revealing low stomatal and biochemical limitations in +Ni plants.

Most studies in the literature associating Ni and photosynthesis are related to its toxic effect by inhibiting the electron transport chain, degrading photosystems (Mohanty *et al*. 1989), disrupting chloroplast structure, decreasing Chl synthesis (Polacco *et al*. 2013), and inactivating the Calvin cycle enzymes (Krupa *et al*. 1993, Seregin and Ivanov 2001, Reis *et al*. 2017). However, some studies with corn and oat reported increased photosynthetic rate of plants mainly due to an increase in photosynthetic pigment content (Mishra and Kar 1974). In the present study, novel evidences that adequate doses of Ni probably acted in soybean plants avoiding the damage caused by *P. pachyrhizi* on photosynthetic machinery and maintaining high concentration of Chl and other accessory pigments are presented.

Upon pathogen arrival on plant tissues, they trigger a series of mechanisms to protect themselves against the oxidizing agents arising from their infection process. The plant defense mechanisms operate at a high cost and the energy needed originates from photosynthesis or through plant reserves (Neilson *et al*. 2013). Moreover, pathogen nutrition is an additional drain on the plant's energy. Beyond energy, sugars supply structural material for defense responses of plants against pathogens infection (Morkunas and Ratajczak 2014). Considering that the photosynthetic capacity of the soybean plants was early affected by *P. pachyrhizi* infection, their reserves were used since then. Starch concentration decreased in inoculated plants after 4 dai and was kept higher for +Ni plants in comparison to -Ni plants during the time-course

evaluated. The concentrations of glucose and fructose were similar between inoculated and non-inoculated plants until 12 dai regardless of Ni supply probably due to the starch degradation. At 16 dai, low *A* values and reduced starch concentration for +Ni inoculated plants were not sufficient to supply glucose and fructose at sufficient concentrations in comparison to non-inoculated plants. Similarly, the biotrophic pathogen *P. euvitis* dramatically reduced photosynthesis and altered the dynamics of production and accumulation of carbohydrates in plants of fox grape (Nogueira Júnior *et al*. 2017). For the -Ni inoculated plants, fructose concentration was kept lower before 12 dai while for +Ni plants it was at 16 dai likely due to the lower *A* values and lower starch concentration. Sucrose concentration was also negatively impacted by *P. pachyrhizi* infection earlier for -Ni plants (12 dai) than for +Ni plants (16 dai). Great sucrose and fructose concentrations at 12 and 16 dai, respectively, and glucose concentration at 16 dai for +Ni inoculated plants in comparison to -Ni inoculated plants can be resulted from the high maintenance of their photosynthetic capacity and starch concentration on leaf tissues. Considering that carbohydrate typically accumulate in plant tissues infected by biotrophic fungi (Ayres 1981), additional studies using histological analysis of sugars are required to gain additional insights into the carbohydrate accumulation in Ni-supplied and inoculated soybean plants.

The results of the present study show the beneficial effect of Ni to lower ASR symptoms on soybean plants as indicated by less cellular damage caused by *P. pachyrhizi* infection. Further evidence of this observation is probably linked to the preservation of their photosynthetic machinery as well as the maintenance of an adequate concentration of photosynthetic pigments.

2.5. FIGURES

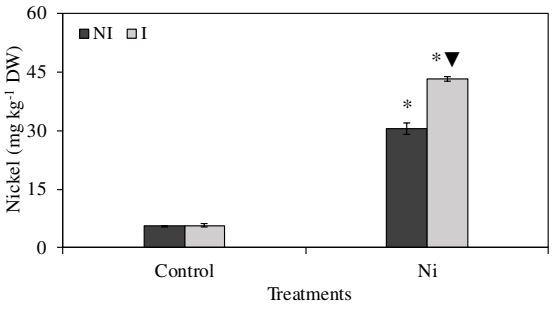

Figure 1. Foliar nickel (Ni) concentration in soybeans plants sprayed with water (control) or with Ni and non-inoculated (NI) or inoculated (I) with *Phakopsora pachyrhizi*. Means for control and Ni treatments followed by an asterisk (*) and for NI and I treatments followed by an inverted triangle (▼) are significantly different ($P \leq 0.05$) according to F test. DW = dry weight. Bars represent the standard error of the means. $n = 8$.

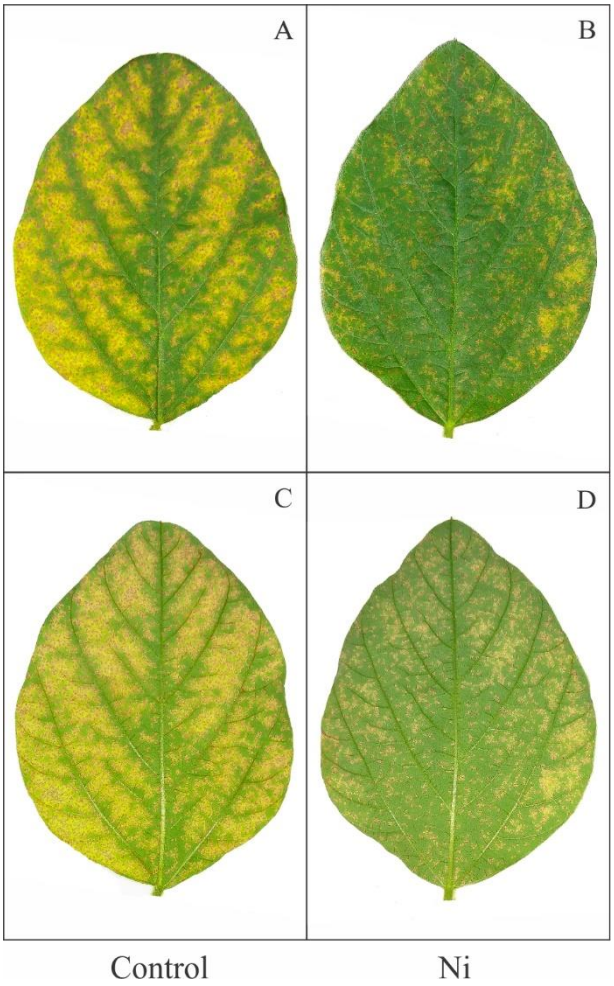

Figure 2. Symptoms of Asian soybean rust in the adaxial (A and B) and abaxial (C and D) leaflets surfaces of soybean plants sprayed with water (control) (A and C) or with nickel (B and D).

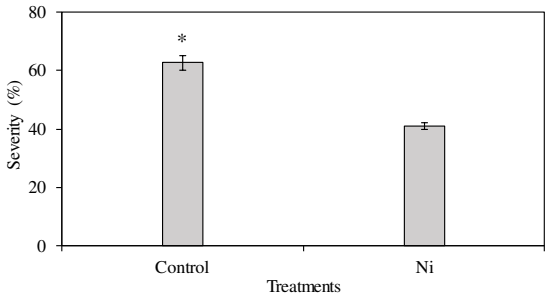

Figure 3. Asian soybean rust severity on leaves of soybean plants sprayed with water (control) or with nickel (Ni). Means for control and Ni treatments followed by an asterisk (*) are significantly different ($P \leq 0.05$) according to F test. Bars represent the standard error of the means. $n = 8$.

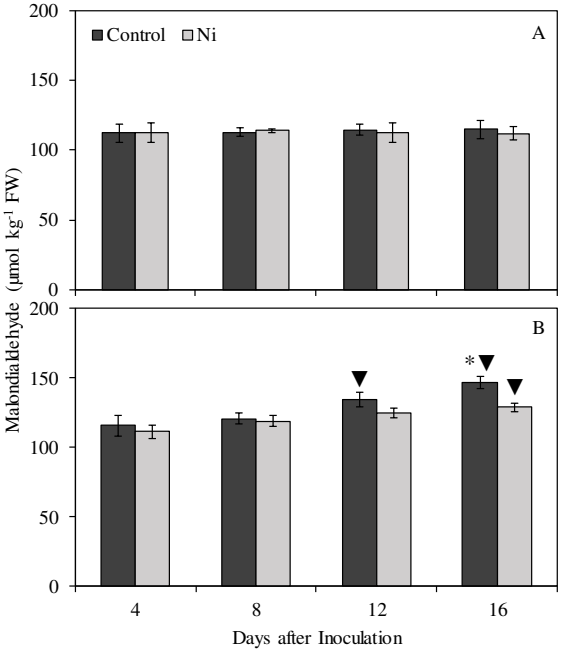

Figure 4. Concentration of malondialdehyde (MDA) in the leaves of soybean plants sprayed with water (control) or with nickel (Ni) and non-inoculated (NI) (A) or inoculated (I) (B) with *Phakopsora pachyrhizi*. For each evaluation time, means for control and Ni treatments followed by an asterisk (*) and for NI and I treatments followed by an inverted triangle (▼) are significantly different ($P \leq 0.05$) according to Tukey's test. FW = fresh weight. dai = days after inoculation. Bars represent the standard error of the means. $n = 8$.

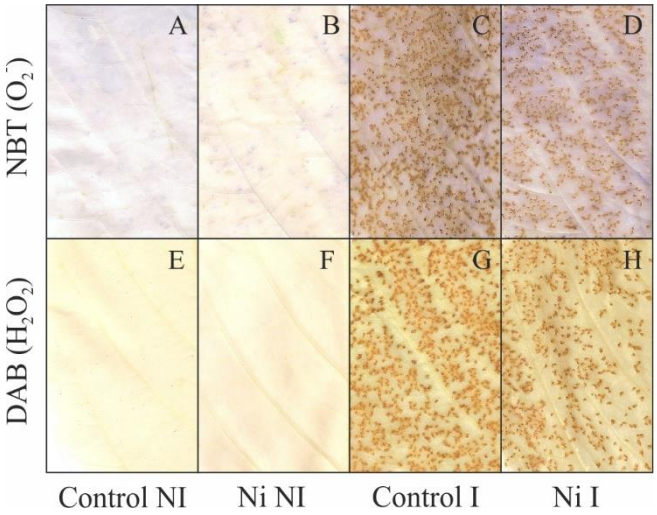

Figure 5. Histochemical detection of superoxide radical (O_2^-) (A-D) and hydrogen peroxide (H_2O_2) (E-H) in the second leaf of soybean plants sprayed with water (control) or with nickel (Ni) and non-inoculated (NI) or inoculated (I) with *Phakopsora pachyrhizi*. The O_2^- and H_2O_2 are detected by blue- and brown-colored tissues, respectively.

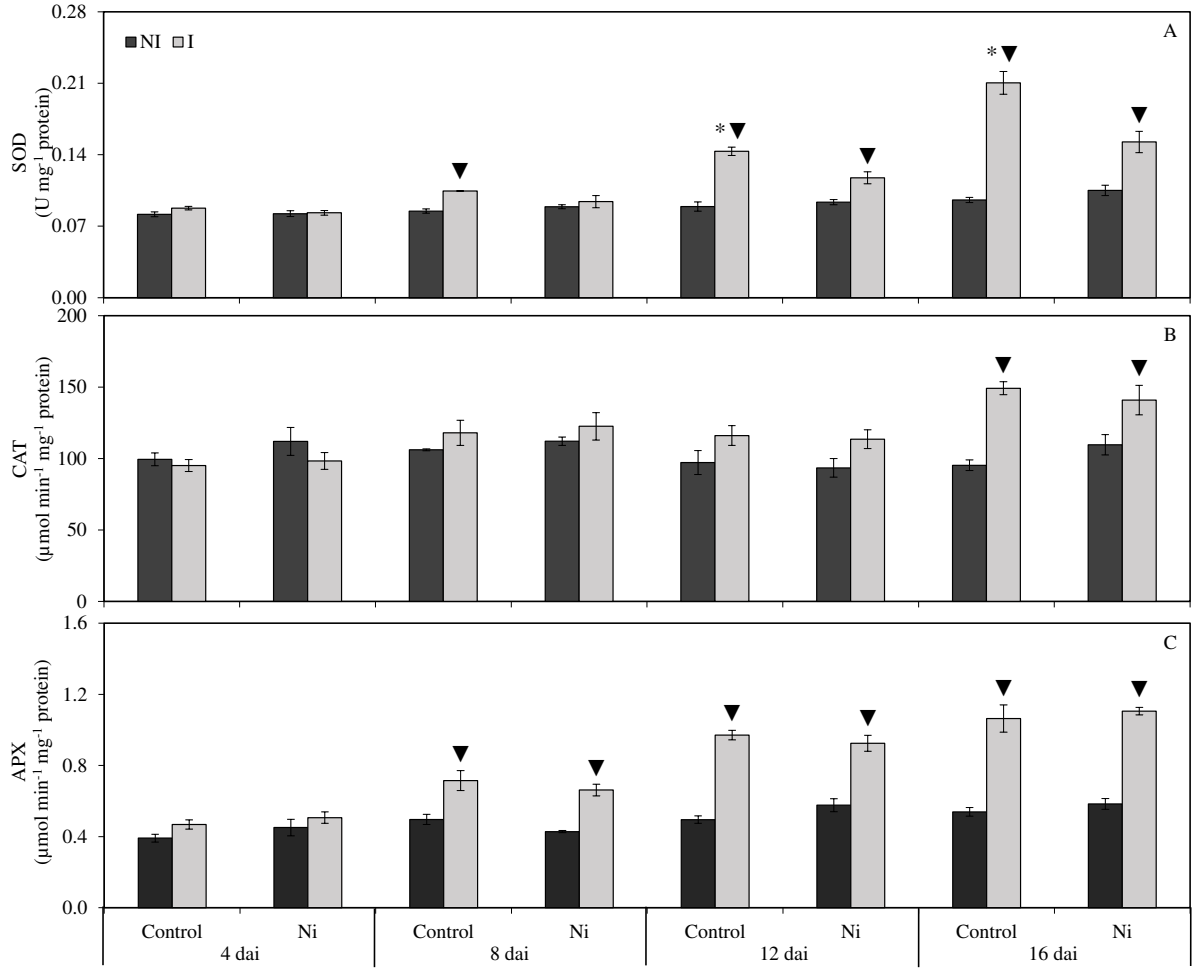

Figure 6. Activities of superoxide dismutase (SOD) (A), catalase (CAT) (B), and ascorbate peroxidase (APX) (C) in the leaves of soybean plants sprayed with water (control) or with nickel (Ni) and non-inoculated (NI) (A) or inoculated (I) (B) with *Phakopsora pachyrhizi*. For each evaluation time, means for control and Ni treatments followed by an asterisk (*) and for NI and I treatments followed by an inverted triangle (▼) are significantly different ($P \leq 0.05$) according to Tukey's test. dai = days after inoculation. Bars represent the standard error of the means. $n = 8$.

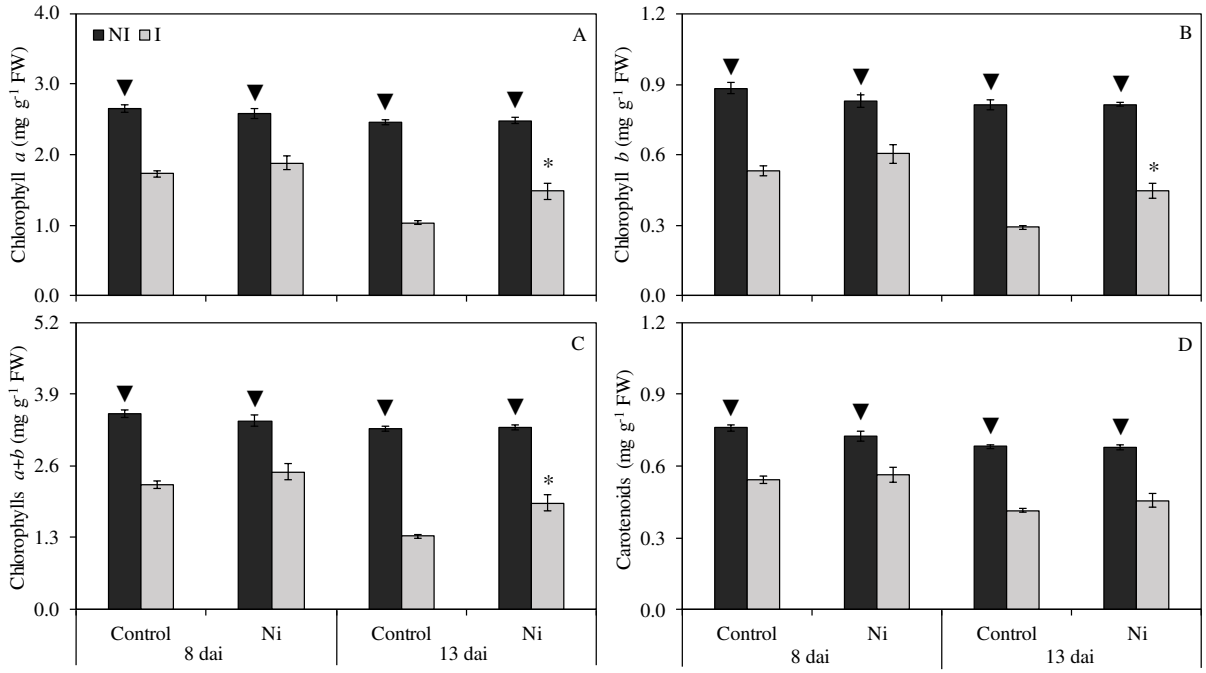

Figure 7. Concentrations of chlorophyll (Chl) *a* (A), chlorophyll *b* (B), total chlorophylls (Chl *a*+*b*) (C), and carotenoids (D) from leaves of soybean plants sprayed with water (control) or with nickel (Ni) and non-inoculated (NI) or inoculated (I) with *Phakopsora pachyrhizi*. For each evaluation time, means for control and Ni treatments followed by an asterisk (*) and for NI and I treatments followed by an inverted triangle (▼) are significantly different ($P \leq 0.05$) according to Tukey's test. FW = fresh weight. dai = days after inoculation. Bars represent the standard error of the means. $n = 8$.

