UNIVERSIDADE FEDERAL DE VIÇOSA

BIANCA APOLÔNIO FONTES

NICKEL IN THE SOYBEAN RESISTANCE AGAINST *Sclerotinia sclerotiorum* INFECTION

VIÇOSA – MINAS GERAIS
2021

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

Adviser: Fabrício Ávila Rodrigues

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Assent:

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BIOGRAPHY

BIANCA APOLÔNIO FONTES, daughter of Marisa Apolônio Fontes and Wilson José Fontes, was born on September 28, 1995, in Viçosa, Minas Gerais State. In May 2013, she started to study Agronomy at the Federal University of Viçosa (UFV). In July 2018, she obtained the title of Agronomist. From August 2018 to February 2019, she received training at the Plant Diagnostic Center at the Louisiana State University. In March 2019, she started her Master's Degree in the Graduate Program of Plant Pathology at UFV under the guidance of Professor Fabrício Ávila Rodrigues. She defended her dissertation on February 26, 2021.

ABSTRACT

FONTES, Bianca Apolônio, M.Sc., Universidade Federal de Viçosa, February, 2021. **Nickel in the soybean resistance against Sclerotinia sclerotiorum infection**. Adviser: Fabrício Ávila Rodrigues.

White mold, caused by the fungus Sclerotinia sclerotiorum, is one of the most destructive diseases impacting soybean yield worldwide. Nickel (Ni) plays an essential role in the metabolism of higher plants because of its involvement in the catalytic process of several enzymes and as a constituent of many biomolecules. This study investigated the potential of spraying Ni to soybean plants to increase their resistance against white mold by accessing the photosynthetic performance (chlorophyll (Chl) a fluorescence parameters and photosynthetic pigments pools (chlorophylls a+b and carotenoids), the concentrations of malondialdehyde (MDA), total soluble phenolics (TSP), and lignin-thioglycolic acid (LTGA) derivatives as well the expression of genes encoding 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), pathogenesis-related protein 1 (PR-1A), nitrate and nitrite reductase (NIR1-1 and INR-2), oxaloacetate acetylhydrolase (Oxalo), and an AP2/ERF type transcription factor (ERF3). The in vitro assay showed that Ni inhibited the mycelial growth of S. sclerotiorum. The higher foliar Ni concentration contributed to decrease white mold severity for Ni-sprayed plants which exhibited less MDA production, maintained great photosynthetic pigments concentration, and had their photosynthetic apparatus much more preserved than the plants non-sprayed with Ni. High concentrations of TSP and LTGA derivatives linked to higher expression of CHI1B1, PAL1.3, PAL2.1, PAL3.1, and PR-1A for Ni-sprayed plants contributed to their resistance against the white mold. Based on the present study results, it is plausible to conclude the potential of using Ni to enhance the resistance of soybean against white mold in the context of a more sustainable agriculture that prizes the mineral nutrition of plants.

Keywords: Glycine max. Aternative Disease Control. Fungal Infection. Host defense Responses. Photosynthesis. Plant nutrition.

RESUMO

FONTES, Bianca Apolônio, M.Sc., Universidade Federal de Viçosa, fevereiro de 2021. **Níquel na resistência da soja contra a infecção por Sclerotinia sclerotiorum**. Orientador: Fabrício Ávila Rodrigues.

O mofo branco, causado pelo fungo Sclerotinia sclerotiorum, é uma das doenças mais destrutivas que afetam a produtividade da soja mundialmente. O níquel (Ni) desempenha um papel essencial no metabolismo das plantas por estar envolvido no processo catalítico de várias enzimas e por ser constituinte de muitas biomoléculas. Este estudo investigou o potencial da pulverização com Ni em plantas de soja para aumentar a resistência delas contra o mofo branco. Foi monitorado o desempenho fotossintético (parâmetros de fluorescência da clorofila (Chl) a e concentração de pigmentos fotossintéticos (clorofila total (a+b) e carotenóides), as concentrações de aldeído malônico (MDA), compostos fenólicos solúveis totais (CFST) e derivados de lignina-ácido tioglicólico (DLATG), bem como a expressão de genes que codificam para fenilalanina amônia-liase (PAL1.1, PAL1.3, PAL2.1 e PAL3.1), quitinase (CHIA1), chalcona isomerase (CHI1B1), lipoxigenase (LOX7), metaloproteinase (MMP2), isochorismato sintase (ICS1 e ICS2), urease (URE), proteína relacionada à patogênese 1 (PR-1A), nitrato e nitrito redutase (NIR1-1 e INR-2), oxaloacetato acetilidrolase (Oxalo) e um fator de transcrição do tipo AP2/ERF (ERF3). O ensaio in vitro mostrou que o Ni inibiu o crescimento micelial de S. sclerotiorum. A maior concentração foliar de Ni contribuiu para diminuir a severidade do mofo branco nas plantas pulverizadas com Ni as quais também apresentaram menor produção de MDA, mantiveram maior concentração de pigmentos fotossintéticos e tiveram o aparato fotossintético preservado do que as plantas não pulverizadas com Ni. Altas concentrações de CFST e DLATG em associação com uma maior expressão dos genes CHI1B1, PAL1.3, PAL2.1, PAL3.1 e PR-1A para as plantas pulverizadas com Ni contribuiu para a resistência delas contra o mofo branco. Com base nos resultados do presente estudo, é plausível concluir que o Ni apresenta potencial para aumentar a resistência da soja ao mofo branco no contexto de uma agricultura mais sustentável onde a nutrição mineral das plantas é valorizada.

Palavras-chave: Glycine max. Controle Alternativo de Doenças. Infecção Fúngica. Respostas de Defesa do Hospedeiro. Fotossíntese. Nutrição de Plantas.

SUMMARY

INTRODUCTION

Soybean (Glycine max (L.) Merrill) is one of the most profitable crops cultivated worldwide, and the occurrence of diseases is the major factor contributing to yield losses. White mold, caused by the necrotrophic fungus Sclerotinia sclerotiorum (Lib.) de Bary, is one of the most important soybean diseases causing great yield losses (Boland and Hall 1994). Water-soaked lesions caused by S. sclerotiorum infection develop on the leaves and stem of soybean plants, which quickly expand and became necrotic with the formation of a white cottony mycelium over them (Bolton et al. 2006; Heffer Link and Johnson 2007). The infected leaf and stem tissues appear bleached and wilted as the white mold develops, causing the death of plants (Purdy 1979; Heffer Link and Johnson 2007). Melanized sclerotia are formed upon the cottony mycelia of S. sclerotiorum and overwinter in the soil and plant debris for several years (Heffer Link and Johnson 2007; Matsuo et al. 2015).

White mold control is difficult to achieve mainly due to the production of many sclerotia that guarantee the fungal survival in the soil, the large host range of S. sclerotiorum, the absence of resistant cultivars besides the fact that some cultural practices such as crop rotation, sowing date, and row spacing are not efficient (Boland 1987; Kim and Diers 2000; Mueller et al. 2002; Bolton et al. 2006). Fungicides have been used with moderate efficiency for white mold management in growing regions with historic of its occurrence (Mueller et al. 2002).

Adequate nutrition of plants plays an essential role in their resistance against diseases (Masood et al. 2012). Nickel (Ni) is an essential micronutrient for higher plants (Dixon and Paiva 1995; Epstein and Bloom 2005) for being part of some enzymes such as urease (Dixon et al. 1975), hydrogenase (Evan et al. 1987), superoxide dismutase, catalase, acetyl coenzyme-A synthase, NiFe hydrogenases, methyl coenzyme-M, RNAse-A, and carbon

monoxide dehydrogenase (Harasim and Filipek 2015), to participate in the nitrogen metabolism (Gerendás 1999) and ethylene production (Smith and Wood-Burn 1984) as well as in seed germination (Brown et al. 1987).

The Ni supply to plants has been shown to reduce the intensities of some diseases. Wood and Reilly (2007) reported the positive effect of Ni for the control of rusts in wheat and rye even before its essentiality to plants was recognized. The application of Ni inhibited the in vitro growth of Fusicladium effusum and reduced the severity of scab on fruit and foliage of pecan trees (Wood et al. 2012). Barcelos et al. (2018) noticed the role of Ni to enhance soybean resistance against powdery mildew by increasing urease, superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) activities. According to Einhardt et al. (2020ab), the foliar application of Ni to soybean plants increased the expression of genes encoding for chalcone isomerase, phenylalanine ammonia-lyase, and urease, resulted in great β-1,3-glucanase activity and lignin concentration in the leaf tissues infected by Phakopsora pachyrhizi. Despite the positive effect of Ni on diseases control, the mechanism by which it potentiates the defense mechanisms of defense of plants against pathogens infection deserves to be investigated.