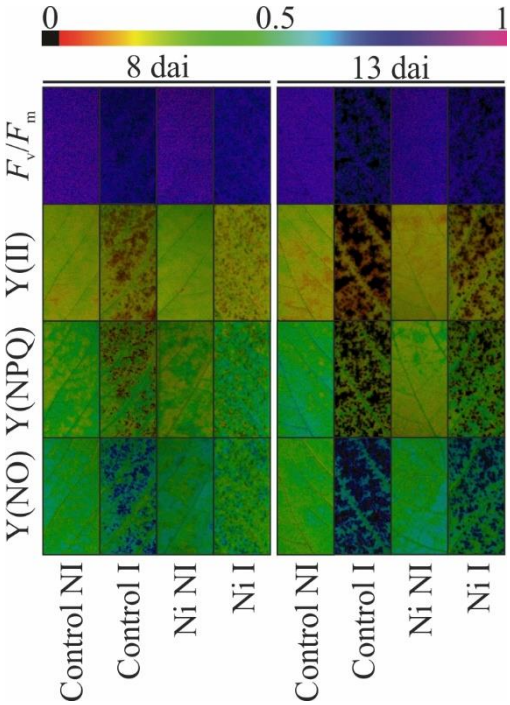

Figure 8. Images of the chlorophyll *a* fluorescence parameters: maximum photochemical efficiency of photosystem II (PSII) (F_v/F_m), effective yield of PSII (Y(II)), yield for dissipation by down-regulation energy (Y(NPQ)), and yield for other non-photochemical (non-regulated) losses (Y(NO)) determined from leaves of soybean plants sprayed with water (control) or with nickel (Ni) and non-inoculated (NI) or inoculated (I) with *Phakopsora pachyrhizi*. Parameter values are displayed by a color scale. dai = days after inoculation.

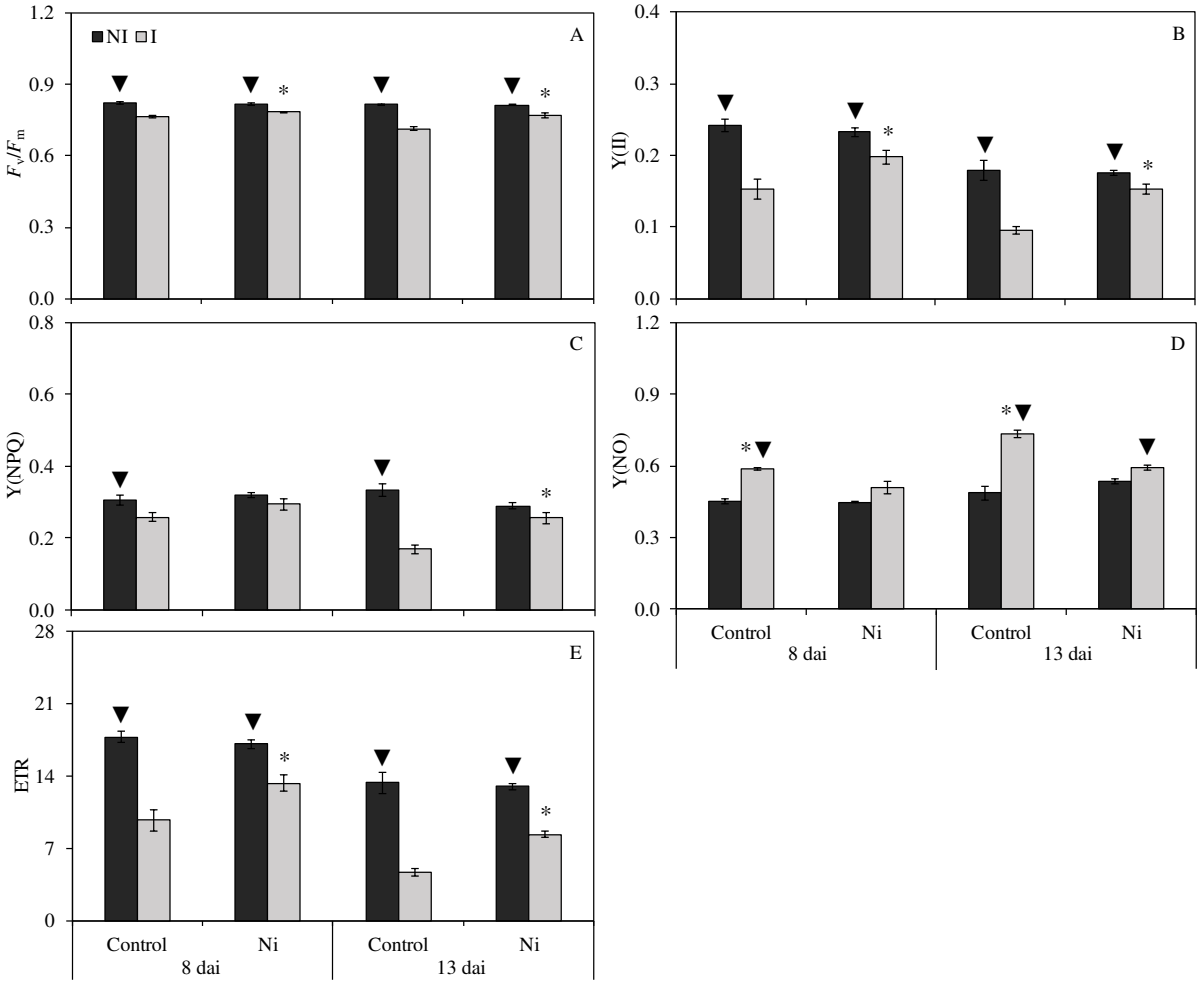

Figure 9. Chlorophyll *a* fluorescence parameters: maximum photochemical efficiency of photosystem II (PSII) (F_v/F_m) (A), effective yield of PSII (Y(II)) (B), yield for dissipation by down-regulation energy (Y(NPQ)) (C), yield for other non-photochemical (non-regulated) losses (Y(NO)) (D), and apparent electron transport rate (ETR) (E) determined from leaves of soybean plants sprayed with water (control) or with nickel and non-inoculated (NI) or inoculated (I) with *Phakopsora pachyrhizi*. For each evaluation time, means for control and Ni treatments followed by an asterisk (*) and for NI and I treatments followed by an inverted triangle (▼) are significantly different ($P \leq 0.05$) according to Tukey's test. dai = days after inoculation. Bars represent the standard error of the means. $n = 8$.

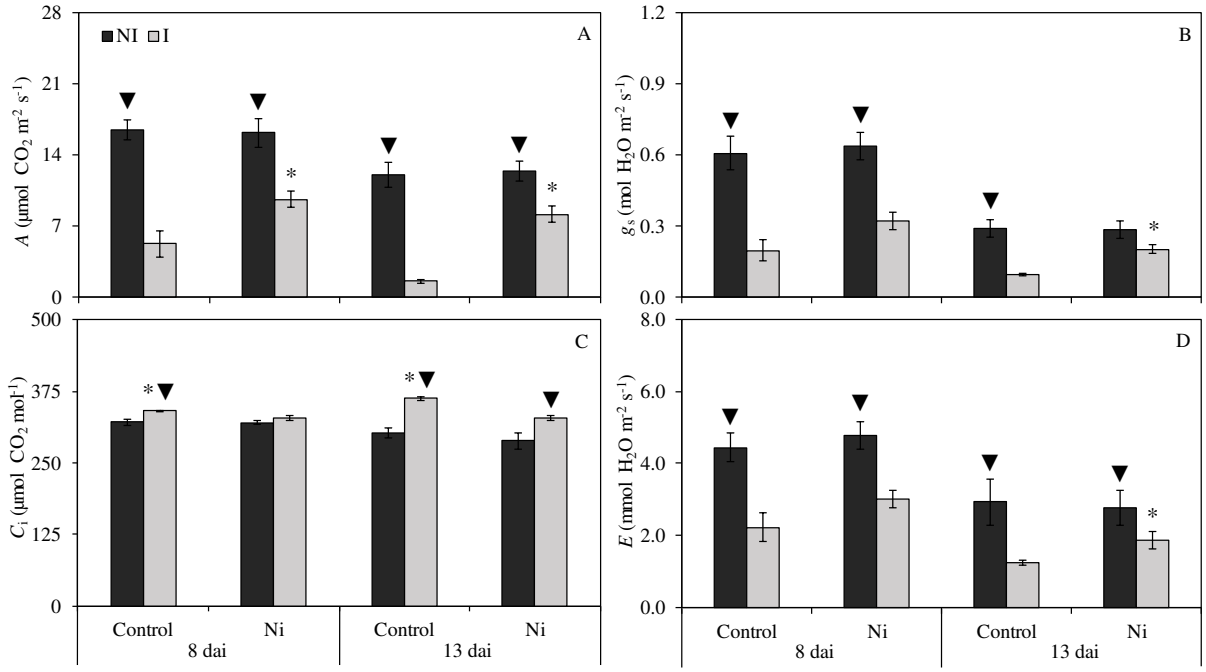

Figure 10. Leaf gas exchange parameters: rate of net CO_2 assimilation (A) (A), stomatal conductance to water vapour (g_s) (B), internal CO_2 concentration (C_i) (C), and transpiration rate (E) (D) determined from leaves of soybean plants sprayed with water (control) or with nickel and non-inoculated (NI) or inoculated (I) with *Phakopsora pachyrhizi*. For each evaluation time, means for control and Ni treatments followed by an asterisk (*) and for NI and I treatments followed by an inverted triangle (▼) are significantly different ($P \leq 0.05$) according to Tukey's test. dai = days after inoculation. Bars represent the standard error of the means. $n = 8$.

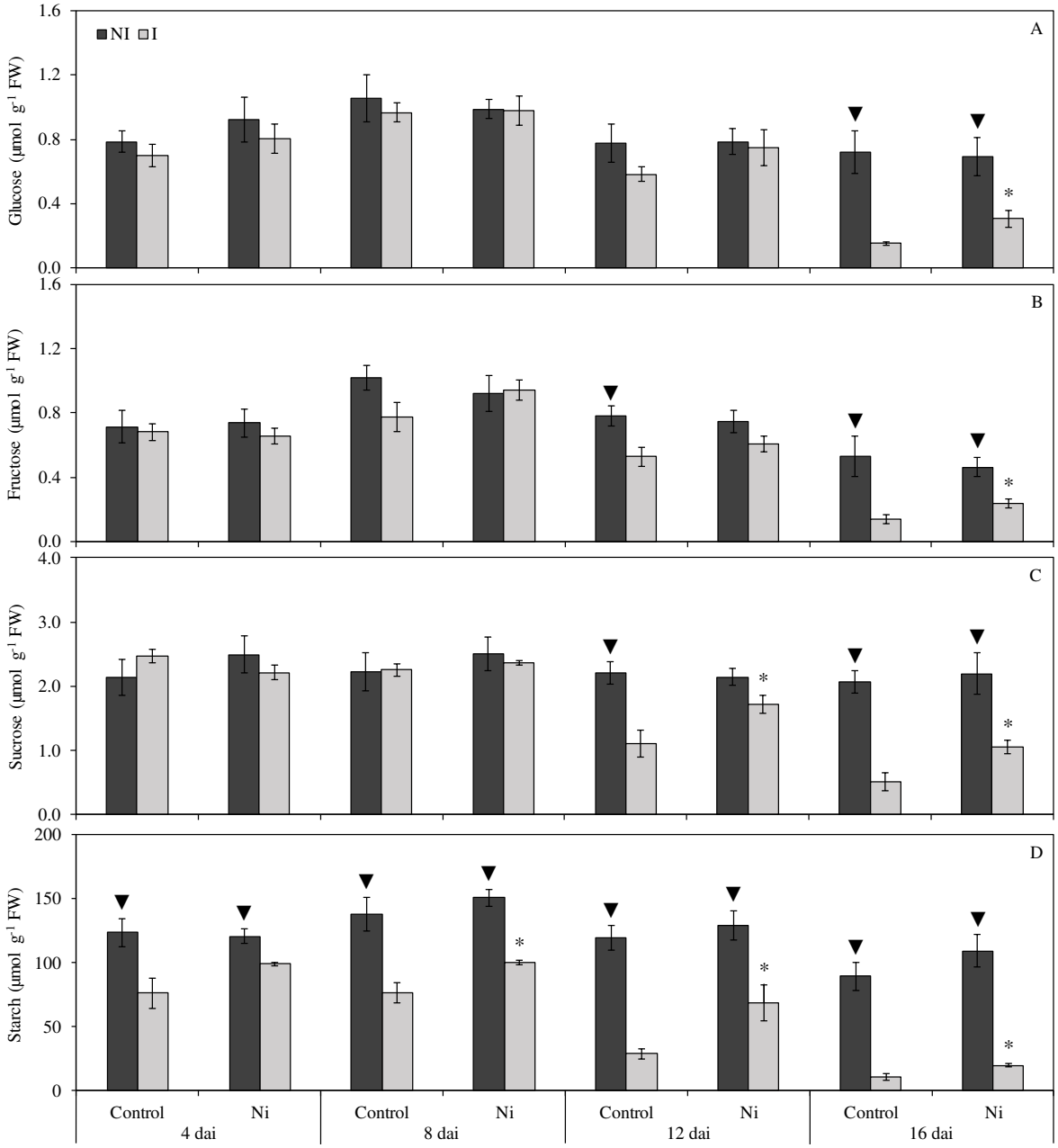

Figure 11. Concentrations of glucose (A), fructose (B), sucrose (C), and starch (D) from leaves of soybean plants sprayed with water (control) or with nickel and non-inoculated (NI) or inoculated (I) with *Phakopsora pachyrhizi*. For each evaluation time, means for control and Ni treatments followed by an asterisk (*) and for NI and I treatments followed by an inverted triangle (▼) are significantly different ($P \leq 0.05$) according to Tukey's test. FW = fresh weight. dai = days after inoculation. Bars represent the standard error of the means. $n = 8$.

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3. CHAPTER 3: CHANGES IN DEFENSE AND OXIDATIVE METABOLISM PROMOTED BY GLYPHOSATE, NICKEL, AND THEIR INTERACTION IN GLYPHOSATE-RESISTANT SOYBEAN PLANTS INFECTED BY *Phakopsora pachyrhizi*

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Abstract: Several factors are related to the non-race specific resistance of soybean plants to Asian Soybean Rust (ASR) such as environmental conditions, the basal level of host resistance, and the nutrition status of plants. Beneficial effects of nickel (Ni) and glyphosate (Gl) on ASR control have been reported. However, the possible mechanism(s) involved on ASR control promoted by Gl and how the combination of this herbicide with Ni may impact disease development requires further elucidation. This study aimed to investigate the alterations promoted by Ni, Gl and their combination in the defense and antioxidative metabolisms of soybean plants from a Gl-resistant cultivar infected by *Phakopsora pachyrhizi*. The ASR severity was lower by 37, 68, and 77%, respectively, in plants supplied with Ni, Gl, and Ni+Gl in comparison to control treatment (plants sprayed with water). Infected plants from the control treatment showed earlier and great concentration of reactive oxygen species and malondialdehyde in comparison to plants sprayed either with Ni or Gl. For inoculated plants sprayed with either Ni or Gl, phenylalanine ammonia lyase and β-1,3-glucanase activities and phenolics concentration were greater. Additionally, Ni-sprayed plants showed earlier production of lignin than plants from the control treatment. Polyphenoloxidase activity was great at 5 days after inoculation for Gl-sprayed plants regardless of *P. pachyrhizi* infection. Based on the results of this study, it can be concluded that Ni and Gl differently regulated the activities of defense enzymes with no alteration on the antioxidant system in soybean plants infected by *P. pachyrhizi*.

Keywords: *Glycine max*. Herbicide. Biotrophic pathogen. Host defense. Rust.

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3.1. INTRODUCTION

Asian soybean rust (ASR), caused by fungus *Phakopsora pachyrhizi* H. Sydow & P. Sydow, is one of the most important diseases that affect the soybean crop. ASR promote energetic impairment on plant growth such limitations to the photosynthesis and drain of the plant energy to the activation of host defense mechanisms against fungal infection and also for pathogen nutrition (Langenbach *et al.* 2016, Rios *et al.* 2018).

Due to biotrophic lifestyle of *P.-pachyrhizi*, during the initial colonization of plants by the fungus usually occur a modest cellular damage in plant tissues (Hématy *et al.* 2009). However, at advanced stage of fungal infection there is an increase on the production of reactive oxygen species (ROS) by plants that result in membranes lipid peroxidation that generates cellular damage (Waszczak *et al.* 2018). Superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) are moderately reactive ROS that can be transformed in highly reactive ROS forms such as hydroperoxyl (HO_2^-) and hydroxyl radicals (OH) (Demidchik 2015). The antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and peroxidase (POX) plays a key role in the ROS detoxification (Demidchik 2015). Another important group of enzymes is involved more directly on the plant defense to pathogens acting on the degradation of fungal cell wall (chitinases, CHI; and β-1,3-glucanase, GLU) (Malik 2019), synthesis of quinones with antimicrobial action (polyphenoloxidase, PPO) (Taranto *et al.* 2017), oxidation of phenolics (POX) (Almagro *et al.* 2009), synthesis of compounds with signaling functions (lipoxygenases, LOX) (Wasternack and Feussner 2018), and in the phenylpropanoids pathway (phenylalanine ammonia-lyase, PAL) (Hossain *et al.* 2018).

A balanced nutrition of plants should always be the first line of defense against the infection by pathogens (Elmer and Datnoff 2014). Nickel (Ni), a micronutrient constituent of various biomolecules, is required for the catalytic process of several enzymes and its deficiency can disrupt the amino acid and organic acid metabolisms (Bai *et al.* 2006, Harasim and Filipek 2015). The positive effect of Ni on diseases control have been reported for tomato-*Fusarium oxysporum* f. sp. *lactucae* and -*F. oxysporum* f. sp. *lycopersici* (Ahmed *et al.* 2016), soybean-*Microsphaera diffusa* (Barcelos *et al.* 2018), and soybean-*P. pachyrhizi* (Chapter 1) interactions. Despite the fact that low Ni concentration is usually sufficient to avoid visual symptoms of deficiency, the effect of additional Ni supply to soybean plants aiming to control ASR is an interesting approach because its deficiency might occur in soybean plants showing no visual symptoms of deficiency (Freitas *et al.* 2019).

Glyphosate (Gl) is the most successful herbicide used in agriculture (Duke 2018). It has been reported the effect of Gl to control anthracnose, spring black stem, and leaf spot on alfalfa

(Samac and Foster-Hartnett 2012), wheat leaf rust and wheat yellow rust on wheat (Feng *et al.* 2005), rust on eucalyptus (Santos *et al.* 2019), and ASR on soybean (Feng *et al.* 2008). It is known that Gl has the ability to interact with divalent cations and to form poorly soluble compounds (Duke *et al.* 2012), alter the hormonal balance of plants in sublethal doses (Baylis, 2000), increase PAL activity, and promote alterations on the synthesis of amino acids (Hoagland *et al.* 1979). However, the mechanism(s) by which Gl is involved to reduce the ASR symptoms on soybean need to be investigated.

Considering the positive effect of either Ni or Gl on ASR control, this study aimed to elucidate the alterations promoted by Gl and its combination with Ni in the potentiation of host defense mechanisms and on the antioxidative metabolism of soybean plants from a Gl-resistant cultivar in response to *P. pachyrhizi* infection.

3.2. MATERIAL AND METHODS

3.2.1. Experimental design

A 4 × 2 factorial experiment, consisting of plants sprayed with water (control), Ni, Gl, and the combination of Ni and Gl (Ni+Gl) and non-inoculated or inoculated with *P. pachyrhizi*, was arranged in a completely randomized design with twelve replications. Each experimental unit consisted of a plastic pot containing four plants. The experiment was carried out twice.

3.2.2. Plant growth

Plants of the cultivar 'TMG 132', resistant to Gl and susceptible to ASR, were cultivated in 2 L plastic pots (four plants per pot) containing substrate made from a 1:1:1 mixture of pine bark, peat and expanded vermiculite (Tropstrato®, Vida Verde, Mogi Mirim, São Paulo, Brazil). The mixture had 0.67 ± 0.14 mg dm^{-3} of disponible Ni. Plants were kept in greenhouse (temperature of 25 ± 3°C and relative humidity of 80 ± 5%) and irrigated with deionized water daily.

3.2.3. Application of Ni and Gl and plant inoculation

Plants at the V4 growth stage (three fully expanded leaves) were sprayed with a solution (7.2 mL per plant) of 0.19 g L^{-1} $NiSO_4.6H_2O$ (equivalent to 60 g ha^{-1} Ni). This dose was based on preliminary experiments realized (unpublished data). At 24 hours after Ni spray, plants were sprayed with Roundup Original (Monsanto) (7.2 mL per plant of a solution of 0.667 g L^{-1} of acid equivalent of N-(phosphonomethyl) glycine (Gl), equivalent to 960 g ha^{-1} a.e. Gl). Plants

sprayed with water (control) served as the control treatment. At 24 hours after Gl spray, corresponding to 48 hours after Ni spray, plants were inoculated as described in chapter 1. The urediniospores were collected from soybean plants (cv. 'TMG132') previously inoculated with the monouredinial isolate UFV-DFP *Pp*25. The urediniospores were collected, preserved at -80ºC (Furtado *et al.* 2008), and their viability determined after plant inoculation. Inoculated plants were kept in a plastic mist growth chamber (temperature of 25 ± 3°C and relative humidity of 90 ± 5%) inside a greenhouse. At 16 hours after inoculation, plants were transferred to another greenhouse with the same conditions as previously described.

3.2.4. Evaluation of ASR severity

The second and third trifoliate leaves, from base to top, of four plants per replication (four replications and 16 plants total) of the control, Ni, Gl, and Ni+Gl treatments were collected at 15 days after inoculation (dai). The abaxial surface of the leaves was scanned at 600 dpi resolution and the obtained images were processed using the software QUANT (Fagundes-Nacarath *et al.* 2018) to quantify ASR severity.

3.2.5. Determination of soil and foliar Ni concentration

Four soils samples were collected before plant cultivation and the disponible Ni was extracted by Mehlich-1 method (Nelson *et al.* 1953) and determined by inductively coupled plasma-optical emission spectrometry (ICP-OES). For determination of foliar Ni concentration, a bulk of the second and third trifoliate leaves from plants used to evaluated ASR severity (15 dai) and also from non-inoculated plants were collected (16 plants and a total of 32 leaves per treatment), washed in deionized water, and dried in a drying oven with forced ventilation. The foliar Ni was extracted by nitric-perchloric digestion method and determined by inductively coupled plasma-optical emission spectrometry (ICP-OES).

3.2.6. Biochemical analysis

The second and third trifoliate leaves, from base to top, of two plants from replication of each treatment (four replications and 8 plants per sampling time) were collected at 5, 10, and 15 dai. Leaf samples were kept in liquid nitrogen during sampling and stored at -80°C until further analysis. Plants that had their leaves collected were discarded after each sampling.

3.2.7. Determination of O_2^- concentration

The O_2^- concentration was determined according to Chaitanya and Naithani (1994). A total of 200 mg of leaf tissue was ground into a fine powder with liquid nitrogen using a vibration ball mill and homogenized in 2 mL of a solution containing 100 mM sodium phosphate buffer (pH 7.2) and sodium diethyldithiocarbamate 1 mM. The homogenate was centrifuged at 22,000 *g* for 20 min at 4°C and an aliquot of the supernatant was added to the reaction medium consisting of 100 mM sodium phosphate buffer (pH 7.2), 1 mM sodium diethyldithiocarbamate, and 10 mM p-nitro-tetrazolium blue. The kinetic absorbance was read at 480 nm.

3.2.8. Determination of H_2O_2 concentration

The H_2O_2 concentration was determined according to Debona *et al.* (2012). A total of 100 mg of leaf tissue was ground into a fine powder with liquid nitrogen using a vibration ball mill and homogenized in 2 mL of a solution containing 50 mM potassium phosphate buffer (pH 6.5) and 1 mM hydroxylamine. The homogenate was centrifuged at 10,000 *g* for 15 min at 4°C, and an aliquot of the supernatant was added to the reaction medium consisting of 100 μM $FeNH_4(SO_4)$, 25 mM sulfuric acid, 250 μM xylenol orange, and 100 mM sorbitol. Samples were kept in the dark for 30 min and the absorbance determined at 560 nm. The controls were prepared in parallel and the H_2O_2 concentration was estimated by the standard curve of H_2O_2.

3.2.9. Determination of malondialdehyde (MDA) concentration

Cellular oxidative damage was estimated based on the production of total 2-thiobarbituric acid (TBA) reactive substances and expressed as equivalents of MDA according to Hodges *et al.* (1999) with a few modifications. A total of 100 mg of leaf tissue was ground into a fine powder using a vibration ball mill (Retsch, Haan, Germany) with liquid nitrogen and homogenized in 2 mL of 0.1% (w/v) trichloroacetic acid (TCA) solution. The homogenate was centrifuged at 12,000 *g* for 15 min at 4°C. After centrifugation, 0.25 mL of the supernatant was added to 0.75 mL of TBA solution (0.5% in 20% TCA) and held for 60 min at 99°C in a thermomixer (Eppendorf, Hamburg, Germany). The reaction was stopped in an ice bath. After the solution reached room temperature, the absorbance was read at 532 nm and discounting the non-specific absorbance at 600 nm. A stand curve of sucrose was used to correct the interference of soluble sugars in the samples. The molar extinction coefficient of 155 mM^{-1} cm^{-1} was used to calculate the MDA concentration.

3.2.10. Determination of antioxidant enzyme activities

A total of 200 mg of leaf tissue was ground into a fine powder using a vibration ball mill with liquid nitrogen. The fine powder was homogenized in 2 mL of a solution containing 100 mM potassium phosphate buffer (pH 7.8), 0.1 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, and 0.5% (m/v) polyvinylpyrrolidone. The homogenate was centrifuged at 13,000 *g* for 15 min at 4°C and the supernatant was collected to be used to determine SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), and APX (EC 1.11.1.1) activities. The activities of the antioxidant enzymes were determined as previously described by Debona *et al.* (2012) and expressed on the basis of protein.

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

A total of 100 mg of leaf tissue was ground into a fine powder with liquid nitrogen using a vibration ball mill 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. TSP concentration was determined in the methanolic extract and the pellet was kept at 20°C to determine the LTGA derivatives concentration following the procedures of Fortunato *et al.* (2015).

3.2.12. Determination of defense enzymes activities

A total of 200 mg of leaf tissue was ground into a fine powder using a vibration ball mill with liquid nitrogen. The fine powder was homogenized in 2 mL of a solution containing 50 mM potassium phosphate buffer (pH 6.8), 0.1 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, and 0.5% (m/v) polyvinylpyrrolidone. The homogenate was centrifuged at 13,000 *g* for 15 min at 4°C and the supernatant was collected and used to determine the activities of CHI (EC 3.2.1.14), GLU (EC 3.2.1.39), PPO (EC 1.10.3.1), POX, LOX (EC 1.13.11.12), and PAL (EC 4.3.1.24). The activities of these enzymes were determined as described by Fortunato *et al.* (2015) and expressed on the basis of protein.

3.2.13. Data analysis

Data from the variables evaluated from the two experiments were analyzed using the MIXED procedure of the SAS software (Release8.02Level02M0for Windows, SAS Institute) to determine if data from the experiments could be combined (Moore and Dixon 2015). Data were checked for normality and homogeneity of variance and subjected to analysis of variance

(ANOVA) thereafter. Treatments means were compared by F test ($P \leq 0.05$). For ASR severity, ANOVA considered plants sprayed with water, Ni, Gl, and Ni+Gl with four replications. For biochemical analysis and foliar Ni concentration, ANOVA was considered to be a 2×4 factorial experiment consisting of non-inoculated and inoculated plants and four spray conditions (water, Ni, Gl, and Ni+Gl) with four replications. Data were analyzed using the Minitab software (version 18, Minitab Corporation).

3.3. RESULTS

3.3.1. Foliar Ni concentration

In non-inoculated plants, the foliar Ni concentration was higher by 174 and 114% in Ni and Ni+Gl plants, respectively, in comparison to that in control plants (Fig. 1). In inoculated plants, the foliar Ni concentrations were higher by 273 and 213% in Ni and Ni+Gl plants, respectively, in comparison to that in control plants. The foliar Ni concentrations were higher by 6, 45, 13, and 55% in control, Ni, Gl, and Ni+Gl inoculated plants, respectively, in comparison to non-inoculated plants.

3.3.2. Severity of ASR

Many necrotic lesions containing several uredinia were observed on the leaves of plants from the control treatment while they were less developed on the leaves of Ni plants and much less developed on the leaves of Gl and Ni+Gl plants (Fig. 2A-H). The ASR severity in Ni, Gl, and Ni+Gl plants was significantly reduced by 37, 68, and 77%, respectively, in comparison to control plants (Fig. 3). Moreover, the ASR severity was lower by 50 and 65% in Gl and Ni+Gl plants, respectively, in comparison to Ni plants.

3.3.3. Concentrations of O_2^-, H_2O_2, and MDA

The O_2^-, H_2O_2, and MDA concentrations were not affected by either Ni or Gl in non-inoculated plants (Fig. 3A-C). The O_2^- concentration was higher at 5 dai in control inoculated plants, at 10 dai in control, Ni, and Gl inoculated plants, and at 15 dai in control and Ni inoculated plants in comparison to that in non-inoculated plants. The O_2^- concentration was significantly higher by 22 and 32% at 10 dai in control inoculated plants in comparison to Gl and Ni+Gl inoculated plants, respectively, and by 32% at 15 dai in control inoculated plants in comparison to Ni+Gl inoculated plants (Fig. 4A). For inoculated plants, the H_2O_2 concentration was significantly higher in Gl plants at 5 dai, in control, Ni, and Ni+Gl plants at 10 dai, and in

control and Gl plants at 15 dai in comparison to that in non-inoculated plants. The H_2O_2 concentration was significantly higher by 34 and 67% at 15 dai in control inoculated plants in comparison to Ni and Ni+Gl inoculated plants, respectively (Fig. 4B). The MDA concentration was significantly higher at 10 dai in control inoculated plants and at 15 dai in control and Ni inoculated plants in comparison to that in non-inoculated plants. The MDA concentration was significantly higher by 29% at 10 dai in control inoculated plants in comparison to Ni+Gl inoculated plants, and by 26 and 29% at 15 dai in control inoculated plants in comparison to Gl and Ni+Gl inoculated plants, respectively (Fig. 4C).

3.3.4. Antioxidant enzymes activities

For non-inoculated plants, there was no significant difference among the control, Ni, Gl, and Ni+Gl treatments regardless of the antioxidant enzymes studied and the evaluation time (Fig. 5A-C). SOD activity was higher at 5 and 10 dai in inoculated plants in comparison to non-inoculated plants regardless of the treatment. At 15 dai, SOD activity was higher in control, Ni, and Gl inoculated plants in comparison to that in non-inoculated plants (Fig. 5A). Following this same comparison, CAT activity was significantly higher at 5 dai in Ni and Ni+Gl inoculated plants and at 15 dai in control and Ni inoculated plants in comparison to that in non-inoculated plants. CAT activity was significantly higher by 45% at 15 dai in control inoculated plants in comparison to Ni+Gl inoculated plants (Fig. 5B). APX activity was significantly higher at 10 and 15 dai in control, Ni, Gl, and Ni+Gl inoculated plants in comparison to that in non-inoculated plants. APX activity was significantly lower by 22 and 23% at 15 dai in Ni+Gl inoculated plants in comparison to control and Ni inoculated plants, respectively (Fig. 5C).

3.3.5. Concentrations of TSP and LTGA derivatives

The concentrations of TSP and LTGA derivatives were not affected by Ni and Gl treatments in non-inoculated plants (Fig. 6A-B). The TSP concentration was higher at 10 dai in Ni and Ni+Gl inoculated plants, and at 15 dai in Ni, Gl, and Ni+Gl inoculated plants in comparison to that in non-inoculated plants. The TSP concentration was higher by 23% at 10 dai in Ni+Gl inoculated plants and by 17, 15, and 26% at 15 dai in Ni, Gl, and Ni+Gl inoculated plants, respectively, in comparison to control inoculated plants (Fig. 6A). The LTGA derivatives concentration was higher in Ni and Ni+Gl inoculated plants at 5 dai and in control, Ni, Gl, and Ni+Gl inoculated plants at 10 and 15 dai in comparison to that in non-inoculated plants. For inoculated plants at 5 dai, the concentration of LTGA derivatives in Ni and Ni+Gl

inoculated plants was significantly higher by 41 and 45%, respectively, in comparison to that in control plants (Fig. 6B).

3.3.6. Activities of defense enzymes

For non-inoculated plants, there was no significant difference among the control, Ni, Gl, and Ni+Gl treatments regardless of the defense enzymes studied and the evaluation time (Fig. 7A, B, D, E, and F), except for PPO, which showed high activity of 30 and 35% at 5 dai in Gl and Ni+Gl plants, respectively, and of 28% at 15 dai in Ni+Gl plants in comparison to that in control plants (Fig. 7C). Comparing non-inoculated and inoculated plants at each evaluation time, CHI activity was significantly higher in inoculated plants than in non-inoculated plants regardless of the treatment and the evaluation time (Fig. 7A). Following this same comparison, GLU activity was higher at 10 and 15 dai in inoculated plants in comparison to non-inoculated plants regardless of the treatment. GLU activity was higher by 44, 45, and 33% at 10 dai in Ni, Gl, and Ni+Gl inoculated plants, respectively, in comparison to that in control inoculated plants (Fig. 7B). PPO activity was higher at 10 dai in control, Ni and Ni+Gl inoculated plants, and at 15 dai in control, Ni, Gl, and Ni+Gl inoculated plants in comparison to that in non-inoculated plants. PPO activity was higher by 37 and 33% at 5 dai in Gl and Ni+Gl inoculated plants, respectively, in comparison to control inoculated plants (Fig. 7C). POX activity was higher at 5 dai in Ni, Gl, and Ni+Gl inoculated plants, and at 10 and 15 dai in control, Ni, Gl, and Ni+Gl inoculated plants in comparison to that in non-inoculated plants (Fig. 7D). LOX activity was not affected by either Ni or Gl and by *P. pachyrhizi* infection (Fig. 7E). PAL activity was higher at 5 dai in Ni, Gl, and Ni+Gl inoculated plants, and at 10 and 15 dai in control, Ni, Gl, and Ni+Gl inoculated plants in comparison to that in non-inoculated plants. The PAL activity was higher by 31 and 32% at 5 dai in Ni and Ni+Gl inoculated plants, respectively, in comparison to control inoculated plants. For inoculated plants at 15 dai, PAL activity was increased by 30 and 39% in Ni and Ni+Gl plants, respectively, in comparison to that in control plants (Fig. 7F).