Given the importance of white mold to impact soybean yield and the potential of Ni for diseases management, this study aimed to investigate the potential of supplying this micronutrient to soybean plants to reduce white mold symptoms through the potentiation of defense mechanisms against S. sclerotiorum infection.

MATERIAL AND METHODS

Plant growth

Ten seeds of soybean from cultivar TMG 135, susceptible to S. sclerotiorum, were sown in each plastic pot containing 2 kg of sand that was previously washed with a solution of HCl 1 N. Each pot was thinned to four seedlings at 10 days after emergence. Plants were kept in greenhouse (temperature of 30 ± 5ºC, relative humidity of 65 ± 5%, and natural photosynthetically active radiation). Plants in each pot were fertilized every two days with 100 mL of the Hoagland and Arnon (1950) nutritive solution and watered as needed.

Fungal growth, plant inoculation, and treatments

Plants were inoculated with the isolate UFV-DFP Ss22 of S. sclerotiorum. This isolate was preserved in the form of sclerotia. For sclerotia production, carrots were washed, peeled, cut in cubes of approximately 1 × 1 × 1 cm, transferred to Erlenmeyer flasks, and autoclaved at 121°C for 20 min (Bae and Knudsen 2007). Next, five plugs (5 mm in diameter) of potato-dextrose-agar (PDA; 20 g of dextrose, 20 g of agar and 200 g of fresh potato per L of distilled water) obtained from the edge of three-days old colony of S. sclerotiorum were transferred into each Erlenmeyer flasks containing the carrots cubes and incubated in a growth chamber (25 ± 2°C and 12 h photoperiod) until the sclerotia were formed. Sclerotia produced in carrots were placed into glass tubes and kept in refrigerator at 4°C for preservation. For inoculum production, one sclerotia was transferred to each Petri dish containing PDA medium. After 5 days, PDA plugs containing fungal mycelia were transferred to new Petri dishes containing PDA medium, which were kept in a growth chamber (20°C and 12 h photoperiod) for 3 days.

The three leaflets of the second and third leaves of each plant (V4 growth stage) per replication of each treatment were inoculated with one plug (5 mm in diameter) of PDA

medium containing fungal mycelia obtained from the edge of three-days old colony of S. sclerotiorum. Each PDA plug was placed between the main vein and the edge of the leaflet and gently pressed with the index finger. Inoculated plants were transferred to a plastic mist growth chamber (MGC) inside a greenhouse for the duration of the experiment. The temperature inside the MGC ranged from 20 ± 2°C (day) to 17 ± 2°C (night). The relative humidity was maintained at 92 ± 3% using a misting system with nozzles (model NEB-100; KGF Co.) that sprayed mist every 20 min above the plant canopy. At 72 h before inoculation with S. sclerotiorum, four plants per replication of each treatment were sprayed with water (control treatment) and with a solution of $NiSO_4.6H_2O$ L^{-1} (10 ml per plant; 0,0013488 g $NiSO_4.6H_2O$ L^{-1}) using a VL Airbrush atomizer (Paasche Airbrush Co.).

In vitro assays

The sensitivity of S. sclerotiorum to Ni was evaluated in vitro using different concentrations of this micronutrient as follow: 0, 0.00375, 0.0075, 0.015, 0.030, 0.045, and 0.060 g L^{-1}. Each Ni concentration was amended to 20 ml L^{-1} of PDA medium. The $NiSO_4.6H_2O$ was used as the Ni source. The fungicide Fluazinam, known to inhibit the mycelial growth of S. sclerotiorum, at the concentration of 5 ml L^{-1} was considered the positive control. The fungicide Kumulus, at a concentration of 0.0168 g sulfur (S) L^{-1}, was used as a source of S considering its presence in the $NiSO_4.6H_2O$. Both Fluazinam and Kumulus at the above mentioned rates were added to the PDA medium and a total of 20 mL of PDA was poured in each Petri dish. A PDA plug (5 mm in diameter) containing mycelia of S. sclerotinia obtained from the edge of a three-days old colony of the fungus was placed in the center of each Petri dish. The Petri dishes were kept in a growth chamber (20°C and 12 h photoperiod). Fungal

colony growth was measured in two orthogonal directions using a digital caliper at 24, 36, and 48 h thereafter.

Evaluation of white mold severity

The leaflets of the second and third trifoliate leaves of each plant per replication of each treatment were collected at 24, 48, 72, and 96 hours after inoculation (hai), scanned at 600 dpi resolution, and the obtained images were processed using the software QUANT (Vale et al. 2003) to quantify the white mold severity (WMS). The area under disease progress curve (AUDPC) for each leaflet of the second and third trifoliate leaves of each plant per replication of each treatment was calculated using the trapezoidal integration of disease progress curves according to Shaner and Finney (1977).

Determination of foliar Ni concentration

The second and third trifoliate leaves from each plant per replication of each treatment were collected at 96 hai, 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).

Chlorophyll (Chl) a fluorescence imaging

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 in the three leaflets of the second trifoliate leaf of each plant per replication of each treatment at 24, 48, 72, and 96 hai. Plants were dark-adapted for 1 h and leaflets were placed individually in the CCD (“charge-coupled device”) camera to obtain the images at the

resolution of 640 × 480 pixels in support at a distance of 18.5 cm from the CCD camera that was used to capture the Chl a fluorescence emission. The leaflets were illuminated with a weak modulated measuring beam (0.5 μmol m^{-2} s^{-1}, 100 μs, 1 Hz) to obtain the initial fluorescence (F_0). A saturating white light pulse of 2,400 μmol m^{-2} s^{-1} (10 Hz) was emitted for 0.8 s to determine the maximum fluorescence emission (F_m). Based on these initial measurements, the maximum photosystem II (PSII) photochemical efficiency of the dark-adapted leaflets was estimated through the variable-to-maximum Chl a fluorescence ratio, $F_v/F_m = [(F_m - F_0)/F_m)]$. Next, the leaflets were exposed to actinic photon irradiance (531 μmol m^{-2} s^{-1}) for 120 s to obtain the steady-state fluorescence yield (F_s), after which a saturating white light pulse (2,400 μmol m^{-2} s^{-1}; 0.8 s) was applied to achieve the light-adapted maximum fluorescence (F_m'). The light-adapted initial fluorescence (F_0') was estimated according to Oxborough and Baker (1997). Based on Kramer et al. (2004), the energy that was absorbed by the PSII for the following three yield components for dissipative processes was calculated as follows: the photochemical yield [$Y(II) = (F_m' - F_s)/F_m')$], the yield for dissipation by down-regulation [$Y(NPQ) = (F_s/F_m') - (F_s/F_m)$] and the yield for other non-photochemical (non-regulated) losses [$Y(NO) = F_s/F_m$]. The apparent electron transport rate was calculated as $ETR = Y(II) \times PPFD \times f \times \alpha$ according to Baker (2008).

Determination of photosynthetic pigments concentration

Five squared pieces of leaflets (1 cm^2) were punched from one leaflet of the second trifoliate leaf of each plant per replication of each treatment at 24, 48, 72, and 96 hai to determine the concentrations of Chl a, Chl b, and carotenoids. Leaflets samples were immersed in glass tubes containing 5 ml of dimethyl sulfoxide (DMSO) solution (saturated with calcium carbonate ($CaCO_3$), 5 g L^{-1}) and kept in the dark at room temperature for 24 h. The

absorbance of the extracts was read at 480, 649, and 665 nm in a spectrophotometer using the $CaCO_3$ saturated solution of DMSO as a blank. The concentrations of Chl a, Chl b, and carotenoids were calculated according to Wellburn (1994).

Biochemical assays

For all biochemical assays, the second and third trifoliate leaves of each plant per replication of each treatment were collected at 24, 48, 72, and 96 hai. Leaves were also collected from non-inoculated plants at these same sampling times. Leaf samples were kept in liquid nitrogen during sampling and stored at -80°C until further analysis.

Determination of 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 tissues collected at 10 and 15 dai was ground into a fine powder using a vibration ball mill (Retsch) with liquid nitrogen and homogenized in 2 ml of 0.1% (wt/vol) 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 thiobarbituric acid solution (0.5% in 20% trichloroacetic acid) and held for 60 min at 99°C in a thermomixer (Eppendorf). The reaction was stopped in an ice bath. After the solution reached the room temperature, the absorbance was read at 532 nm and the nonspecific absorbance at 600 nm was discounted. The molar extinction coefficient of 155 mM^{-1} cm^{-1} was used to calculate the MDA concentration.