3.4. DISCUSSION

The present study brings novel evidences of the Gl effect and its combination with Ni on the potentiation of soybean resistance against *P. pachyrhizi* infection. The great foliar Ni concentration in Ni-treated plants confirmed its absorption. The foliar Ni concentration higher in inoculated plants than in non-inoculated plants regardless of Ni-supply can be related with its mobilization from non-inoculated for inoculated areas of plant leaf tissues. Interestingly, the

fungal infection minimized the negative effect of Gl on foliar Ni concentration in Ni-supplied plants possible due the mobilization of this micronutrient.

The different types of defense mechanisms mounted by the plants associated with their intensity and time of occurrence determine their level of resistance against pathogens infection (Glazebrook 2015, Mauch-Mani *et al.* 2017, Mazid *et al.* 2011, Torres 2010). Mineral nutrients and others compounds affect the plant metabolism and regulate the defense mechanisms resulting, therefore, in alterations of the level of basal resistance in response to pathogens attack (Elmer and Datnoff 2014). The ASR control by Ni and Gl observed in the present study corroborate with the findings of Feng *et al.* (2008) and the results presented in chapter 1. The MDA result from the oxidative fragmentation of polyunsaturated fatty acid-rich lipids and is a robust marker of non-enzymatic lipid oxidation in plants (Schmid-Siegert *et al.* 2016). The great ASR severity in control inoculated plants corroborated with an increase on MDA concentration starting at 10 dai in comparison to non-inoculated plants. The MDA concentration in Ni inoculated plants increased only at 15 dai revealing lower oxidative damage on these plants in comparison to inoculated plants from the control treatment. The absence of significance for MDA concentration between non-inoculated and inoculated plants from the Gl and Ni+Gl treatments at 15 dai also reveals the important effect of Gl in affecting the infection of *P. pachyrhizi*.

Lipid peroxidation results from the action of highly ROS such as peroxyl (OH^-) and hydroperoxyl radicals (HO_2^-). Considering that O_2^- and H_2O_2 will be converted into these highly reactive species (Waszczak *et al.* 2018), the great accumulation of O_2^- and H_2O_2 observed in control inoculated plants at 5, 10, and 15 dai and in Ni inoculated plants at 10 and 15 dai corroborate with the great MDA concentration observed for these plants.

ROS generation is directly related to the type of stress imposed to plants (Demidchik 2015) and has been reported to occur in plants infected by pathogens of different lifestyles (Rodrigues *et al.* 2017, Künstler *et al.* 2018, Fagundes-Nacarath *et al.* 2018). The composition and functionality of the antioxidant system are pivotal to cellular ROS detoxification. Among these systems, the enzymatic antioxidant system plays a crucial role. The Ni effect on the activities of plant antioxidant enzymes was reported in wheat (Gajewska and Skłodowska 2007) and soybean (Sirhindi *et al.* 2016, Barcelos *et al.* 2018). The Gl application also affects the activities of antioxidant enzymes in peanut (Radwan and Fayez 2016) and in different species of *Acer* (Percival 2017). The results of the present study reveal no significant effect of Ni and Gl on the SOD, CAT and APX activities in non-inoculated plants and in inoculated plants until 10 dai. At 15 dai, CAT and APX activities were higher in control inoculated plants in

comparison to Ni+Gl inoculated plants. The great O_2^- and H_2O_2 concentrations at 15 dai in control inoculated plants revealed that SOD, CAT, and APX activities in these plants was not enough to avoid ROS accumulation resulting, therefore, in cellular damage that corroborated with the MDA concentration. By contrast, the non-accumulation of H_2O_2 in Ni inoculated plants may be explained by the great CAT and APX activities similarly to what was obtained for control inoculated plants. Indeed, the great O_2^- obtained for Ni inoculated plants revealed that the great SOD activity was not enough to avoid the O_2^- accumulation in Ni-supplied plants similarly to what was noticed for plants from the control treatment.

Phenolics are of pivotal importance to limit the colonization of soybean leaf tissues by *P. pachyrhizi* (Lygin *et al.* 2009). Phenolics exert a direct effect against the fungi or indirectly limit the colonization of their hosts tissues due to lignin deposition. For a better comprehension of the alterations on phenolics metabolism, PAL activity and the determination of phenolics and lignin concentrations must be analyzed together. The higher PAL activity at 5 dai in Ni and Ni+Gl inoculated plants than in inoculated plants for the control treatment theoretically would result in a great TSP concentration. However, the increase on TSP concentration was not observed at 5 dai probably due to the polymerization of phenolics to form lignin corroborating with the great LTGA derivatives concentration noticed for Ni-treated plants. Differently of what was reported by Zobiole *et al.* (2010), Gl did not affect lignin concentration in Gl-resistant plants. However, the effect of Gl itself in the phenolics metabolism was observed at 15 dai resulting in an increase on TSP concentration in inoculated plants. Moreover, an additional effect of fungal infection on the increase of TSP concentration was observed in Ni+Gl plants in comparison to plants from the control, Ni, and Gl treatments. Cell wall lignification has a protective role in plants limiting ASR development (Lygin *et al.* 2009). The faster increase of PAL activity and LTGA derivatives concentration in Ni and Ni+Gl inoculated plants in comparison to control inoculated plants may be related to the lower ASR severity in Ni-treated plants than in plants from the control treatment. Additionally, the higher TSP concentration observed at 10 and 15 dai for Ni inoculated plants in comparison to that in non-inoculated plants corroborate with the positive effect of Ni on phenolics concentration which could have limited the *P. pachyrhizi* infection.

Besides the PAL, the interaction of fungal pathogens with their hosts results in the induction of others pathogenesis-related defense proteins including CHI, GLU, PPO, POX, and LOX (Fagundes-Nacarath *et al.* 2018). In the present study, CHI activity increased on the leaves of infected plants regardless of control, Ni, Gl, and Ni+Gl treatments and the evaluation time. The higher GLU activity at 10 dai in Ni, Gl, and Ni+Gl inoculated plants in comparison to

control inoculated plants may have contributed to limit the colonization of leaf tissues by *P. pachyrhizi* due to the degradation of *β*-l,3-glucan found in fungal cell wall. The increase on CHI and GLU activities and the up-regulation of their corresponding coding genes were associated with an increase on the resistance of soybean plants against *P. pachyrhizi* infection (Tremblay *et al.* 2010, Chapter 1).

The Gl-treated plants showed higher PPO activity in comparison to plants from the control treatment at 5 dai regardless of the inoculation with *P. pachyrhizi*. The positive effect of Gl on PPO activity was reported on plants of *Cyperus esculentus* by Cañal *et al.* (1988). In the present study, Ni had not effect on PPO activity. Considering that PPO oxide phenolics to quinones, which are often more toxic to fungi than original phenolics (Taranto *et al.* 2017), the faster increase on PPO activity can be associated with the low ASR severity observed in Gl-treated plants.

The POX is involved in different physiological processes on plants such as oxidation of phenolics, lignin formation, synthesis of phytoalexins, and in the antioxidative metabolism (Almagro *et al.* 2009). The faster increase on POX activity for inoculated plants from the Ni, Gl, and Ni+Gl treatments in comparison to plants from the control treatment can be related to the high lignin concentration in Ni-supplied plants. The increase on POX activity in response to Ni has already been observed in wheat (Gajewska and Sklodowska 2007) and soybean (Chapter 1). The LOX is linked to the synthesis of compounds with signaling functions on plants submitted to biotic stress (Wasternack and Feussner 2018). However, the LOX activity was no affected on plants from the control, Ni, Gl, and Ni+Gl treatments regardless of inoculation with *P. pachyrhizi*.

It is plausible to conclude that both Ni and Gl regulated the activities of defense enzymes in a different way in a scenario where the participation of the antioxidant system was less important to counteract the infection by *P. pachyrhizi*. In general, the lower ASR symptoms on the leaves of Ni plants was linked with high PAL and GLU activities and great concentrations of TSP and LTGA derivatives while for Gl plants, PAL, GLU and PPO activities and TSP concentration were of greater importance.

3.5. FIGURES

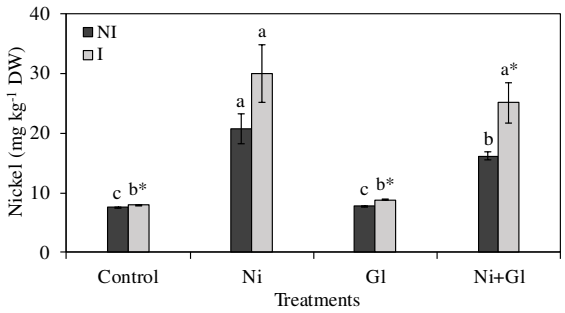

Figure 1. Nickel concentration in the leaves of soybeans plants sprayed with water (control), nickel (Ni), glyphosate (Gl), and with the combination of Ni and Gl (Ni+Gl) and non-inoculated (NI) or inoculated (I) with *Phakopsora pachyrhizi*. Means for each treatment (control, Ni, Gl, and Ni+Gl) followed by different letters and for the NI and I treatments followed by an asterisk (*) are significantly different ($P \leq 0.05$) according to Tukey's test. Bars represent the standard error of the means. $n = 8$.

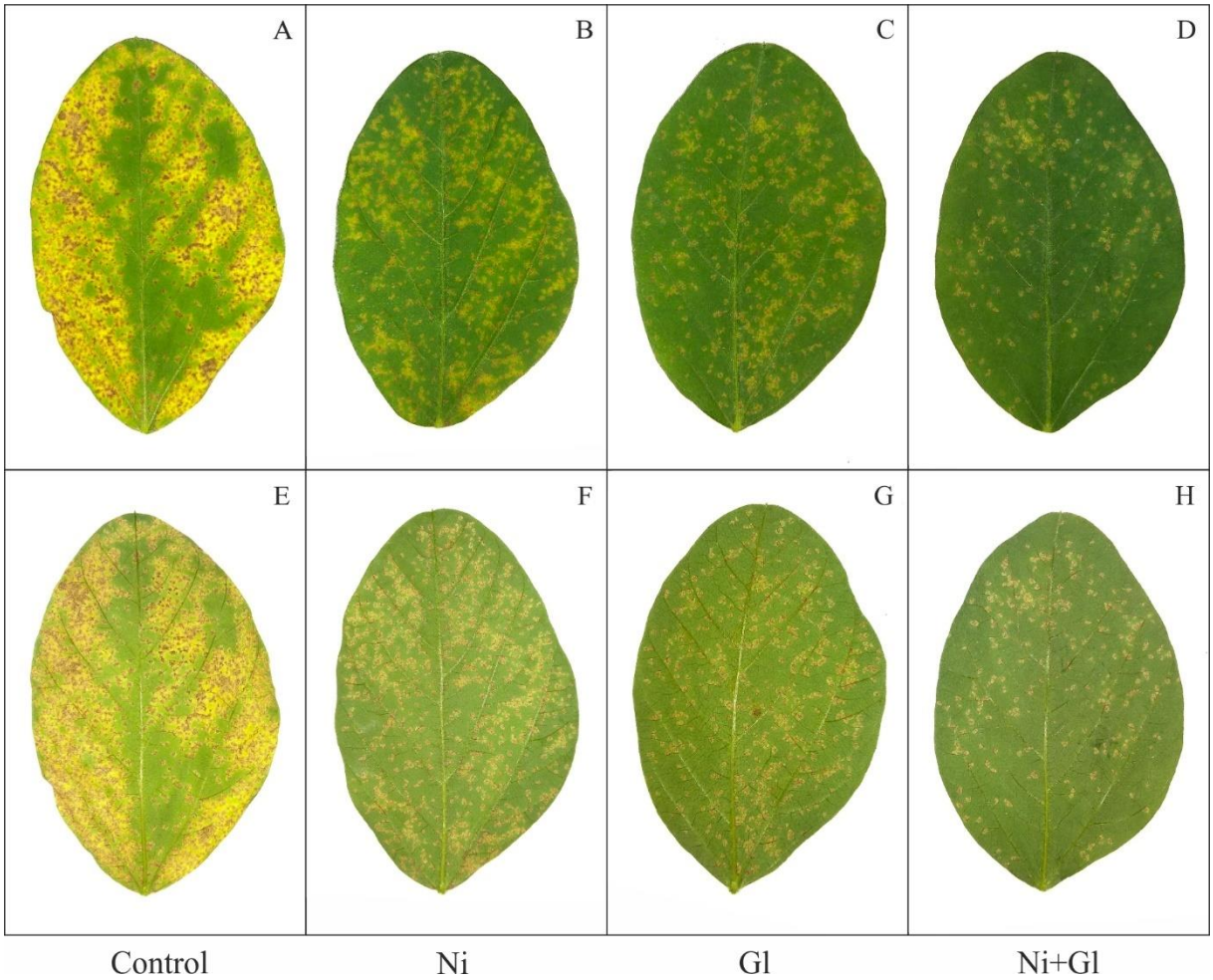

Figure 2. Symptoms of Asian soybean rust in the adaxial (A-D) and abaxial (E-H) surfaces of leaflets of soybean plants sprayed with water (control) (A and E), nickel (Ni) (B and F), glyphosate (Gl) (C and G), and with the combination of Ni and Gl (Ni+Gl) (D and H).

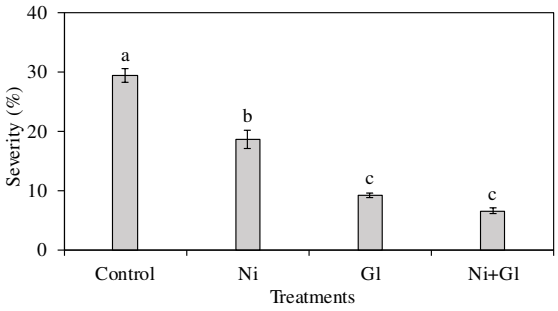

Figure 3. Asian soybean rust severity in leaves of soybean plants sprayed with water (control), nickel (Ni), glyphosate (Gl), and with the combination of Ni and Gl (Ni+Gl). Means for each treatment followed by different letters are significantly different ($P \leq 0.05$) according to Tukey's test. Bars represent the standard error of the means. $n = 8$.

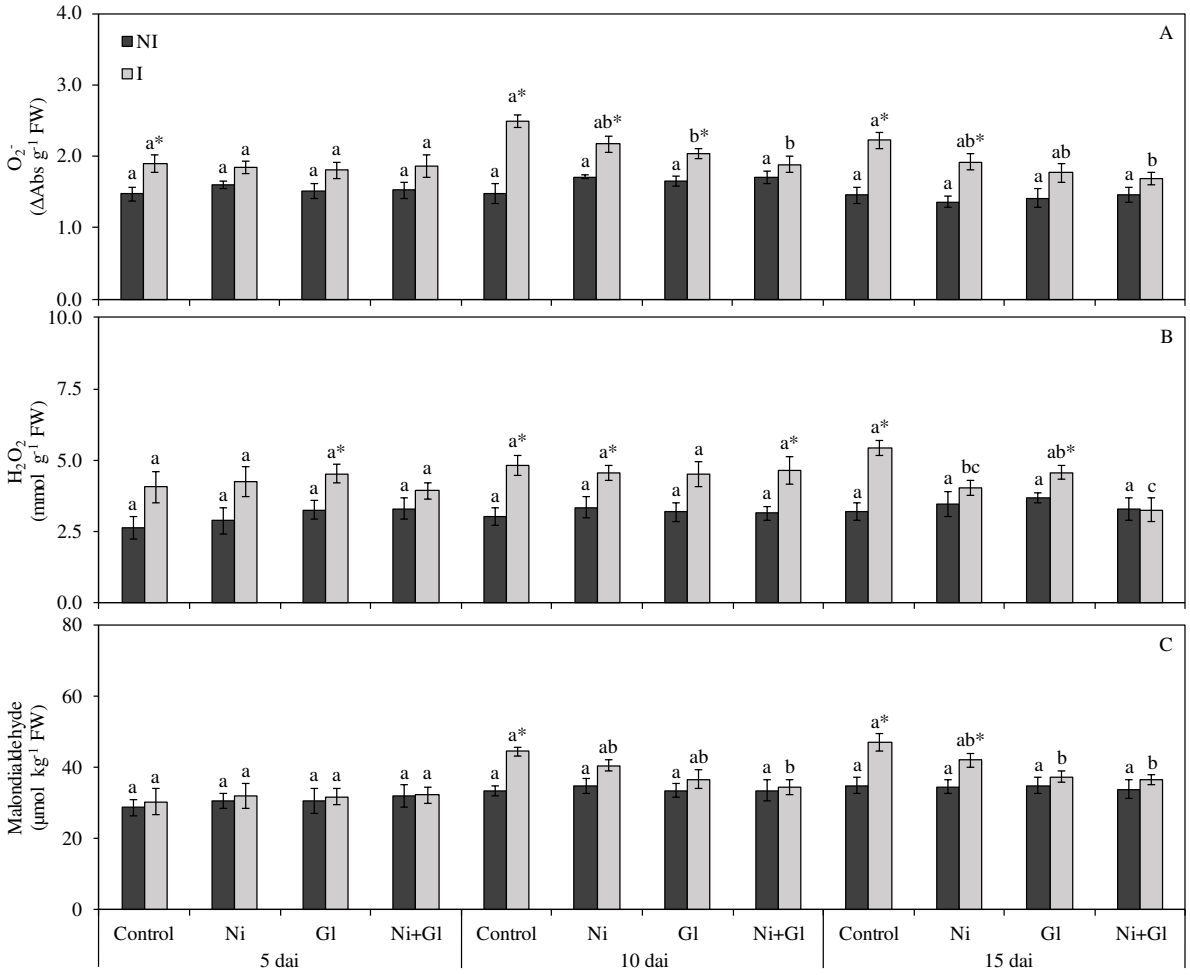

Figure 4. Concentrations of superoxide (O_2^-) (A), hydrogen peroxide (H_2O_2) (B), and malondialdehyde (C) in the leaves of soybean plants sprayed with water (control), nickel (Ni), glyphosate (Gl), and with the combination of Ni and Gl (Ni+Gl) and non-inoculated (NI) or inoculated (I) with *Phakopsora pachyrhizi*. For each evaluation time, means for each treatment (control, Ni, Gl, and Ni+Gl) followed by different letters and for the NI and I treatments followed by an asterisk (*) are significantly different ($P \leq 0.05$) according to Tukey's test. FW = fresh weight. dai = days after inoculation. Bars represent the standard error of the means. $n = 8$.