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

A total of 100 mg of leaf tissues collected at 24, 48, 72, and 96 hai was ground into a fine powder with liquid nitrogen using a vibration ball mill (Retsch) and homogenized in 1 ml of 80% (vol/vol) 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).

Quantitative real-time PCR

The second and third trifoliate leaves of one plant per replication of each treatment were collected at 24, 48, 72, and 96 hai. 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 from each replication was ground into a fine powder using a vibration ball mill with liquid nitrogen. The RNA was extracted with Trizol (Invitrogen®). Contamination in the DNA was removed with RQ1 RNase-free DNase (Promega). The amount of RNA was measured in a Qubit fluorometer using Qubit RNA HS Assay Kit (Invitrogen®) and its quality and integrity 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®). Quantitative reverse transcription PCR (RT-qPCR) was performed on a CFX Real Time Thermal Cycler (Bio-Rad) 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), pathogenesis-related protein (PR-1A), nitrate and nitrite reductase (NIR1-1 and INR-2), oxaloacetate acetylhydrolase (Oxalo), and an AP2/ERF type transcription factor (ERF3) was performed using specific primers (Table 1). The expression of EF1, corresponding to the translation elongation factor 1α of S. sclerotiorum, was also quantified to determine its presence in the plant tissues. The Ubiquitin-3 gene was used as a reference for normalization according to Mortel et al. (2007).

Experimental design and statistical analysis

A 2 × 2 factorial experiment, consisting of plants sprayed with water (control, referred as -Ni plants) or with Ni (referred as +Ni plants) and non-inoculated or inoculated with S. sclerotiorum), was arranged in a completely randomized design with four replications. Each replication corresponded to a plastic pot containing four plants. The experiment was repeated once. All variables and parameters evaluated were subjected to analysis of variance (ANOVA) and means were compared by F test ($P \leq 0.05$). Data were analyzed using the Minitab software (version 18; Minitab Corporation).

RESULTS

Analysis of variance

The factors Ni treatments (Ni) and plant inoculation (PI) were significant for most of the parameters and variables evaluated. The Ni × PI interaction was significant for ETR, concentrations of Ni and Chl a+b, as well as for the expression of PAL 1.1, PAL 3.1, and EF1 (Table 2).

In vitro assay

Fungal mycelial growth was reduced by 10, 16, 50, 66, 85, and 86% on PDA medium amended with 0.00375, 0. 0075, 0.015, 0.030, 0.045, and 0.060 g Ni L^{-1}, respectively, in comparison to the control treatment (Fig. 1A-G). For Kumulus treatment, fungal mycelial growth was reduced by 25% compared to the control (Fig. 1H). There was no fungal growth on PDA medium amended with Fluazinam (Fig. 1I). The EC_{50} value was 0.016555 g Ni L^{-1} (Fig. 1J).

Disease evaluation

On the leaves of +Ni plants, white mold symptoms were reduced compared to the -Ni plants during the time-course evaluated (Fig. 2A-H). Decreases of 77, 70, 64, and 57% for white mold severity at 24, 48, 72, and 96 hai, respectively, were recorded for +Ni plants compared to -Ni plants (Fig. 3A). The AUDPC was significantly reduced by 61% for +Ni plants in contrast to -Ni plants (Fig. 3B).

Foliar Ni concentration

The foliar Ni concentration for non-inoculated +Ni and inoculated +Ni plants significantly increased by 561 and 139% compared to non-inoculated -Ni and inoculated -Ni plants, respectively (Fig. 4).

Chl a fluorescence parameters

There was no difference between inoculated -Ni and inoculated +Ni plants in terms of color patterns of the images of Chl a fluorescence and quantitative analysis for the parameters F_v/F_m, Y(II), Y(NPQ), Y(NO), and ETR regardless of the evaluation time, except for ETR at 48 hai (Figs. 5 and 6A, C, E, G, and I). For inoculated plants, variations in the images of Chl a fluorescence parameters occurred at 24 hai for both -Ni and +Ni plants. However, for +Ni plants, the alterations in the images were less expressive compared to -Ni plants (Fig. 5). As the white mold developed, there was a progressive loss of the photosynthetic capacity of the leaflets from -Ni and +Ni plants as indicated by the black areas, but such loss was remarkably high for -Ni plants than for +Ni plants. For inoculated plants, there were significant increases of 11, 46, and 21% for Y(II) at 24, 72, and 96 hai, respectively, of 22 and 15% for Y(NPQ), and of 83 and 41% for ETR at 72 and 96 hai, respectively, for +Ni plants in comparison to -Ni plants (Fig. 6D, F, and J). Conversely, there were significant decreases of 17 and 3% for F_v/F_m at 24 and 72 hai, respectively, of 10% for Y(NPQ) at 24 hai, and of 13% for Y(NO) at 96 hai for +Ni plants in comparison to -Ni plants (Fig. 6B, F, and H). For inoculated -Ni plants, F_v/F_m significantly decreased by 4, 12, 12, 7%, Y(II) by 25, 45, 54, 45%, and ETR by 27, 40, 59, 52% at 24, 48, 72, and 96 hai, respectively, in comparison to non-inoculated -Ni plants (Fig. 6A-D and I-J). Also, for inoculated -Ni plants, Y(NO) significantly increased by 8, 21, 44, and 79% at 24, 48, 72, and 96 hai, respectively, and Y(NPQ) by 27 and 22% at 24

and 48 hai, respectively, in comparison to their non-inoculated counterparts (Fig. 6E-H). Inoculated +Ni plants showed significant increases of 24 and 45% for Y(NO) at 48 and 96 hai, respectively, and of 22, 46, 26, and 11% for Y(NPQ) at 24, 48, 72, and 96 hai, respectively, in comparison to non-inoculated +Ni plants (Fig. 6E-H). The F_v/F_m significantly decreased by 20, 14, 9, and 8%, Y(II) by 19, 42, 35, and 32%, and ETR by 23, 42, 24, and 30%, respectively, at 24, 48, 72, and 96 hai for inoculated +Ni plants in comparison to non-inoculated +Ni plants (Fig. 6A-D and I-J).

Photosynthetic pigments

There was no significant difference between non-inoculated -Ni and non-inoculated +Ni plants regardless of the evaluation time and between inoculated -Ni and inoculated +Ni plants at 24 and 48 hai for the concentrations of total Chl a+b and carotenoids (Fig. 7A-D). For inoculated plants, significant differences in the concentrations of total Chl a+b and carotenoids were observed between -Ni and +Ni plants at 72 and 96 hai. Carotenoids concentration significantly increased by 29 and 55% and the total Chl a+b concentration by 43 and 62%, respectively, at 72 and 96 hai for inoculated +Ni plants in comparison to inoculated -Ni plants (Fig. 7B and D). For inoculated -Ni plants, total Chl a+b concentration was significantly lower by 38 and 44% and the carotenoids concentration by 25 and 37%, respectively, at 72 and 96 hai in comparison to their non-inoculated counterparts (Fig. 7B and D). For non-inoculated +Ni plants, total Chl a+b concentration was significantly higher by 6 and 13% and the carotenoids concentration by 6 and 8%, respectively, at 72 and 96 hai in comparison to their inoculated counterparts (Fig. 7A and C).

Concentration of MDA

There was no significant difference between non-inoculated -Ni and non-inoculated +Ni plants regardless of the evaluation time and for inoculated -Ni and inoculated +Ni plants at 24 and 72 hai (Fig. 8A-B). For inoculated plants, MDA concentration significantly increased by 27% at 48 hai and significantly decreased by 50% at 96 hai for +Ni plants in comparison to -Ni plants (Fig. 8B). Comparing non-inoculated and inoculated plants, MDA concentration was significantly higher by 21, 53, and 46% at 48, 72, and 96 hai, respectively, and significantly lower by 7 and 31% at 24 and 96 hai, respectively, for -Ni plants compared to +Ni plants (Fig. 8A-B).

Concentrations of TSP and LTGA derivatives

Regardless of the evaluation time, there was no significant difference for TSP and LTGA derivatives concentrations between non-inoculated -Ni and non-inoculated +Ni plants (Fig. 8C and E). For inoculated +Ni plants, the concentrations of TSP (35 and 12% at 72 and 96 hai, respectively) and LTGA derivatives (44% at 96 hai) were significantly higher in comparison to inoculated -Ni plants (Fig. 8D and F). Comparing non-inoculated and inoculated plants, the TSP concentration was significantly lower by 8 and 4% for -Ni and +Ni plants, respectively, at 24 hai, by 13% for +Ni plants at 48 hai, and by 25 and 2% for -Ni and +Ni plants, respectively, at 72 hai (Fig. 8C and D). For inoculated +Ni plants, the concentration of LTGA derivatives was significantly higher by 26, 55, 36, and 54% at 24, 48, 72, and 96 hai, respectively, in comparison to non-inoculated +Ni plants (Fig. 8E and F). For inoculated -Ni plants, the concentration of LTGA derivatives significantly increased by 32, 67, 32, and 6% at 24, 48, 72, and 96 hai, respectively, compared to non-inoculated -Ni plants (Fig. 8E and F).

Genes expression

There was no significant difference between non-inoculated -Ni and non-inoculated +Ni plants for the expression of the genes studied regardless of the evaluation time (Fig. 9A-B). Therefore, the fold change was based on the medium values of $2^{-\Delta CT}$ for non-inoculated -Ni plants at 24 hai. There was no expression of EF1 for non-inoculated plants; therefore, its fold change was based on the medium values of $2^{-\Delta CT}$ for inoculated -Ni plants at 24 hai. The expression of defense-related genes (PAL1.1, PAL1.3, LOX7, and MMP2) was higher at 24 hai for both inoculated -Ni and inoculated +Ni plants in comparison to their non-inoculated counterparts (Fig. 9C-D). The expression of CHIB1 was significantly higher for inoculated +Ni plants in comparison to inoculated -Ni plants regardless of the evaluation time (Fig. 9C-D). At 24 hai, the expression of the PAL1.3, PAL2.1, and PAL3.1 significantly increased for inoculated +Ni plants compared to inoculated -Ni plants. Conversely, the expressions of PAL1.1, URE, MMP2, and INR-2 were significantly reduced at 24 hai for inoculated +Ni plants in comparison to inoculated -Ni plants. For inoculated +Ni plants, the expression of PAL1.1 and PR1-A significantly increased at 48, 72, and 96 hai compared to inoculated -Ni plants. The PAL2.1 expression was significantly higher at 48 and 72 hai and the PAL3.1 at 96 hai for inoculated +Ni plants compared to inoculated -Ni plants (Fig. 9C-D). The EF1 expression was significantly increased by 1.20, 1.23, and 1.46 times at 48, 72, and 96 hai, respectively, for inoculated -Ni plants compared to inoculated +Ni plants (Fig. 9C-D). There was no significant change in the expression of the CHIA, LOX7, ICS1, ICS2, ERF3, NIR1-1, and Oxalo for both -Ni and +Ni plants regardless of the evaluation time (Fig. 9A-D). For inoculated -Ni plants, the expression of PAL3.1 and CHIA1 was significantly higher at 24, 48, 72 and 96 hai compared to non-inoculated -Ni plants (Fig. 9A and C). Conversely, there was significant increase in the expression of PAL2.1 and URE at 24 hai, of CHIB1 at 48, 72, and

96 hai, of MMP2 and EF1 at 72 and 96 hai, of PAL1.1 at 24, 72, and 96 hai, of PAL1.3 at 24, 48, and 96 hai, of ERF3 at 24 and 96 hai and of PR-1A at 72 hai for inoculated -Ni plants compared to non-inoculated -Ni plants (Fig. 9A and C). By contrast, the expression of PAL1.1, ICS1, NIR1-1, and INR-2 at 48 hai, of ERF3 at 48 and 72 hai, and of PAL2.1 at 96 hai were significantly lower for inoculated -Ni plants in comparison to non-inoculated -Ni plants (Fig. 9A and C). For inoculated +Ni plants, the expression of PAL1.3 at 24, 48, 72, and 96 hai, of PAL1.1 and CHIB1 at 24, 48, and 72 hai, of MMP2 and PR-1A at 48 and 72 hai, of PAL2.1 and EF1 at 72 and 96 hai, of PAL3.1 at 48hai, and of CHIA1 and Oxalo at 96 hai significantly increased in comparison to non-inoculated +Ni plants. Only the expression of LOX7 was significantly lower at 72 hai for inoculated +Ni plants compared to non-inoculated +Ni plants (Fig. 9B and D).

DISCUSSION

The present study provides novel insights into the biochemical, physiological, and molecular aspects of foliar Ni application to enhance the resistance of soybean plants against S. sclerotiorum infection. It is known that pathogens of different lifestyles are directly affected upon contact with Ni (Wiebke-Strohm et al. 2012; Wood et al. 2012). Mycelia of S. sclerotiorum were inhibited in the in vitro assay by the same Ni concentration used to spray the soybean leaves. Ahamed et al. (2016) and Sharma et al. (2017) reported a reduction in mycelial growth of Fusarium oxysporum f. sp. lactucae, F. oxysporum f. sp. lycopersici, Dematophora necatrix, and Colletotrichum gloeosporioides with the application of Ni nanoparticles. Urease is a Ni-dependent metalloenzyme that may directly or indirectly damage fungal mycelial growth due to an increase in the osmotic pression (Wiebke-Strohm et al. 2012). Becker-Ritt et al. (2007) showed a decrease in the mycelial growth of Curvularia lunata, Fusarium solani, Penicillium herguei, and three species of Trichoderma as well as inhibition of conidia germination by urease from soybean seeds. Furthermore, mycelia of P. herguei treated with urease obtained from seeds of soybean and jack bean provoked plasmolysis and injuries in the cell wall of the fungus (Becker-Ritt et al. 2007).

White mold symptoms were less expressive in the leaflets of Ni-sprayed plants, and the lower EF1 expression may have contributed to that achievement. The foliar application of Ni, at small concentrations, enhanced the resistance of some crops against diseases (Reis et al. 2014; Langenbach et al. 2016). In this regards, it has been reported the positive effect of Ni to decrease the severities of rust in daylily plants (Wood and Reilly 2007), scab in pecan (Wood et al. 2012), powdery mildew in soybean (Barcelos et al. 2018), and Asian soybean rust in soybean (Einhardt et al. 2020a,b).

Ojeda-Barrios et al. (2016) demonstrated that the Ni application increased its foliar concentration and concomitantly the urease activity. In the present study, the foliar Ni concentration increased for Ni-sprayed plants regardless of inoculation with S. sclerotiorum confirming, therefore, its absorption at the leaf level. Conversely, the foliar Ni concentration was lower for inoculated Ni-sprayed plants in comparison to non-inoculated Ni-sprayed plants, probably because of the mobilization of this micronutrient to other parts of the leaf as the white mold symptoms progressed. Einhardt et al. (2020a,b) reported that the foliar Ni concentration increased for soybean plants sprayed with this micronutrient and contributed to decreasing the severity of Asian soybean rust.

Photosynthesis is one of the main physiological processes impaired in plants infected by pathogens of different lifestyles (Aucique-Pérez et al. 2014; Silveira et al. 2015; Dias et al. 2018; Einhardt et al. 2020a,b). The Chl a fluorescence imaging is a great tool to assess the photosynthetic performance of plants at the cellular, leaf, and whole-plant levels when challenged by pathogens (Pérez-Bueno et al. 2019). In the present study, the photosynthetic apparatus of soybean leaves was dramatically affected at the early stages of S. sclerotiorum infection. This finding corroborates with the reports of Novaes et al. (2019) and Chaves et al. (2020) for the soybean and tomato-S. sclerotiorum interactions. The F_v/F_m parameter represents the maximum photochemical efficiency of PSII, and it is used as a physiological indicator of the level of stress imposed to plants (Rolfe and Scholes 2010; Sharma et al. 2015). In the present study, the F_v/F_m, Y(II), and ETR values decreased over time, while the values of Y(NO) and Y(NPQ) increased, suggesting, thus, photoinhibition and damage of the PSII, especially in the leaves of plants non-sprayed with Ni. Conversely, an increase in the values of Y(II), Y(NPQ), and ETR at advanced stages of S. sclerotiorum infection and decreases in the values of F_v/F_m at the early stage of fungal infection occurred for Ni-sprayed

plants implying that the photochemical dysfunction was attenuated by Ni. The lower severity of white mold for Ni-sprayed plants mightily contributed to preserving the efficient use of light energy through carbon fixation reactions. Consistent with the results of the present study, Einhardt et al. (2020b) found that Ni helped to preserve the proper operation of the photosynthetic apparatus of soybean plants infected by P. pachyrhizi as denoted by the higher F_v/F_m and ETR values and lower Y(NO) values compared to inoculated and non-sprayed Ni plants. The pool of photosynthetic pigments (total Chl a+b and carotenoids) was lowered in the infected leaflets of soybean plants non-sprayed with Ni and hampered the functionality of the photosynthetic apparatus in clear contrast to what was noticed for the infected and Ni-sprayed plants. A more robust nitrogen metabolism and the maintenance of great concentration of photosynthetic pigments in soybean plants infected by P. pachyrhizi was gained with Ni supply (Einhardt et al. 2020b).