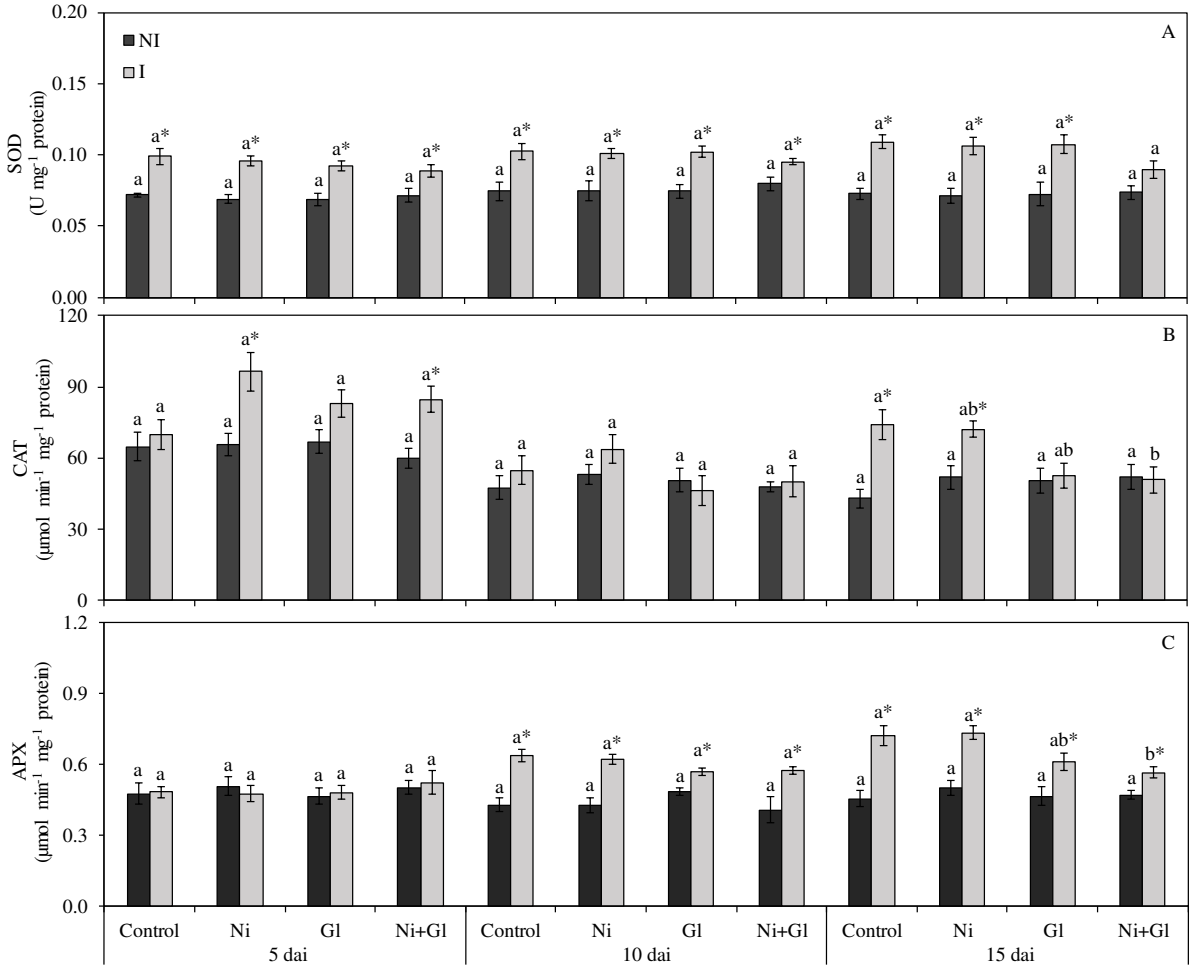

Figure 5. Activities of superoxide dismutase (SOD) (A), catalase (CAT) (B), and ascorbate peroxidase (APX) (C) in the leaves of soybean plants sprayed with water (control), nickel (Ni), glyphosate (Gl), and with the combination of Ni and Gl (Ni+Gl) and non-inoculated (NI) or inoculated (I) with *Phakopsora pachyrhizi*. For each evaluation time, means for each treatment (control, Ni, Gl, and Ni+Gl) followed by different letters and for the NI and I treatments followed by an asterisk (*) are significantly different ($P \leq 0.05$) according to Tukey's test. dai = days after inoculation. Bars represent the standard error of the means. $n = 8$.

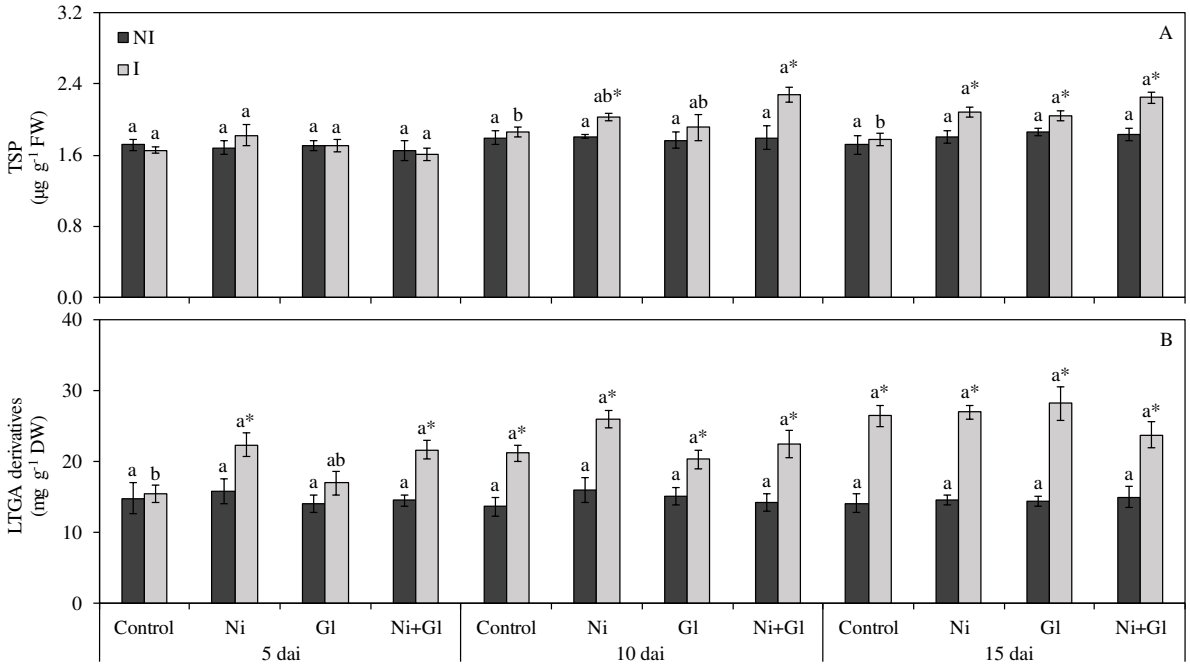

Figure 6. Concentrations of total soluble phenolics (TSP) (A) and lignin-thioglycolic acid (LTGA) derivatives (B) in the leaves of soybean plants sprayed with water (control), nickel (Ni), glyphosate (Gl), and with the combination of Ni and Gl (Ni+Gl) and non-inoculated or inoculated (I) with *Phakopsora pachyrhizi*. For each evaluation time, means for each treatment (control, Ni, Gl, and Ni+Gl) followed by different letters and for the NI and I treatments followed by an asterisk (*) are significantly different ($P \leq 0.05$) according to Tukey's test. FW = fresh weight. DW = dry weight. dai = days after inoculation. Bars represent the standard error of the means. $n = 8$.

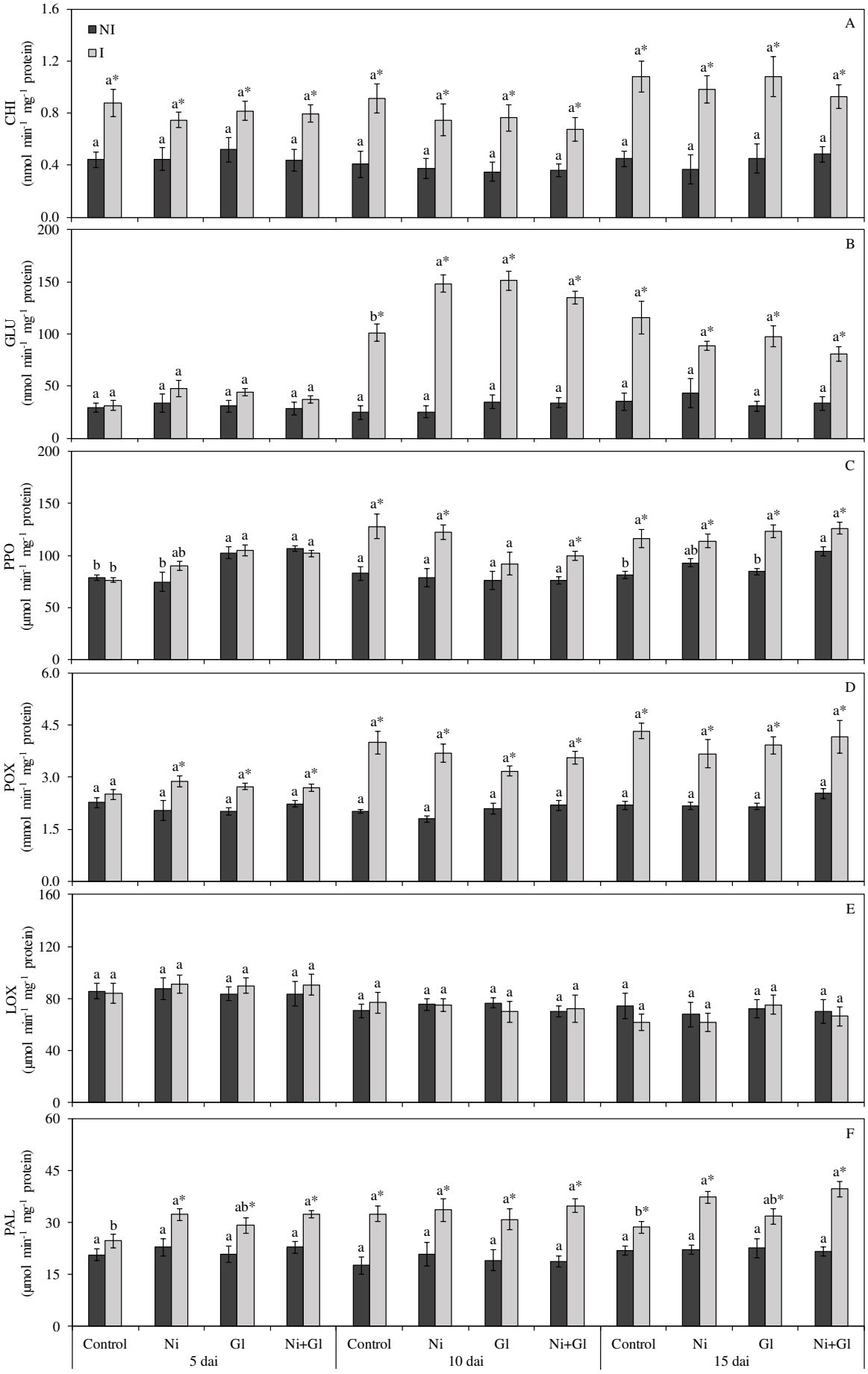
A
B
C
D
E
F
NI
I
CHI (nmol min-1 mg-1 protein)
GLU (nmol min-1 mg-1 protein)
PPO (µmol min-1 mg-1 protein)
POX (mmol min-1 mg-1 protein)
LOX (µmol min-1 mg-1 protein)
PAL (µmol min-1 mg-1 protein)
Control
Ni
Gl
Ni+Gl
5 dai
10 dai
15 dai

Figure 7. Activities of chitinase (CHI) (A), β-1,3-glucanase (GLU) (B), polyphenoloxidase (PPO) (C), peroxidase (POX) (D), lipoxygenase (LOX) (E), and phenylalanine ammonia lyase (PAL) (F) in the leaves of soybean plants sprayed with water (control), nickel (Ni), glyphosate (Gl), and with the combination of Ni and Gl (Ni+Gl) and non-inoculated (NI) or inoculated (I) with *Phakopsora pachyrhizi*. For each evaluation time, means for each treatment (control, Ni, Gl, and Ni+Gl) followed by different letters and for the NI and I treatments followed by an asterisk (*) are significantly different ($P \leq 0.05$) according to Tukey's test. dai = days after inoculation. Bars represent the standard error of the means. $n = 8$.

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4. CHAPTER 4: PHOTOSYNTHESIS AND ETHYLENE ARE DIFFERENTLY AFFECTED IN GLYPHOSATE-RESISTANT SOYBEAN PLANTS SPRAYED WITH GLYPHOSATE AND NICKEL AND INFECTED BY *Phakopsora pachyrhizi*

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Abstract: It is known that both nickel (Ni) and glyphosate (Gl) may result in reduction of the Asian soybean rust (ASR), caused by *Phakopsora pachyrhizi*, symptoms on soybean. However, the effect of both Ni and Gl associated with the energetic balance and ethylene metabolism of soybean plants infected by *P. pachyrhizi* need to be elucidated. This study aimed to fill out this gap trying to elucidate the effect of Ni, Gl and their combination (Ni+Gl) on ASR development, photosynthetic capacity, sugars concentration, and ethylene concentration in Gl-resistant soybean plants infected or not with *P. pachyrhizi*. Plants sprayed with water served as a control treatment. The ASR severity was reduced on plants sprayed with Ni and Gl. Carotenoids and chlorophylls concentrations were higher for Ni, Gl, and Ni+Gl inoculated plants in comparison to that in control plants. Parameters of chlorophyll *a* fluorescence revealed photosynthetic apparatus damage and lowest destination of energy to photochemistry process on inoculated plants from the control treatment. Limitations on the photosynthetic machinery capacity of inoculated plants to capture light and use the absorbed energy by photosystem II reflected on their capacity to reduce the CO_2 as indicated by the high values for internal CO_2 concentration and low values for rate of net carbon assimilation. Low sugars concentration on inoculated plants from the control treatment was linked to their reduced photosynthetic capacity due to the high ASR severity. For non-inoculated plants, ethylene concentration was not affected by Ni and Gl, but its concentration decreased for inoculated plants being more pronounced for plants from the control treatment. In conclusion, this study sheds light into the role played by both Ni and Gl on ASR control from a physiological point of view. Soybean plants exposed to Ni and Gl were able to maintain their photosynthetic capacity and the great ethylene concentration during the infection process of *P. pachyrhizi*.

Keywords: *Glycine max*. Herbicide. Plant nutrition. Rust. Photosynthesis. Plant hormones.

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4.1. INTRODUCTION

Traditionally, soybean (*Glycine max* (L.) Merrill) is the major source of protein used worldwide (Ravindran 2013). The well-balanced essential amino acid profile of soybean enables it to balance most cereal-based diets (Beski *et al.* 2015). Asian soybean rust (ASR), caused by *Phakopsora pachyrhizi* H. Sydow & P. Sydow, is one of the most important soybean diseases. Significant yield losses are caused by ASR on soybean and disease control management considerably increases the cost of production (Godoy *et al.* 2016).

The symptoms of ASR are mainly characterized by the appearance of small chlorotic lesions that progress to larger necrotic lesions associated with the uredinia eruption from leaf epidermis mainly in the abaxial leaf surface (Goellner *et al.* 2010). The impairments caused by *P. pachyrhizi* infection in soybean plants are related to the energetic cost originated from the activation of host defense responses and by pathogen consumption of nutrients as well as to the limited photosynthetic capacity caused by decreases of the photosynthetic pigments concentration, damage at photosystems levels, impairments on gas exchange regulations, and biochemical limitations (Rios *et al.* 2018, Chapter 2).

Nickel (Ni) was the last mineral element recognized as an nutrient to plants (Brown *et al.* 1987). Ni is required for the catalytic process of several enzymes, binding to high number of proteins and peptides, and is constituent of several biomolecules (Harasim and Filipek 2015). Deficiency of this micronutrient might occur in soybean plants without visual symptoms, reducing the foliar iron concentration and affecting the photosynthetic process (Freitas *et al.* 2019). Ni has shown potential to control powdery mildew (Barcelos *et al.* 2018) and ASR (Chapters 1 and 2) on soybean. In soybean-*Microsphaera diffusa* interaction, the lowest powdery mildew severity in Ni-treated plants was related to a more robust antioxidant metabolism (Barcelos *et al.* 2018). For the soybean-*P. pachyrhizi* interaction, Ni potentiated host defense responses against fungal infection by increasing β-1,3-glucanase activity, expression of genes coding for urease, chalcone isomerase, and phenylalanine ammonia lyase as well leaf tissue lignification (Chapter 1).