The MDA is a biochemical marker to indicate lipids peroxidation in plant cells exposed to abiotic and biotic stress (Mittler 2002). In the present study, the MDA concentration increased for infected and non-supplied Ni plants at advanced stages of S. sclerotiorum infection, but its concentration lowered when plants were sprayed with Ni, reflecting the lower white mold severity. Einhardt et al. (2020b) found less MDA concentration in the leaflets of soybean plants sprayed with Ni in response to P. pachyrhizi infection.

Phenolics and lignin precursors contribute to reducing the colonization of plant tissues by some fungal pathogens for having a direct effect against their cell wall and making difficult the diffusion of the hydrolytic enzymes and nonhost-selective toxins released by them to the plant tissues (Tomás-Barberán et al. 1990; Yadav et al. 2020). The concentrations of TSP and LTGA derivatives were higher for Ni-sprayed plants at advanced stages of S. sclerotiorum infection, becoming another factor in enhancing the resistance of plants against

white mold. According to Einhardt et al. (2020a), a higher concentration of LTGA derivatives in the leaves of soybean plants sprayed with Ni was of detrimental importance to reduce the severity of Asian soybean rust.

The chalcone isomerase, encoded by the CHI gene, is an important enzyme of the flavonoids pathway that results in the production of several antimicrobial compounds against pathogens (Zhou et al. 2018). In the present study, the CHIB1 expression was higher for Ni-sprayed plants during the time-course of S. sclerotiorum infection, suggesting its involvement in soybean resistance against white mold. Zhou et al. (2018) showed that the overexpression of GmCHI1A gene increased the resistance against of soybean plants to Phytophthora sojae infection.

The PAL plays an important role in plant defense against pathogens because it participates in the synthesis of many phenolics, including the salicylic acid that is involved in the systemic acquired resistance of plants (Mauch-Mani and Slusarenko 1996; Nugroho et al. 2002; Chaman et al. 2003). In the present study, the transcript levels of PAL1.3, PAL2.1, and PAL3.1 at 24 hai and PAL1.1 at 48, 72, and 96 hai were higher for Ni-supplied plants infected by S. sclerotiorum. According to Shine et al. (2016), increased expressions of PAL1.1, PAL1.3, and PAL3.1 were associated with the resistance of soybean plants to Pseudomonas syringae and P. sojae infections. Soybean plants infected with P. pachyrhizi and supplied with Ni also showed higher PAL1.1 and PAL 2.1 expressions that increased their resistance against Asian soybean rust (Einhardt et al. 2020a).

Urease, a Ni-dependent enzyme, is responsible for converting urea into ammonia and carbon dioxide (Dixon et al. 1975; Bai et al. 2006). In the present study, the URE expression was low for inoculated and Ni-sprayed plants at the early stages of S. sclerotiorum infection, indicating that the ammonia pool in the infected leaf tissues and the generation of compounds

containing nitrogen was compromised in favor of a better fungal nutrition. Despite being considered important in the resistance of plants to diseases, in the present study, urease did not contribute to enhancing the resistance of soybean plants against S. sclerotiorum infection. Lower urease activity in Ni-deficient soybean plants resulted in urea accumulation in the leaf tissues (Witte 2011). In contrast to the findings of the present study, Einhardt et al. (2020a) reported that the URE expression was high for Ni-sprayed soybean plants infected by P. pachyrhizi.

The LOX catalyzes the oxidation of polyunsaturated fatty acids to start the synthesis of oxylipins, which have distinct cellular functions (Porta and Rocha-Sosa 2002). In the present study, the LOX7 expression was high for infected plants regardless of Ni supply. Higher LOX activity in plants tissues in response to infection by pathogens has been reported (Slusarenko 1996). Furthermore, in plants infected by pathogens, LOX is linked to the synthesis of different compounds with signaling roles to increase their resistance against diseases (Slusarenko 1996; Porta and Rocha-Sosa 2002).

The matrix metalloproteinase MMP2 gene is transcriptionally up-regulated in infected plant tissues and plays a significant role in defense of plants against pathogens (Liu et al. 2001). In the present study, regardless of Ni supply, the MMP2 expression increased in response to S. sclerotiorum infection implying its possible contribution to soybean resistance. Consistent with the results of the present study, soybean plants showed a rapid increase in MMP2 expression in response to infections by P. sojae and Pseudomonas syringae pv. glycinea (Liu et al. 2001).

Plants respond to pathogens infection and environmental stresses by a rapid change in genes expression to synthesize key proteins for their better performance (Jiang et al. 2015; Boccardo et al. 2019). The PR proteins are induced and accumulate in host tissues of plants in

response to pathogens infection or even when they are exposed to abiotic stresses (Kim and Hwang 2000). High expression of PR-1A for Ni-sprayed plants infected with S. sclerotiorum was an indicator of their resistance against white mold. The CHI, one of the major PR-related proteins, plays an important role in host defense responses against pathogens by degrading their cell wall, of which chitin is the main structural component (Petruzzelli et al. 1999; Ebrahim et al. 2011). An increase in the CHIA1 expression occurred for Ni-sprayed plants in response to S. sclerotiorum infection. Einhardt et al. (2020a) reported a high expression of CHIA1 in soybean plants infected by P. pachyrhizi. Interestingly, the CHIA1 expression was similar regardless of S. sclerotinia inoculation and Ni supply.

The isochorismate synthase (ICS) catalyzes the conversion of isochorismate to produce salicylic acid that is considered a signaling molecule to external stresses (Sadeghi et al. 2013). There was no change in the ICS1 and ICS2 expressions regardless of Ni supply. In Arabidopsis, the biosynthesis of ICS1-derived contributed to about 98% of the pathogen-induced salicylic acid production, while in soybean the ICS and PAL pathways are likewise more important for pathogen-induced salicylic acid biosynthesis (Shine et al. 2016). No significant change was detected in ERF3 expression regardless of S. sclerotinia inoculation and Ni supply. Conversely, the ERF3 expression occurred at 1 hour after the Arabidopsis plants were infected with Pseudomonas syringae pv. tomato suggesting its involvement in host resistance at the transcription level (Liang et al. 2010).

The principal source of mineral nitrogen for plants are nitrate (NO_3^-) and ammonium (Baker and Pilbeam 2007). Once absorbed by roots, NO_3^- is reduced to nitrite by nitrate reductase (NR), and nitrite is reduced to ammonium by nitrite reductase (NiR) (Forde 2000). The status of Ni in plant tissues influences the concentration of ammonium, nitrate, and glutamine as well as the activities of NiR, NR, and glutamine synthetase (GS) (Gajewska and

Skłodowska 2009). Reduced INR-2 expression was noticed at the early stages of S. sclerotiorum infection for Ni-sprayed plants, and no change in NIR1-1 expression occurred for either non-sprayed and Ni-sprayed plants. Consistent with the results of the present study, Gajewska and Sklodwska (2009) reported a decrease of 80% in NiR activity in wheat leaves that received Ni application.

The oxaloacetate acetylhydrolase (OAH), encoded by the Oxalo gene, is important to catalyze oxalate into oxalic acid (OA). The OA is a nonhost-selective toxin produced by S. sclerotiorum with great capacity to damage the tissues of its hosts (Andrew et al. 2012) and reported to be a pathogenicity factor for this fungus (Rollins 2003; Liang et al. 2015). In addition to OA, S. sclerotiorum produces some hydrolytic enzymes (e.g., pectinases, glycosidases, xylanases, proteases, and cutinases) that degrade the plant tissues in favor of its successful infection process (Marciano et al. 1983). In the present study, the Oxalo expression was higher for Ni-sprayed plants at advanced fungal infection stages. The production of OA only at advanced stages of S. sclerotiorum infection may suggest that hydrolytic enzymes were of earlier importance to damage the soybean leaf tissues.