Glyphosate (Gl) has become the world's most widely used agrochemical (Duke 2018). Along with the increased use of Gl, the number of studies related to its effect on plant diseases has dramatically increased nowadays (Hammerschmidt 2017, Martinez *et al.* 2018). The Gl has shown potential to control ASR (Feng *et al.* 2005, 2008). However, the mechanism which Gl acts on ASR control requires further elucidation. Non-lethal doses of Gl increases ethylene production on plants of common beans (Abu-irmaileh *et al.* 1979) and paper birch (Stasiak *et al.* 1992). On cotton plants, Gl acts disrupting auxin transport leading, therefore, to an increase

on ethylene synthesis (Beyer and Morgan 1969). Distinctly, Pennazio and Roggero (1992) reported that Ni inhibited ethylene production in soybean plants by depressing ethylene-forming enzyme activity although it stimulated the production of free ACC (1-Aminocyclopropane-1-carboxylic acid), precursor of ethylene. It is commonly accepted that ethylene/jasmonic acid-dependent defenses are generally active against necrotrophic pathogens whereas salicylic acid-inducible defenses predominantly effective against the biotrophics (Zhang *et al.* 2009, Sun *et al.* 2017, Van Der Ent and Pieterse 2012). Therefore, the effect of both Ni and Gl on the regulation of ethylene biosynthesis in soybean plants infected by *P. pachyrhizi* becomes one interesting topic to be investigated.

Thus, this study aimed to evaluate the effect of Ni, Gl, and their combination, on the photosynthesis and ethylene production of soybean plants infected by *P. pachyrhizi*.

4.2. MATERIAL AND METHODS

4.2.1. Experimental design

A 4 × 2 factorial experiment, consisting of plants sprayed with water (control), Ni, Gl, and the combination of Ni and Gl (Ni+Gl) and non-inoculated or inoculated with *P. pachyrhizi*, was arranged in a completely randomized design with sixteen replications. Each experimental unit consisted of a plastic pot containing four plants. The experiment was carried out twice. Data from the variables and parameters evaluated from the two experiments were analyzed using the MIXED procedure of the SAS software (Release8.02Level02M0for Windows, SAS Institute) to determine if data from the experiments could be combined (Moore and Dixon 2015).

4.2.2. Plant growth

Plants of the cultivar 'TMG 132', resistant to Gl and susceptible to ASR, were cultivated in 2 L plastic pots (four plants per pot) containing substrate made from a 1:1:1 mixture of pine bark, peat and expanded vermiculite (Tropstrato®, Vida Verde, Mogi Mirim, São Paulo, Brazil). The mixture had 0.69 ± 0.09 mg dm^{-3} of disponible Ni. Plants were kept in greenhouse (temperature of 25 ± 3°C and relative humidity of 80 ± 5%) and irrigated with deionized water daily.

4.2.3. Application of Ni and Gl and plant inoculation

Plants (V4 growth stage and with three fully expanded leaves), were sprayed with a solution (7.2 mL per plant) of 0.19 g L^{-1} $NiSO_4.6H_2O$ (equivalent to 60 g ha^{-1} Ni). This dose was based on preliminary experiments realized (unpublished data). At 24 hours after Ni spray, plants were sprayed with Roundup Original (Monsanto) (7.2 mL per plant of a solution of 0.667 g L^{-1} of acid equivalent of N-(phosphonomethyl) glycine (Gl), equivalent to 960 g ha^{-1} a.e. Gl). Plants sprayed with water (control) served as the control treatment. At 24 hours after Gl spray, corresponding to 48 hours after Ni spray, plants were inoculated with *P. pachyrhizi* as described in chapter 1. The urediniospores were collected from soybean plants (cv. 'TMG132') previously inoculated with the monouredinial isolate UFV-DFP *Pp*25. The urediniospores were collected, preserved at -80ºC (Furtado *et al.* 2008), and their viability determined after plant inoculation. Inoculated plants were kept in a plastic mist growth chamber (temperature of 25 ± 3°C and relative humidity of 90 ± 5%) inside a greenhouse. At 16 hours after inoculation, plants were transferred to another greenhouse with the same conditions as previously described.

4.2.4. Evaluation of ASR severity

The second and third trifoliate leaves, from base to top, of four plants per replication (four replications and 16 plants total) of the control, Ni, Gl, and Ni+Gl treatments were collected at 15 days after inoculation (dai). The abaxial surface of the leaves was scanned at 600 dpi resolution and the obtained images were processed using the software QUANT (Fagundes-Nacarath *et al.* 2018) to quantify ASR severity.

4.2.5. Determination of soil and foliar Ni concentration

Four soils samples were collected before plant cultivation and the disponible Ni was extracted by Mehlich-1 method (Nelson *et al.* 1953) and determined by inductively coupled plasma-optical emission spectrometry (ICP-OES). For determination of foliar Ni concentration, a bulk of the second and third trifoliate leaves from plants used to evaluated ASR severity (15 dai) and also from non-inoculated plants were collected (16 plants and a total of 32 leaves per treatment), washed in deionized water, and dried in a drying oven with forced ventilation. The foliar Ni concentration was determined by nitric-perchloric digestion method and inductively coupled plasma-optical emission spectrometry (ICP-OES).

4.2.6. Photosynthetic pigments measurement

Photosynthetic pigments were determined in the lateral leaflet of the third trifoliate leaf, from base to top, of one plant from replication of each treatment (four replications and four

plants per each sampling time) at 8 and 13 dai. The photosynthetic pigments of six-leaf discs (0.8 cm^3 each) were extracted with dimethyl sulfoxide (DMSO) saturated with calcium carbonate (Santos *et al.* 2008). The concentrations of chlorophyll (Chl) *a*, Chl *b*, Chl *a*+*b*, and carotenoids were calculated based on the absorbance at 480, 649, and 665 nm (Sumanta *et al.* 2014).

4.2.7. Gas-exchange parameters

Leaf gas exchange parameters were determined in the lateral leaflet of the third trifoliate leaf, from base to top, of one plant from replication of each treatment (four replications and four plants per each sampling time) at 8 and 13 dai. The rate of net carbon assimilation (*A*), stomatal conductance to water vapor (g_s), internal CO_2 concentration (C_i), and transpiration rate (*E*) were estimated from 09:00 to 12:00 h under artificial and saturating photon irradiance (1200 μmol m^{-2} s^{-1}) and an external CO_2 concentration of 400 μmol mol^{-1} using a portable open-system infrared gas analyzer (LI-6400, LI-COR Inc., Lincoln, NE, USA). All measurements were performed by setting the block temperature at 25°C.

4.2.8. Chl *a* fluorescence parameters measurement

The third trifoliate leaf used for the assessment of the gas-exchange parameters (8 and 13 dai) were also used to evaluate the Chl *a* fluorescence parameters (four replications and one plant per each sampling time). The Imaging-PAM fluorometer and the Imaging Win software MAXI version (Heinz Walz GmbH, Effeltrich, Germany) were used to obtain the images and parameters of Chl *a* fluorescence. The methodology used was according to Fagundes-Nacarath *et al.* (2018) changing only the time of actinic photon irradiance to obtain the steady-state fluorescence yield that was fixed in 5 min.

4.2.9. Determination of sugars concentrations

The second and third trifoliate leaves, from base to top, of one plant per replication of each treatment (four replications and four plants per each sampling time) were collected at 5, 10, and 15 dai. Leaf samples were kept in liquid nitrogen during sampling and stored at -80°C until further analysis. Plants that had their leaves collected were discarded after each sampling. Approximately 50 mg of fresh ground material was used for sugars analyses. Samples were subjected to methanol extraction according to Medeiros *et al.* (2017). The ethanol soluble phase was used to the quantification of sugars and the pellet was used to quantify starch (Fernie *et al.*

2001). The starch and soluble sugars (glucose, fructose, and sucrose) were analyzed as described by Daloso *et al.* (2015).

4.2.10. Ethylene measurement

The ethylene was measured according to Silva *et al.* (2014). The first, second, and third trifoliate leaves, from base to top, of one plant per replication of each treatment (four replications and four plants per each sampling time) were collected at 5, 10, and 15 dai, placed in 50 mL Erlenmeyer flasks and immediately sealed with serum rubber. The flasks were maintained in a Biochemical Oxygen Demand (BOD) incubator at 28ºC during 24 hours when an air sample (1 mL) was taken from the flask headspace and injected in a gas chromatograph (Hewlett Packard 5890, Series II) equipped with a stainless-steel column (1.0 m × 6.0 mm) packed with Porapak-N 80-100 mesh. The analysis was carried at flux of 30 mL min^{-1} of nitrogen carrier gas and hydrogen and 320 mL air min^{-1}. Column, injector, and detector temperatures were 60, 110, and 150ºC, respectively. The software Peak Simple (Version 3.92), coupled to the chromatograph, was used to register the ethylene peaks and the quantification was realized by comparison with authentic ethylene standards.

4.2.11. Data analysis

Data from the variables and parameters evaluated were checked for normality and homogeneity of variance and subjected to analysis of variance (ANOVA) thereafter. Treatments means were compared by F test ($P \leq 0.05$). For ASR severity, ANOVA considered plants sprayed with water, Ni, Gl, and Ni+Gl with four replications For foliar Ni concentrations, sugars, photosynthetic pigments, parameters of Chl *a* fluorescence, and leaf gas exchange, ANOVA was considered to be a 2 × 4 factorial experiment consisting of non-inoculated and inoculated plants and four spray conditions (water, Ni, Gl, and Ni+Gl) with four replications. Data were analyzed using the Minitab software (version 18, Minitab Corporation).

4.3. RESULTS

4.3.1. Foliar Ni concentration

For non-inoculated plants, the foliar Ni concentration was higher by 199 and 114% in Ni and Ni+Gl plants, respectively, in comparison to that in control plants (Fig. 1). For inoculated plants, the foliar Ni concentration was higher by 270 and 57% in Ni and Ni+Gl plants, respectively, in comparison to that in control plants. The foliar Ni concentrations were higher

by 8, 34, 9, and 57% in control, Ni, Gl, and Ni+Gl inoculated plants, respectively, in comparison to that in non-inoculated plants.

4.3.2. Severity of ASR

The ASR severity in Ni, Gl, and Ni+Gl plants was significantly reduced by 34, 72, and 80%, respectively, in comparison to control plants (Fig. 2 and 3). The ASR severity was lower by 57 and 70% in Gl and Ni+Gl plants, respectively, in comparison to Ni plants.

4.3.3. Photosynthetic pigments concentrations

The concentration of photosynthetic pigments at 8 and 13 dai was lower in control inoculated plants in comparison to that in non-inoculated plants (Fig. 4A-D). At 13 dai, the pigments evaluated were lower in Ni, Gl, and Ni+Gl inoculated plants in comparison to that in non-inoculated plants. In inoculated plants at 13 dai, Chl *a* concentration was higher by 68, 88, and 150%, Chl *b* concentration was higher by 66, 68, and 89%, and Chl *a*+*b* concentration was higher by 67, 81, and 130% in Ni, Gl, and Ni+Gl plants, respectively, in comparison to that in control plants (Fig. 4A-C). The carotenoids concentration was higher by 35, 44, and 69% at 13 dai in Ni, Gl, and Ni+Gl inoculated plants, respectively, in comparison to control inoculated plants (Fig. 4D). Chl *a*, Chl *a*+*b*, and carotenoids concentrations were higher by 49, 37, and 25% in Ni+Gl inoculated plants, respectively, in comparison to Ni inoculated plants (Fig. 4A, B, and D).

4.3.4. Chl *a* fluorescence parameters

Images of Chl *a* fluorescence evidenced changes caused by fungal infection for all parameters examined with minor changes for Ni- and Gl-supplied plants (Fig. 5). Irrespective of the evaluation time, in non-inoculated plants there was no significant difference on the maximum photochemical efficiency of photosystem II (PSII) (F_v/F_m) and effective yield of PSII (Y(II)) values among the control, Ni, Gl, and Ni+Gl treatments (Fig. 6A and B). However, the yield for dissipation by down-regulation energy (Y(NPQ)) values were decreased and the yield for other non-photochemical (non-regulated) losses (Y(NO)) values were increased by Gl in non-inoculated plants regardless of Ni supply. In non-inoculated plants at 8 dai, Y(NPQ) values were lower by 20 and 26% and the Y(NO) values were higher by 23 and 30% in Gl and Ni+Gl plants, respectively, in comparison to that in control plants (Fig. 6C and D). In inoculated plants at 8 dai, F_v/F_m values were higher by 8, 9, and 6% in Ni, Gl, and Ni+Gl plants, respectively, in comparison to that in control plants. F_v/F_m values were higher by 8 and 13% at 13 dai in Gl and

Ni+Gl inoculated plants, respectively, in comparison to control inoculated plants (Fig. 6A). Y(II) values were higher by 24, 42, and 45% at 8 dai and by 25, 34, and 39% at 13 dai in Ni, Gl, and Ni+Gl inoculated plants, respectively, in comparison to control inoculated plants (Fig. 6B). Y(NO) values were lower by 13, 16, and 15% at 8 dai in Ni, Gl, and Ni+Gl inoculated plants, respectively, in comparison to control inoculated plants. In inoculated plants at 13 dai, Y(NO) values were lower by 19 and 13% in Ni and Gl plants, respectively, in comparison to that in control plants (Fig. 6D). The electron transport rate (ETR) values were higher by 40, 69, and 70% at 8 dai and by 64, 102, and 132% at 13 dai in Ni, Gl, and Ni+Gl inoculated plants, respectively, in comparison to control inoculated plants (Fig. 6E).

ASR caused a significant decrease on Y(II) and ETR values regardless of control, Ni, Gl, and Ni+Gl treatments and the evaluation time (Fig. 6B and E). F_v/F_m values were lower by 9% at 8 dai in control inoculated plants and by 11, 10, and 6% at 13 dai in control, Ni, and Gl inoculated plants, respectively, in comparison to that in non-inoculated plants (Fig. 6A). The Y(II) values were lower by 35, 20, 15, and 8% at 8 dai and by 30, 17, 8, and 10% at 13 dai in control, Ni, Gl, and Ni+Gl inoculated plants, respectively, in comparison to that in non-inoculated plants (Fig. 6B). At 8 dai, Y(NPQ) values were lower by 23 and 19% in control and Ni inoculated plants, respectively, and higher by 4% in Ni+Gl inoculated plants in comparison to that in non-inoculated plants. At 13 dai, the Y(NPQ) values were lower by 12% in control inoculated plants and higher by 23% in Gl inoculated plants in comparison to that in non-inoculated plants (Fig. 6C). Y(NO) values were higher by 59 and 23% at 8 and 13 dai, respectively, in control inoculated plants than in non-inoculated plants. Moreover, Y(NO) values were higher by 42 and 9% at 8 dai in Ni and Gl inoculated plants, respectively, in comparison to that in non-inoculated plants. ETR values were lower by 47, 24, 15, and 7% at 8 dai and by 59, 37, 18, and 14% at 13 dai in control, Ni, Gl, and Ni+Gl inoculated plants, respectively, in comparison to that in non-inoculated plants.

4.3.5. Gas-exchange parameters

Irrespective of the evaluation time, there was no significant difference in A, g_s, C_i, and E values among the control, Ni, Gl, and Ni+Gl treatments in non-inoculated plants (Fig. 7A and B). Following this same comparison, spray conditions did not affect g_s and E in inoculated plants (Fig. 7B and D). At each evaluation time, A values were higher by 62% at 8 dai in Ni+Gl inoculated plants and by 130, 125, and 200% at 13 dai in Ni, Gl, and Ni+Gl inoculated plants, respectively, in comparison to control inoculated plants (Fig. 7A). C_i values were lower by 8 and 11% at 8 dai and by 6 and 10% at 13 dai in Gl and Ni+Gl inoculated plants, respectively,

in comparison to control inoculated plants. In Ni+Gl inoculated plants at 13 dai, C_i values were lower by 2% in comparison to Ni inoculated plants (Fig. 7C).

In inoculated plants, A, g_s, and E values significantly decreased in comparison to non-inoculated plants regardless of control, Ni, Gl, and Ni+Gl treatments and the evaluation time (Fig. 7A, B, and D). A values were lower by 64, 46, 42, and 38% at 8 dai and by 76, 45, 48, and 33% at 13 dai in control, Ni, Gl, and Ni+Gl inoculated plants, respectively, in comparison to that in non-inoculated plants (Fig. 7A). C_i values in control inoculated plants were increased by 5 and 7% at 8 and 13 dai, respectively, in comparison to control non-inoculated plants. Increases in C_i values was not observed between inoculated and non-inoculated plants in Ni, Gl, and Ni+Gl treatments (Fig. 7C).

4.3.6. Sugars concentrations

There was no significant difference in the sugars concentrations among the control, Ni, Gl, and Ni+Gl treatments in non-inoculated plants regardless of the evaluation time (Fig. 8A-D). There was no significant difference for glucose and fructose concentrations among the control, Ni, Gl, and Ni+Gl treatments in inoculated plants regardless of the evaluation time (Fig. 8A and B). Comparing the sprays conditions at each evaluation time, the sucrose concentration was higher by 34 and 36% at 15 dai, respectively, in Gl and Ni+Gl inoculated plants in comparison to control inoculated plants (Fig. 8C). The starch concentration was higher by 51 and 57% at 10 dai and by 60 and 80% at 15 dai, respectively, in Gl and Ni+Gl inoculated plants in comparison to control inoculated plants (Fig. 8D). Comparing the inoculation factor at each spray condition and evaluation time, the glucose concentration was higher by 41% at 10 dai in control inoculated plants in comparison to control non-inoculated plants (Fig. 8A). Following this same comparison, the fructose concentration in control inoculated plants was decreased by 27% at 15 dai in comparison to that in non-inoculated plants (Fig. 8B). Moreover, the sucrose concentration was lower by 15 and 32% at 10 and 15 dai, respectively, and the starch concentration was lower by 38 and 49% at 10 and 15 dai, respectively, in control inoculated plants in comparison to control non-inoculated plants (Fig. 8C and D).

4.3.7. Ethylene concentration

Irrespective of the evaluation time, there was no significant difference in the ethylene concentration among the control, Ni, Gl, and Ni+Gl treatments in non-inoculated plants (Fig. 9A). At 5 and 10 dai, ethylene concentration was not affected by the spray conditions (Fig. 9B). Following this same comparison, ethylene concentration was higher by 63 and 67% at 15 dai

in Gl and Ni+Gl inoculated plants, respectively, in comparison to control inoculated plants. The ethylene concentration was decreased by 43% at 10 dai in control inoculated plants and by 55 and 42% at 15 dai in control and Ni inoculated plants, respectively, in comparison to that in non-inoculated plants (Fig. 9A and B).

4.4. DISCUSSION

In this study, we report the effect of Gl and additional Ni foliar supply on the increase of soybean resistance against *P. pachyrhizi* infection. Earlier reports demonstrated that Gl and Ni supply decreased ASR development on soybean plants (Feng *et al.* 2005, 2008, Chapter 1, Chapter 2). The results of the present study corroborate those reports and provide novel evidences of an additional effect of the combination of Ni and Gl on the maintenance of the photosynthetic capacity of infected plants. The high foliar Ni concentration in Ni-supplied plants confirmed its absorption. The highest Ni concentration in inoculated plants in comparison to non-inoculated plants may be explained by Ni mobilization from non-infected areas to infected areas of as result of fungal infection. Gl showed a negative effect on foliar Ni concentration in Ni-supplied plants which was minimized on infected plants. Duke *et al.* (2018) detected minimal and inconsistent effects of Gl on foliar and seed Ni concentration. However, at the conditions of the present study, the ability of Gl to interact with divalent cations and to form poorly soluble compounds (Duke *et al.* 2012) may have affected Ni mobilization between leaves and caused the difference on foliar Ni concentration noticed.

The high level of functionality of the photosynthetic apparatus plays a key role to provide the energy demanded by plant metabolisms (Wu *et al.* 2019). The effect of pathogens infection on the photosynthesis is variable according to their lifestyle. Necrotrophic pathogens generally have higher evasive destruction of the cellular constituents promoting, therefore, a fast and high decrease of the photosynthetic capacity of infected plants (Kumudini *et al.* 2018). Distinctly, biotrophic pathogens such as *P. pachyrhizi* needs its host´s living tissue to reproduce and survive and, consequently, affects photosynthesis later in contrast to necrotrophic pathogens (Kumudini *et al.* 2018). In all cases, the plants' ability to defend themselves from the pathogens, avoiding or delaying the photosynthetic damage, determine their ability to maintain high levels of energy production demanded by the different metabolic pathways on plants (Bolton 2009). Inoculated plants supplied with Ni or Gl maintained carotenoids, Chl *a*, Chl *b*, and Chl *a+b* concentrations higher than control inoculated plants. This effect was even more pronounced in Ni+Gl-supplied plants. In general, as the ASR developed, there was a faster and bigger decrease

on the concentration of photosynthetic pigments for control plants in contrast to plants submitted to the other spray conditions.

Photosynthetic pigments play a key role in the harvest of the light energy and transfer it to the reaction center of the photosystems (Akhtar *et al.* 2015). The energy absorbed by the PSII may be destined to three dissipative processes: photochemistry process, dissipation by down-regulation energy, and dissipation by other non-regulated losses which are represented by the yield components Y(II), Y(NPQ), and Y(NO), respectively (Kramer *et al.* 2004). In the present study, it was noticed that Ni, Gl, and Ni+Gl inoculated plants showed higher destination of the energy to photochemistry process in comparison to control inoculated plants, as indicated by the Y(II) values. Interestingly, the values for Y(NPQ) and Y(NO) decreased and increased, respectively, for non-inoculated plants exposed to Gl indicating, therefore, that the energy dissipation was destined from a regulated process for a non-regulated one. Theoretically, this energy dissipation deregulation would have a negative effect on the plant metabolism due to the production of reactive oxygen species that may damage the PSII (Vass 2011, Krieger-Liszkay *et al.* 2011, Huang *et al.* 2018). However, we found no difference in the values of F_v/F_m, Y(II), and ETR between non-inoculated plants of control and Gl-supplied treatments reveal that the photosynthetic apparatus integrity was maintained. Control inoculated plants showed lowest F_v/F_m, Y(II), and ETR values and higher Y(NO) values in comparison to the inoculated plants revealing a highest damage and lowest functionality of the photosynthetic apparatus in plants non-supplied with both Ni and Gl.

The limitations observed in inoculated plants for the photosynthetic machinery capacity to capture light and to use absorbed energy by PSII reflected on their limited capacity to process CO_2. Additionally, the low g_s values obtained for inoculated plants corroborated with the low *A* values. The no significant difference for g_s values between control, Ni, Gl, and Ni+Gl inoculated plants associated to the high C_i values observed in control inoculated plants revealed biochemical limitations on the photosynthesis in inoculated plants that did not receive neither Ni or Gl (Urban *et al.* 2017). Moreover, the lower damage in the photosynthetic apparatus of Ni+Gl inoculated plants resulted in higher *A* values for these plants in comparison to inoculated plants.

Sugar metabolism play a key role in plant-pathogen interactions (Kanwar and Jha 2019). Considering that photosynthesis is responsive to the control of the biosynthesis and mobilization of several sugars, any alteration on this process may affect the infectious process of pathogens. No significant difference was observed for sugars concentrations between inoculated and non-inoculated plants of Ni, Gl, and Ni+Gl treatments. However, the fungal

infection decreased the starch and sucrose concentrations starting at 10 dai in control plants. This finding may be explained by the limited photosynthesis capacity of control inoculated plants limiting, therefore, the biosynthesis of sugars and reflecting in the increase of reserves consumption. In addition, the consumption of nutrients by the pathogen and the plant's attempt to defend itself against the infection through the activation of defense responses generates a high energetic cost and may increase starch degradation. The lowest sucrose concentration in control inoculated plants may have explained the limited fructose concentration observed at 15 dai. In chapter 2, the *P. pachyrhizi* infection decreased the sugar concentrations in soybean plants and was reported a similar Ni effect in avoiding the negative ASR effect.

The ethylene concentration in non-inoculated plants was not affected by neither Ni or Gl during the time-course evaluated. Non-lethal doses of Gl increased the production of ethylene by Gl non-resistant plants of common beans (Abu-irmaileh *et al.* 1979) and paper birch (Stasiak *et al.* 1992). We found no difference for ethylene concentration reported in the present study between plants treated and non-treated with Gl may be explained by use of the soybean cultivar resistant to Gl. Considering that both Ni and Gl did not affect ethylene concentration for non-inoculated plants, the maintenance of higher ethylene concentration in plants inoculated and treated with Ni, Gl or Ni+Gl in comparison to control inoculated plants probably resulted of the Ni and Gl abilities to suppress ASR development. The great ASR severity observed in control plants caused a drastic decrease on ethylene concentration. Ethylene dependent responses on plants generally are not initiated by biotrophic pathogens (Garcia-Brugger *et al.* 2006, Van Der Ent and Pieterse 2012) despite some exceptions (Pieterse *et al.* 2009). The lowest ASR severity in plants treated with Ni and Gl was not accompanied by an increase in ethylene concentration indicating that fungal infection was not able to start plant ethylene dependent responses.

In summary, the results of the present demonstrated that Gl and additional Ni foliar spray were of considerable effect to lower ASR development on soybean plants. The resistance of soybean plants against *P. pachyrhizi* was potentiated by combining Ni with Gl in a scenario where the pool of photosynthetic pigments and the concentration of sugars were kept higher and the integrity of the photosynthetic apparatus was preserved, Additionally, ethylene concentration at advanced stages of *P. pachyrhizi* infection lowered, but in the presence of Ni and Gl the concentration of this hormone was kept higher than control plants.

4.5. FIGURES

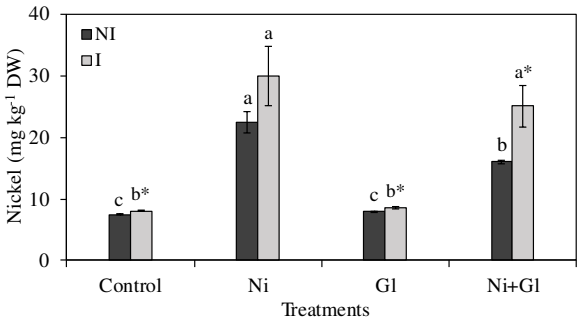

Figure 1. Foliar nickel concentration for soybeans plants sprayed with water (control), nickel (Ni), glyphosate (Gl), and with the combination of Ni and Gl (Ni+Gl) and non-inoculated (NI) or inoculated (I) with *Phakopsora pachyrhizi*. Means for each treatment (control, Ni, Gl, and Ni+Gl) followed by different letters and for the NI and I treatments followed by an asterisk (*) are significantly different ($P \leq 0.05$) according to Tukey's test. Bars represent the standard error of the means. $n = 8$.

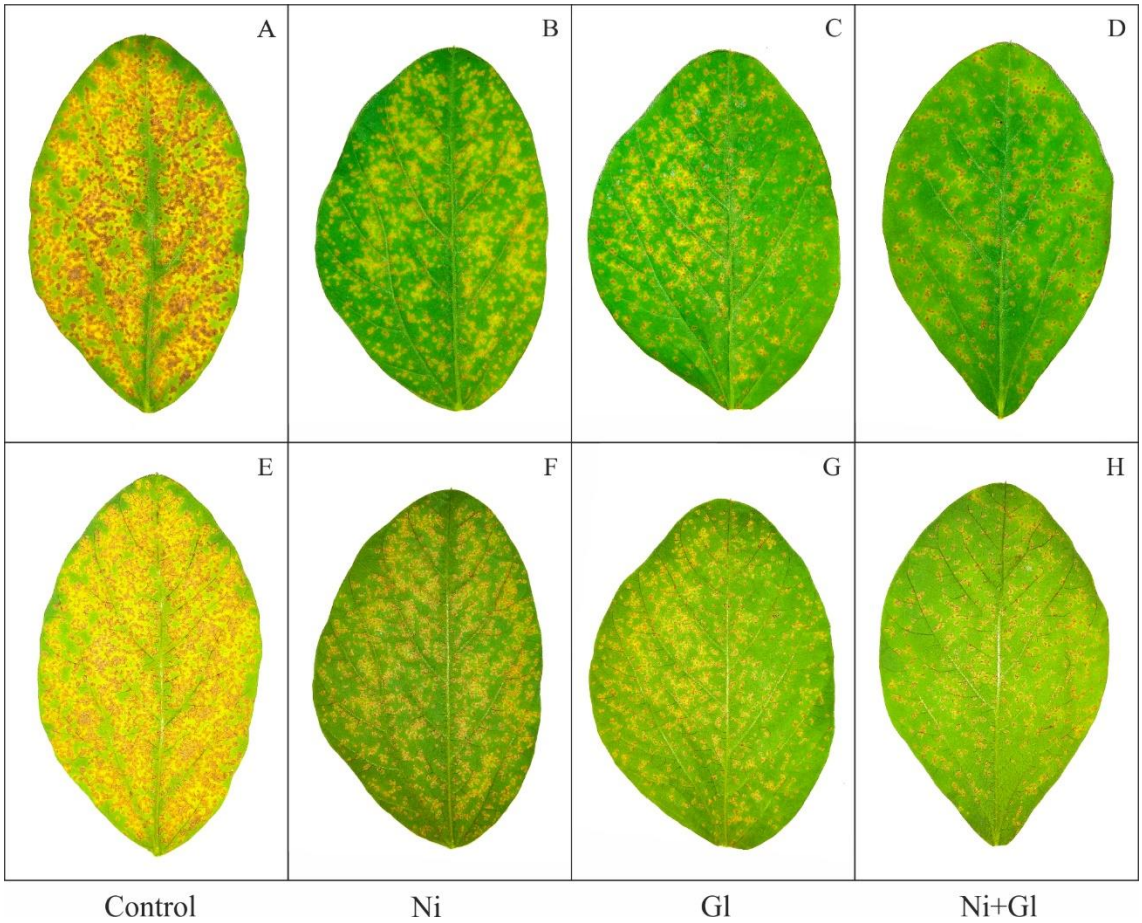

Figure 2. Symptoms of Asian soybean rust in the adaxial (A-D) and abaxial (E-H) surfaces of leaflets of soybean plants sprayed with water (control) (A and E), nickel (Ni) (B and F), glyphosate (Gl) (C and G), and with the combination of Ni and Gl (Ni+Gl) (D and H).

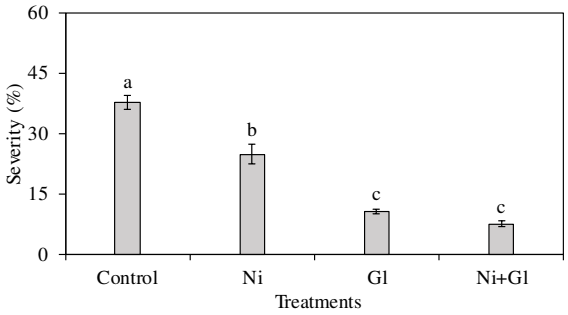

Figure 3. Asian soybean rust severity in the leaves of soybean plants sprayed with water (control), nickel (Ni), glyphosate (Gl), and with the combination of Ni and Gl (Ni+Gl). Means for each spray treatment followed by different letters are significantly different ($P \leq 0.05$) according to Tukey's test. Bars represent the standard error of the means. $n = 8$.

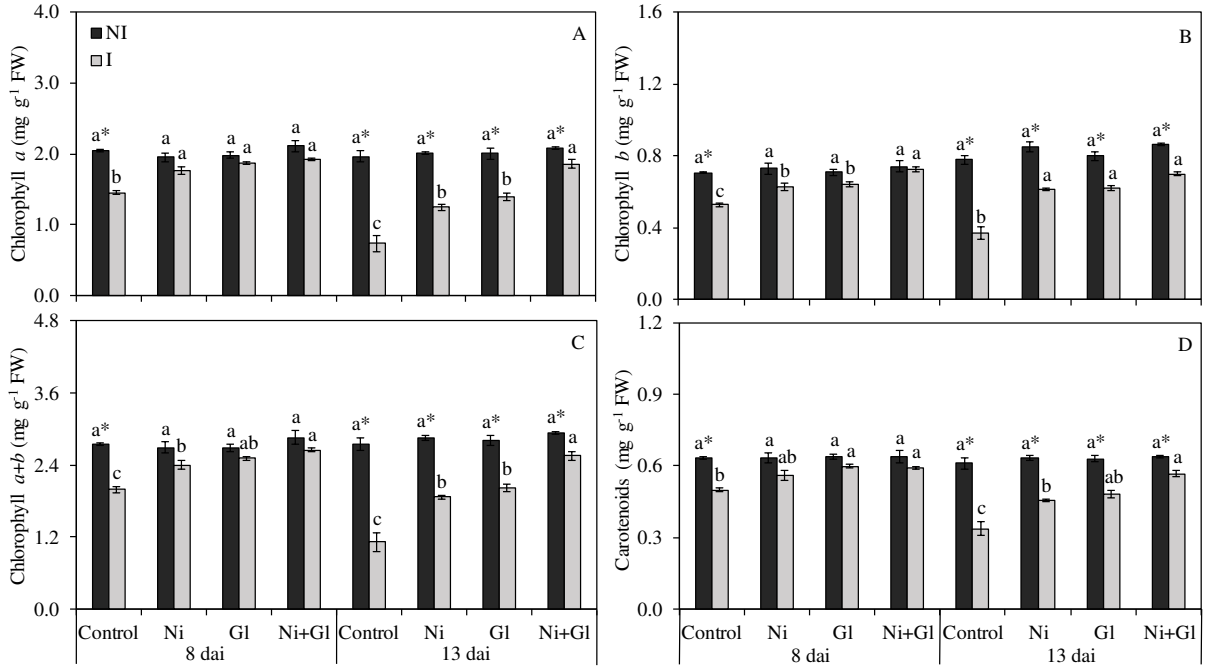

Figure 4. Concentrations of chlorophyll *a* (A), chlorophyll *b* (B), chlorophyll *a+b* (C), and carotenoids (D) in the leaves of soybean plants sprayed with water (control), nickel (Ni), glyphosate (Gl), and with the combination of Ni and Gl (Ni+Gl) and non-inoculated (NI) or inoculated (I) with *Phakopsora pachyrhizi*. For each evaluation time, means for each treatment (control, Ni, Gl, and Ni+Gl) followed by different letters and for the NI and I treatments followed by an asterisk (*) are significantly different ($P \leq 0.05$) according to Tukey's test. FW = fresh weight. dai = days after inoculation. Bars represent the standard error of the means. $n = 8$.