In conclusion, based on the results of the present study, the foliar Ni application played a beneficial role in the resistance of soybean against white mold through the potentiation of the expression of CHI1B1, PAL, and PR-1A linked to a great production of phenolics and lignin and the maintenance of the functionality of the photosynthetic apparatus. It is also important to stress out that the infection of soybean plants by S. sclerotiorum could have been affected earlier due to a possible direct effect of Ni against fungal mycelia.

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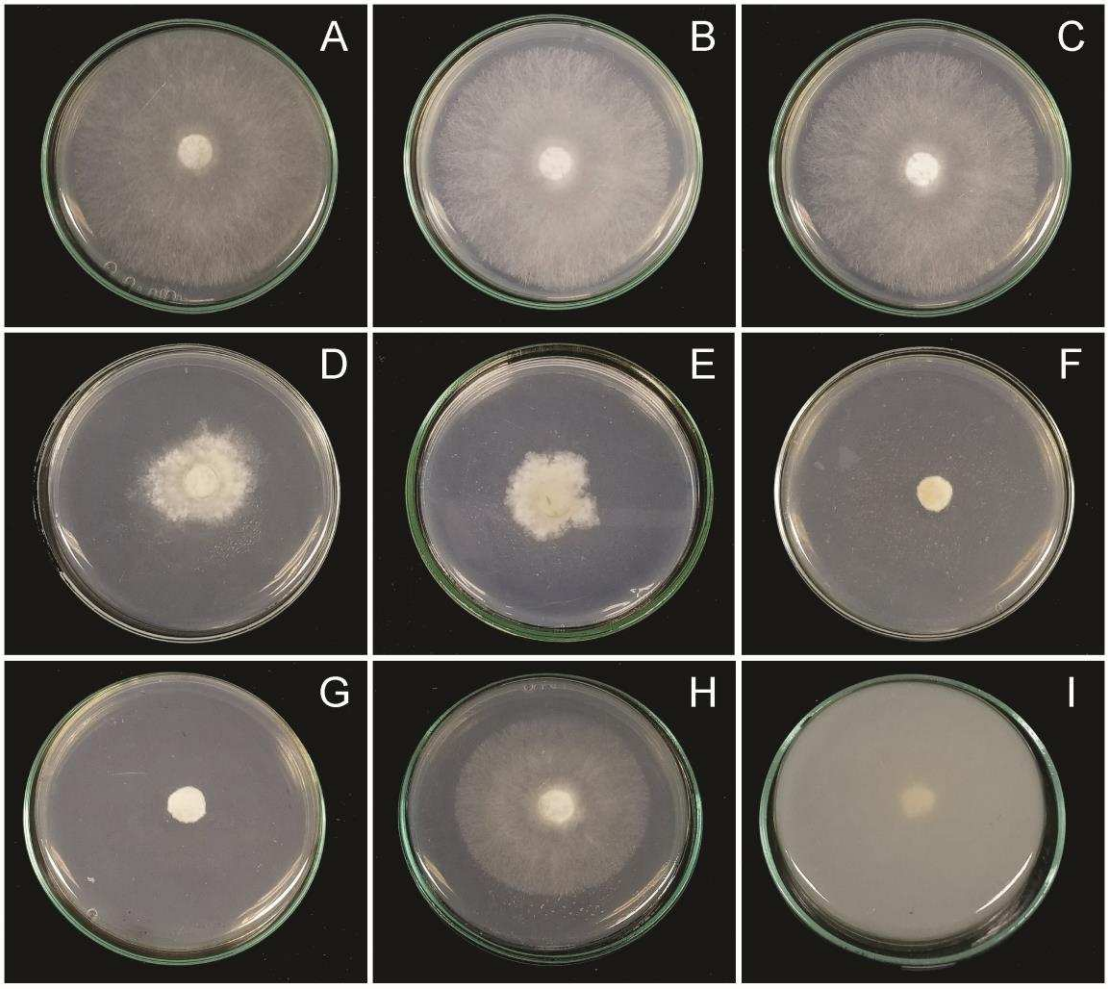
TABLES AND FIGURES

Table 1. Primer sequences for the genes phenylalanine ammonia-lyase (PAL1.1, PAL1.3, PAL2.1, and PAL3.1), chitinase (CHIA1), chalcone isomerase (CHI1B1), lipoxygenase (LOX7), pathogenesis-related protein 1 (PR-1A), metalloproteinase (MMP2), urease (URE) isochorismate synthase (ICS1 and ICS2), nitrate reductase and nitrite reductase (NIR1-1 and INR-2, respectively), an AP2/ERF type transcription factor (ERF3), the translation elongation factor 1α of Sclerotinia sclerotiorum (EF1), and oxaloacetate acetylhydrolase (Oxalo) that were analyzed by quantitative reverse transcription PCR using leaflets of soybean plants non-sprayed or sprayed with nickel and non-inoculated or inoculated with S. sclerotiorum.

Genes	GenBank	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	GTTTCCCCTGCTTTGAAGAGA	GGATTGGCCTCTAACTCTTTGAAG
LOX7	Glyma13G347800	ACAAGCTAGGCACAACAAAA	TTGTTTCCTCCGATGATTCCAA
PR-1A	AF136636.1	GCACTACACACAGGTCGTTTGG	CCTCCGTTATCACATGTCACTTTG
MMP2	Glyma01G0369001	TGGGCTCTTCCCAGTGAAA	TTGCCGCACTCTCCAAGTC
URE	Glyma11G248700	AGTTAGTCACCATTCATGACCT	CAAGTGAAGGTACTGGAAGAAAA
ICS1	Glyma01G104100	GAAACAGTACAGTCCCTGCT	TGTGGCTGGGAAAAGAAAAC
ICS2	Glyma03G070600	GCAACATCCTCGTACCTCTT	CTCTCTGCAACCGTTCATTG
NIR1-1	Glyma.02g132100	TATAGGGACCTTGGCTTCAG	ACAGCAGGCAAAAGTTGTAA
INR-2	Glyma.06g109200	CTGTGGAGGTTGAGGTTTTG	GACAGGAACGTACCATAACATT
ERF3	Glyma03G263700	AACTCGCTCAAGCATCTCTT	CAACAATCGAGATTTCCCCAA
EF1	-	CATCGAGAAGTTCGAGAAGG	TACTTGAAGGAACCCTTACC
Oxalo	-	CCCAATCGTCGAGGACAAGC	TGCCTGCTCCGGTCATGTAA

Table 2. Analysis of variance of the effects of nickel treatments (Ni), plant inoculation (PI), and the interaction Ni × PI for white mold severity (WMS), area under disease progress curve (AUDPC), foliar nickel (Ni) concentration, chlorophyll (Chl) a fluorescence parameters (maximum PSII quantum efficiency (F_v/F_m), photochemical yield (Y(II)), yield for dissipation by down-regulation (Y(NPQ)), yield for other non-photochemical (non-regulated) losses (Y(NO)), and electron transport rate (ETR)), concentrations of total chlorophylls a+b (Chl a+b), carotenoids (CAR), malondialdehyde (MDA), total soluble phenolics (TSP) and lignin-thioglycolic acid (LTGA) derivatives as well as 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), pathogenesis-related protein 1 (PR-1A), AP2/ERF type transcription factor (ERF3), nitrate reductase and nitrite reductase (NIR1-1 and INR-2), translation elongation factor 1α of Sclerotinia sclerotiorum (EF1), and oxaloacetate acetylhydrolase (Oxalo).

Variables/Parameters	Ni	PI	Ni × PI
WMS	<0.001	-	-
AUDPC	<0.001	-	-
Ni concentration	<0.001	0.007	<0.001
F_v/F_m	0.054	<0.001	0.062
Y(II)	<0.001	<0.001	0.001
Y(NPQ)	0.187	<0.001	0.001
Y(NO)	0.378	<0.001	0.020
ETR	<0.001	<0.001	<0.001
Chl a+b	<0.001	<0.001	<0.001
CAR	0.001	0.760	0.140
MDA	0.402	<0.001	0.017
TSP	<0.001	<0.001	0.136
LTGA derivatives	0.037	<0.001	0.436
PAL1.1	<0.001	<0.001	<0.001
PAL1.3	0.637	<0.001	0.111
PAL2.1	0.117	<0.001	0.162
PAL3.1	0.001	<0.001	<0.001
CHIA1	0.954	<0.001	0.924
CHIB1	0.011	<0.001	0.055
LOX7	0.008	0.414	0.069
MMP2	<0.001	<0.001	0.098
ICS1	0.435	0.790	0.062
ICS2	0.956	0.559	0.262
URE	0.283	0.434	0.385
PR-1A	0.004	0.053	0.215
ERF3	0.021	0.686	0.573
NIR1-1	0.853	0.808	0.120
INR-2	0.147	0.441	0.242
EF1	<0.001	<0.001	<0.001
Oxalo	0.229	<0.001	0.229


A
B
C
D
E
F
G
H
I

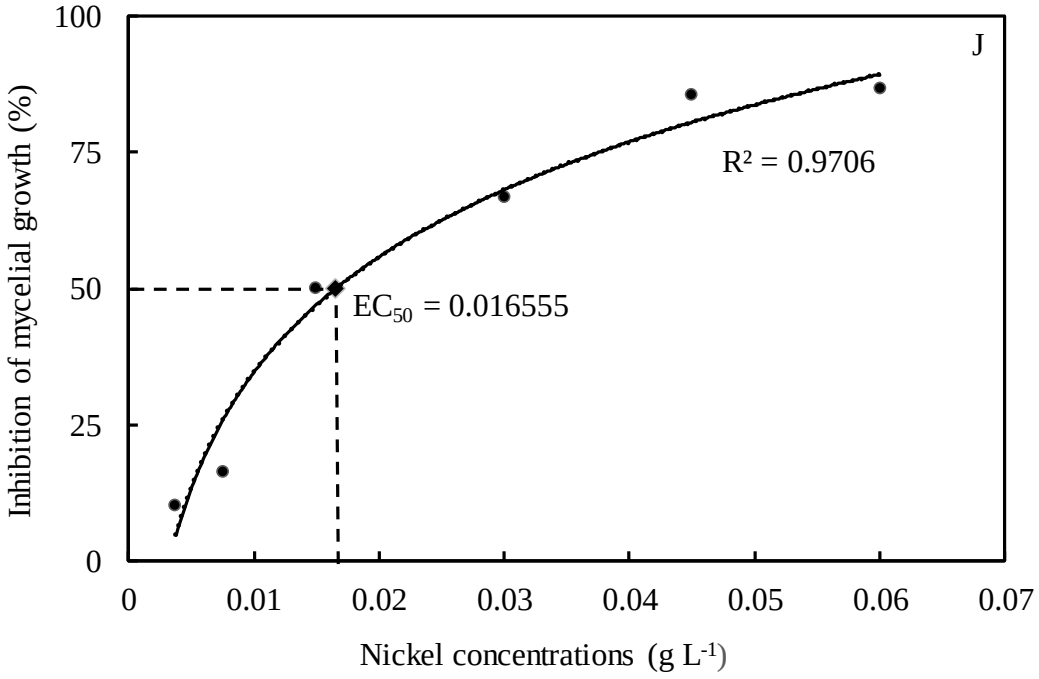

J
100
75
50
25
0
Inhibition of mycelial growth (%)
R² = 0.9706
EC50 = 0.016555
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
Nickel concentrations (g L-1)

Figure 1. Mycelial growth of Sclerotinia sclerotiorum in Petri dishes containing potato-dextrose-agar amended with 0 (A), 0.00375 (B), 0.0075 (C), 0.015 (D), 0.030 (E), 0.045 (F), and 0.060 (G) g L^{-1} of nickel as well as 0.0168 g L^{-1} of sulfur (H) and 5 mL L^{-1} of the fungicide Fluazinam (I). Effective concentration (EC_{50}) of nickel sulfate that inhibited 50% of the mycelial growth of S. sclerotiorum (J).

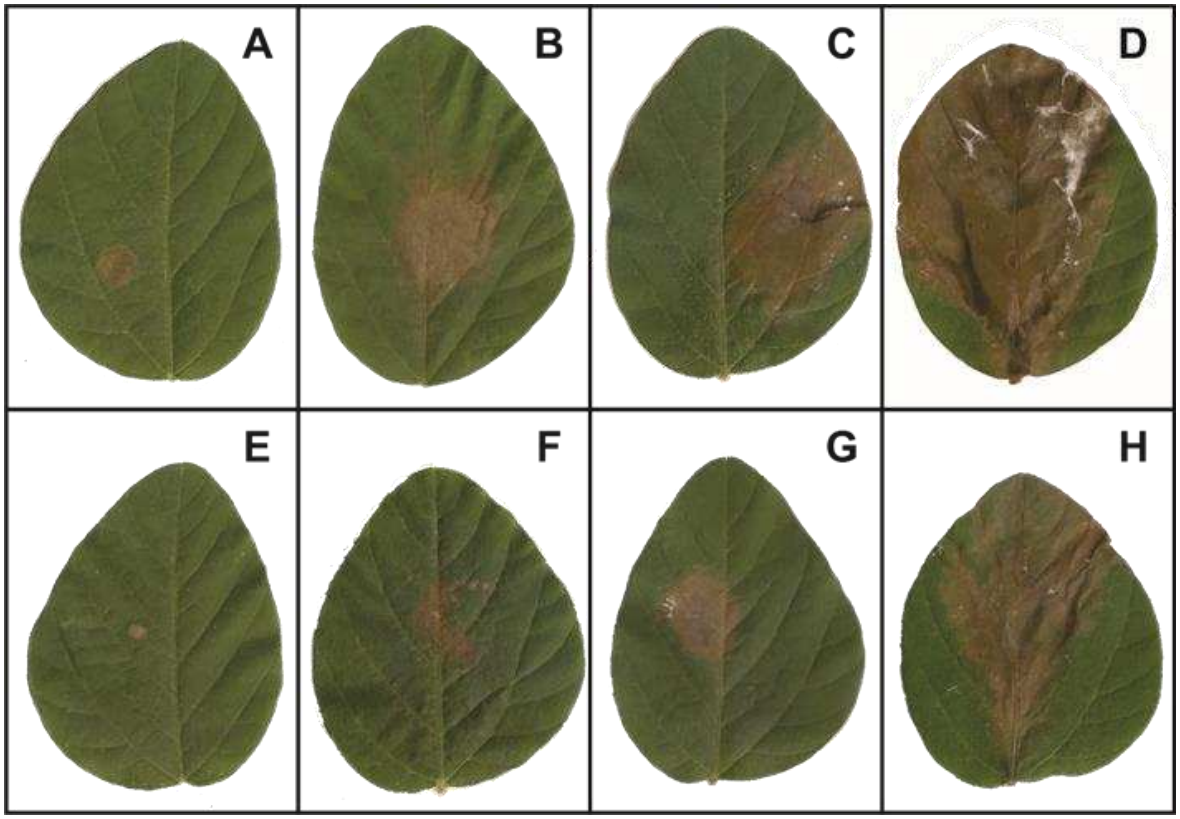


Figure 2. White mold symptoms on the leaflets of soybean plants non-sprayed (-Ni) (A-D) or sprayed (+Ni) (E-H) with nickel (Ni) at 24 (A and E), 48 (B and F), 72 (C and G), and 96 (D and H) hours after inoculation with Sclerotinia sclerotiorum.