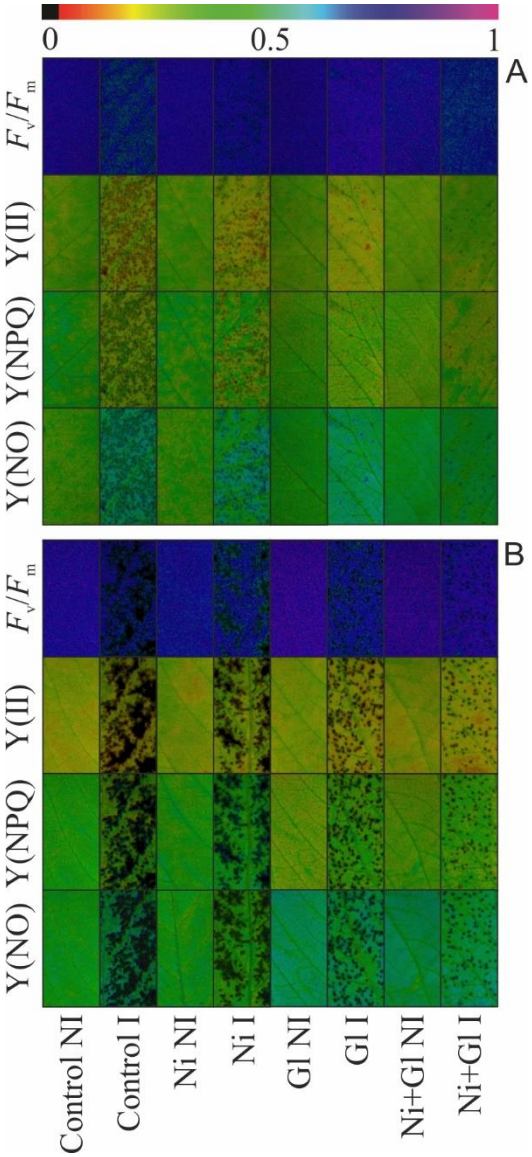

Figure 5. Images of the chlorophyll *a* fluorescence parameters: maximum photochemical efficiency of photosystem II (PSII) (F_v/F_m), effective quantum yield of PSII (Y(II)), yield for dissipation by down-regulation energy (Y(NPQ)), and yield for other non-photochemical (non-regulated) losses (Y(NO)) determined on the leaves of soybean plants sprayed with water (control), nickel (Ni), glyphosate (Gl), and with the combination of Ni and Gl (Ni+Gl) and inoculated (I) with *Phakopsora pachyrhizi* at 8 (A) and 13 (B) days after inoculation. The images of these parameters were also obtained in the leaves of non-inoculated (NI) plants submitted to the control, Ni, Gl, and Ni+Gl treatments.

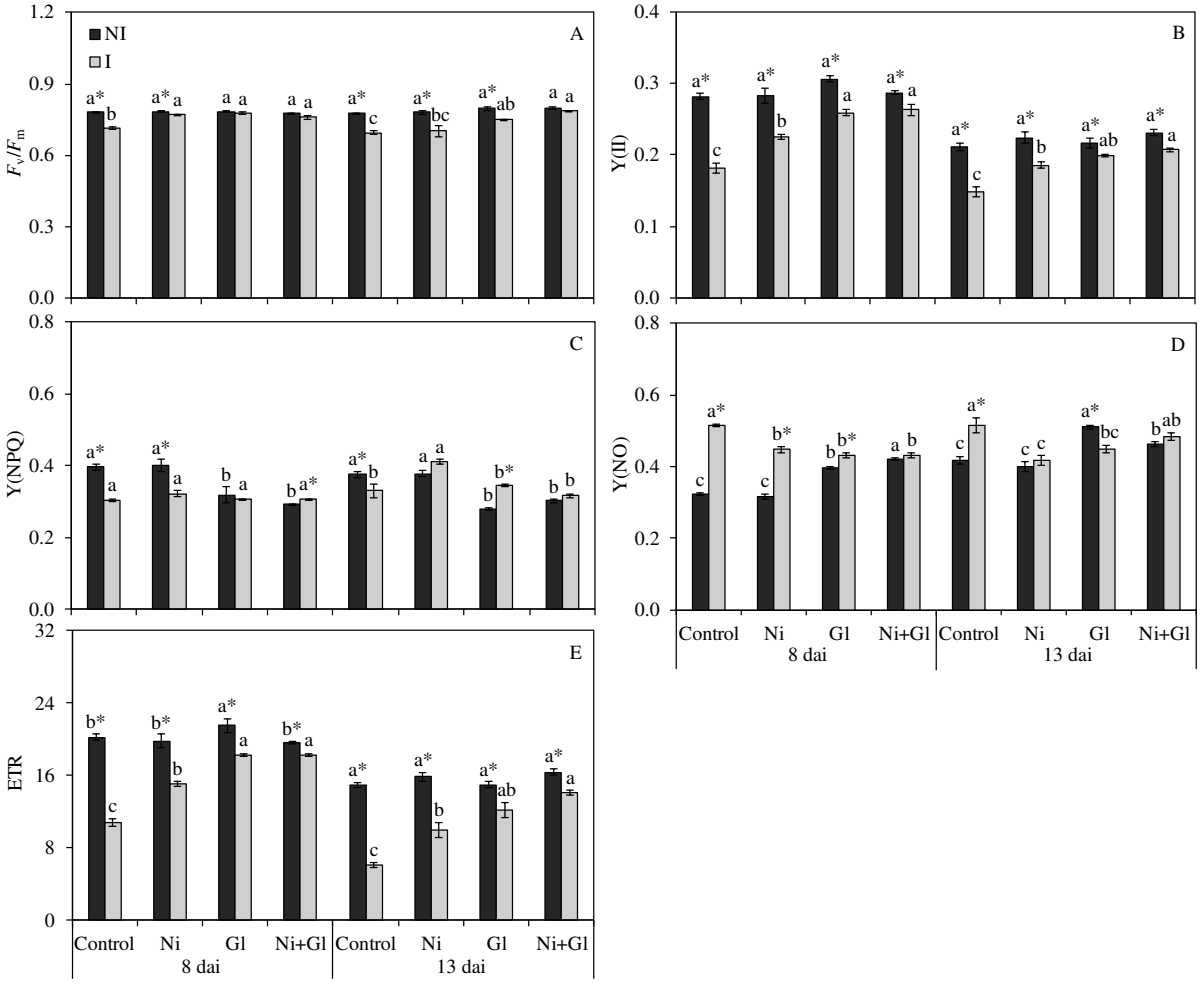

Figure 6. Chlorophyll *a* fluorescence parameters: maximum photochemical efficiency of photosystem II (PSII) (F_v/F_m), effective quantum yield of PSII (Y(II)), yield for dissipation by down-regulation energy (Y(NPQ)), and yield for other non-photochemical (non-regulated) losses (Y(NO)) determined on the leaves of soybean plants sprayed with water (control), nickel (Ni), glyphosate (Gl), and with the combination of Ni and Gl (Ni+Gl) and non-inoculated (NI) or inoculated (I) with *Phakopsora pachyrhizi*. For each evaluation time, means for each treatment (control, Ni, Gl, and Ni+Gl) followed by different letters and for the NI and I treatments followed by an asterisk (*) are significantly different ($P \leq 0.05$) according to Tukey's test. dai = days after inoculation. Bars represent the standard error of the means. $n = 8$.

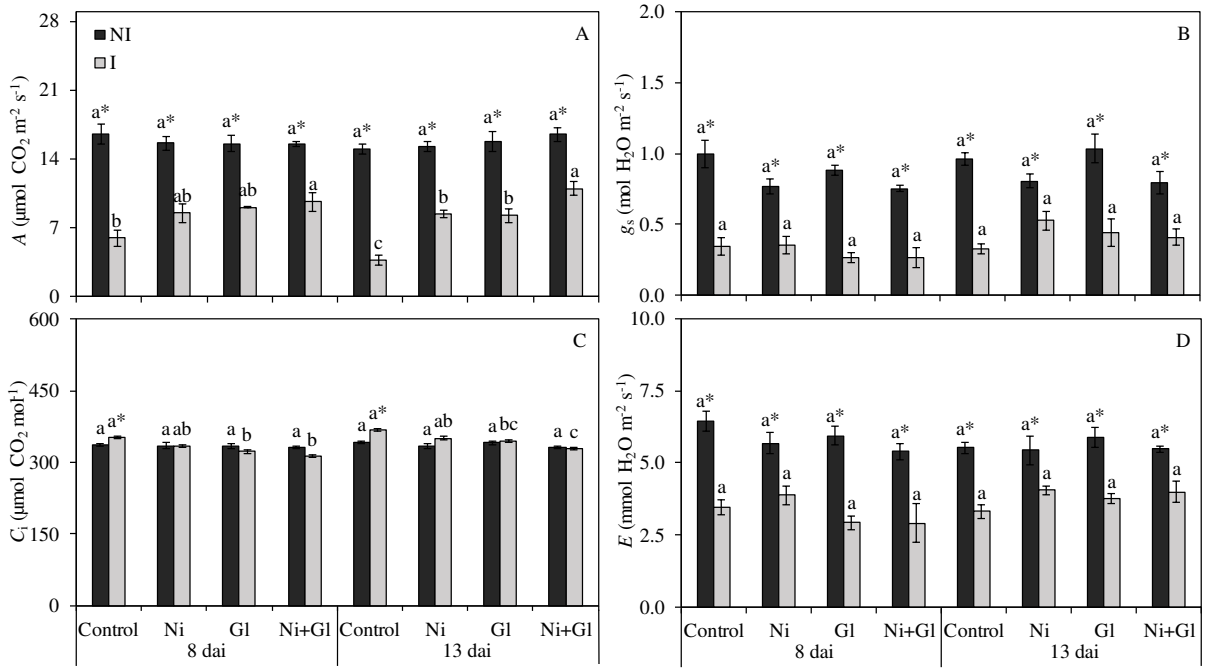

Figure 7. Leaf gas exchange parameters: net carbon assimilation rate (*A*) (A), stomatal conductance to water vapour (g_s) (B), internal CO_2 concentration (C_i) (C), and transpiration rate (*E*) (D) determined on the leaves of soybean plants sprayed with water (control), nickel (Ni), glyphosate (Gl), and with the combination of Ni and Gl (Ni+Gl) and non-inoculated (NI) or inoculated (I) with *Phakopsora pachyrhizi*. For each evaluation time, means for each treatment (control, Ni, Gl, and Ni+Gl) followed by different letters and for the NI and I treatments followed by an asterisk (*) are significantly different ($P \leq 0.05$) according to Tukey's test. dai = days after inoculation. Bars represent the standard error of the means. $n = 8$.

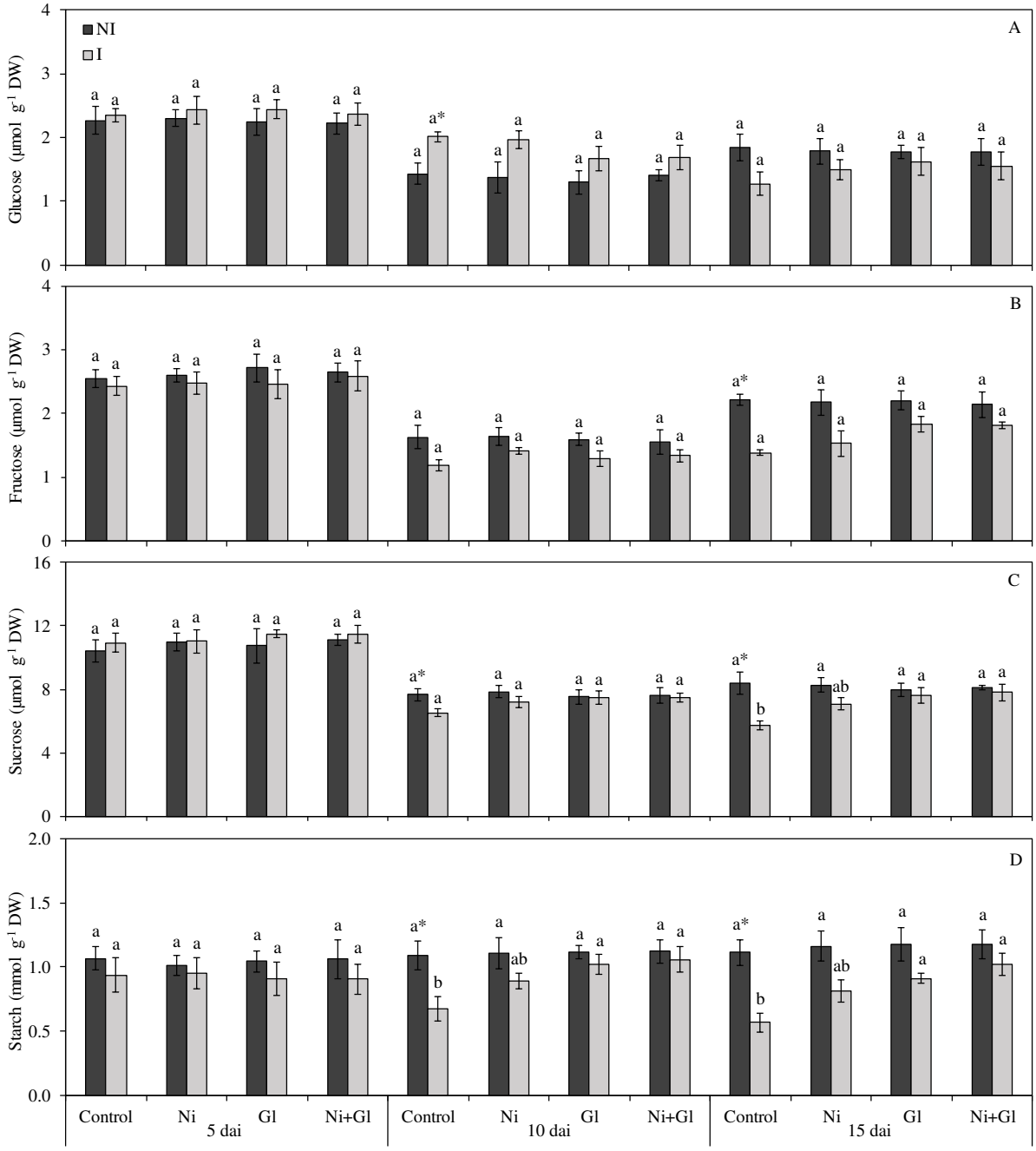

Figure 8. Concentrations of glycose (A), fructose (B), sucrose (C), and starch (D) in the leaves of soybean plants sprayed with water (control), nickel (Ni), glyphosate (Gl), and with the combination of Ni and Gl (Ni+Gl) and non-inoculated (NI) or inoculated (I) with *Phakopsora pachyrhizi*. For each evaluation time, means for each treatment (control, Ni, Gl, and Ni+Gl) followed by different letters and for the NI and I treatments followed by an asterisk (*) are significantly different ($P \leq 0.05$) according to Tukey's test. DW = dry weight, dai = days after inoculation. Bars represent the standard error of the means. $n = 8$.

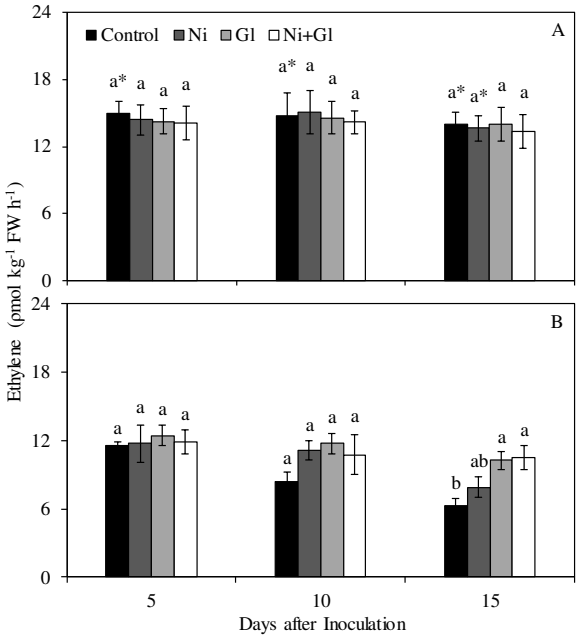

Figure 9. Concentration of ethylene in the leaves of soybean plants sprayed with water (control), nickel (Ni), glyphosate (Gl), and with the combination of Ni and Gl (Ni+Gl) and non-inoculated (NI) (A) or inoculated (I) (B) with *Phakopsora pachyrhizi*. For each evaluation time, means for each treatment (control, Ni, Gl, and Ni+Gl) followed by different letters and for the NI and I treatments followed by an asterisk (*) are significantly different ($P \leq 0.05$) according to Tukey's test. FW = fresh weight. Bars represent the standard error of the means. $n = 8$.

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5. ACKNOWLEDGMENTS

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6. GENERALS CONCLUSIONS

To our knowledge, this is the first study to explore in depth the effects of nickel (Ni) and glyphosate (Gl) on the soybean defense mechanisms against *Phakopsora pachyrhizi*. Thus, four experiments were carried out.

Ni had a beneficial effect on the potentiation of the defense responses of a susceptible soybean genotype against infection by *P. pachyrhizi* mainly by stimulating the expression of the *CHI1B1*, *PAL*, *PR-1A*, and *URE* genes and β-1,3-glucanase (GLU) activity in addition to increasing phenolics and lignin production. The beneficial effect of Ni to decrease the ASR symptoms on soybean plants as indicated by the less cellular damage caused by *P. pachyrhizi* infection linked to the preservation of their photosynthetic machinery as well as the maintenance of an adequate concentration of photosynthetic pigments.

Gl also had a considerable effect to lower ASR development on soybean plants. Gl increased the concentration of TSP, the activity of defense enzymes (GLU, phenylalanine ammonia lyase, and polyphenoloxidase), and did not affected the antioxidant enzymes of soybean plants infected by *P. pachyrhizi*. The resistance of soybean plants against *P. pachyrhizi* was potentiated by combining Ni with Gl in a scenario where the pool of photosynthetic pigments and the concentration of sugars were kept higher and the integrity of the photosynthetic apparatus was maintained. Additionally, ethylene concentration at advanced stages of *P. pachyrhizi* infection decreased, but in the presence of Ni and Gl the concentration of this hormone was kept higher than control plants.

In summary, our results have a theoretical and practical importance providing news insights of the use of Ni to increase the basal level of soybean resistance to ASR and to complement other control methods within the context of sustainable agriculture. However, is necessary caution in the use of this micronutrient in the field due your heavy metal characteristic and your low demand by the plant metabolism. Moreover, the identification of the plant defense mechanism against *P. pachyrhizi* affected by Gl elucidated how this herbicide acts on the control of this pathogen.