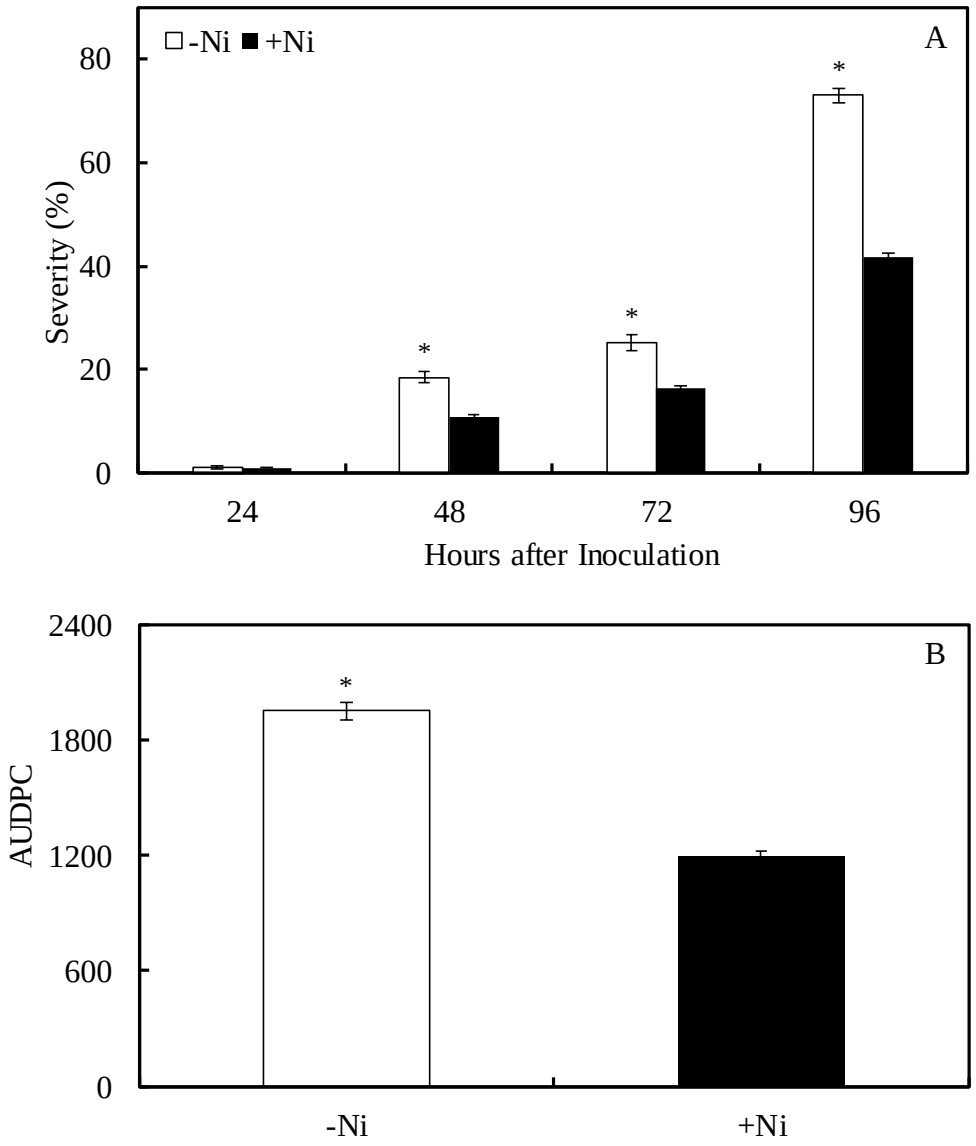


Figure 3. White mold severity (A) and area under disease progress curve (AUDPC) (B) for soybean plants non-sprayed (-Ni) or sprayed (+Ni) with nickel (Ni). Means for the -Ni and +Ni treatments followed by an asterisk (*), at each evaluation time (A), and between these treatments (B) are significantly different ($P \leq 0.05$) by F test. Bars represent the standard error of the means. n = 4.

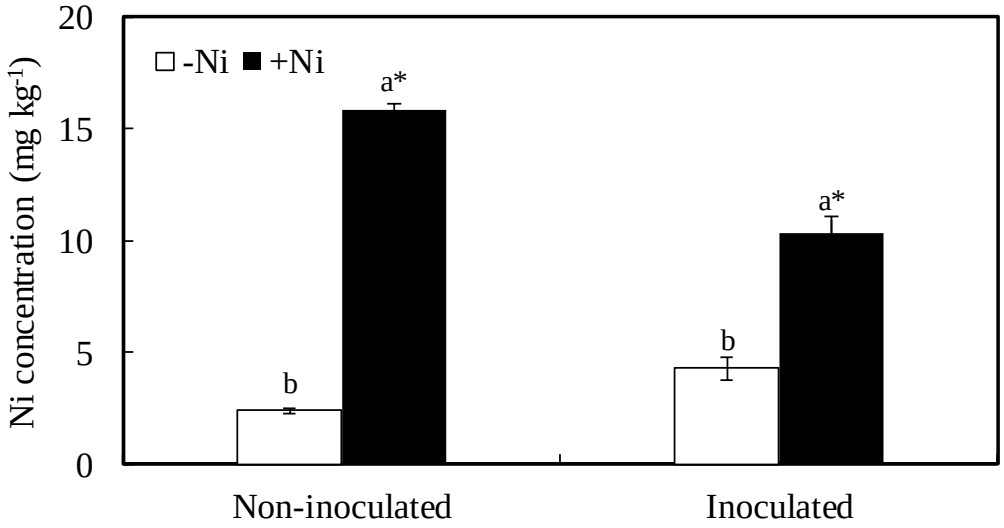


Figure 4. Foliar nickel (Ni) concentration for soybean plants non-inoculated (NI) or inoculated (I) with Sclerotinia sclerotiorum and non-sprayed (-Ni) or sprayed (+Ni) with Ni. Means for the -Ni and +Ni treatments followed by an asterisk (*) and for the NI and I treatments followed by different letters are significantly different ($P \leq 0.05$) according to F test. Bars represent the standard error of the means. n = 4.

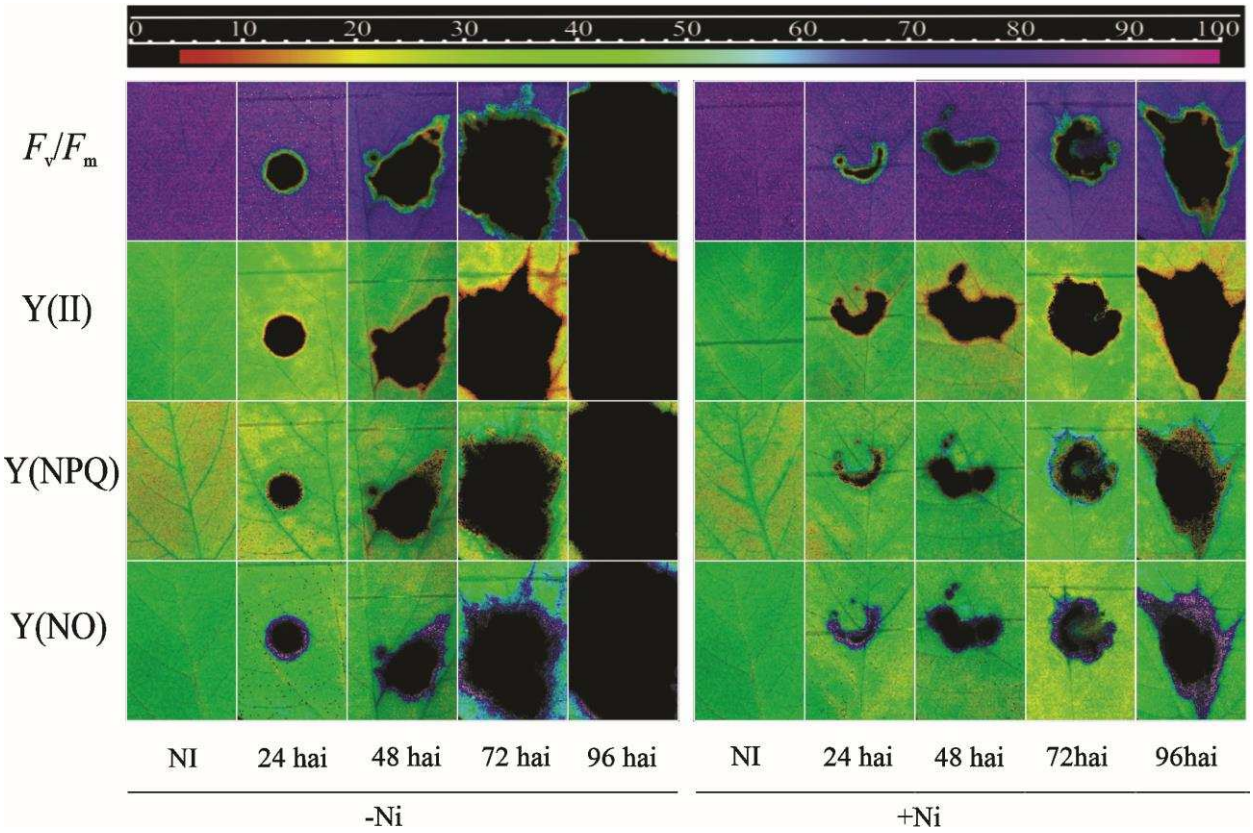


Figure 5. Images of chlorophyll a fluorescence parameters: maximum PSII quantum efficiency (F_v/F_m), photochemical yield (Y(II)), yield for dissipation by down-regulation (Y(NPQ)), and yield for non-regulated dissipation (Y(NO)) determined on the leaflets of soybean plants non-sprayed (-Ni) or sprayed (+Ni) with nickel (Ni) and non-inoculated (NI) or at 24, 48, 72, and 96 hours after inoculation (hai) with Sclerotinia sclerotiorum.

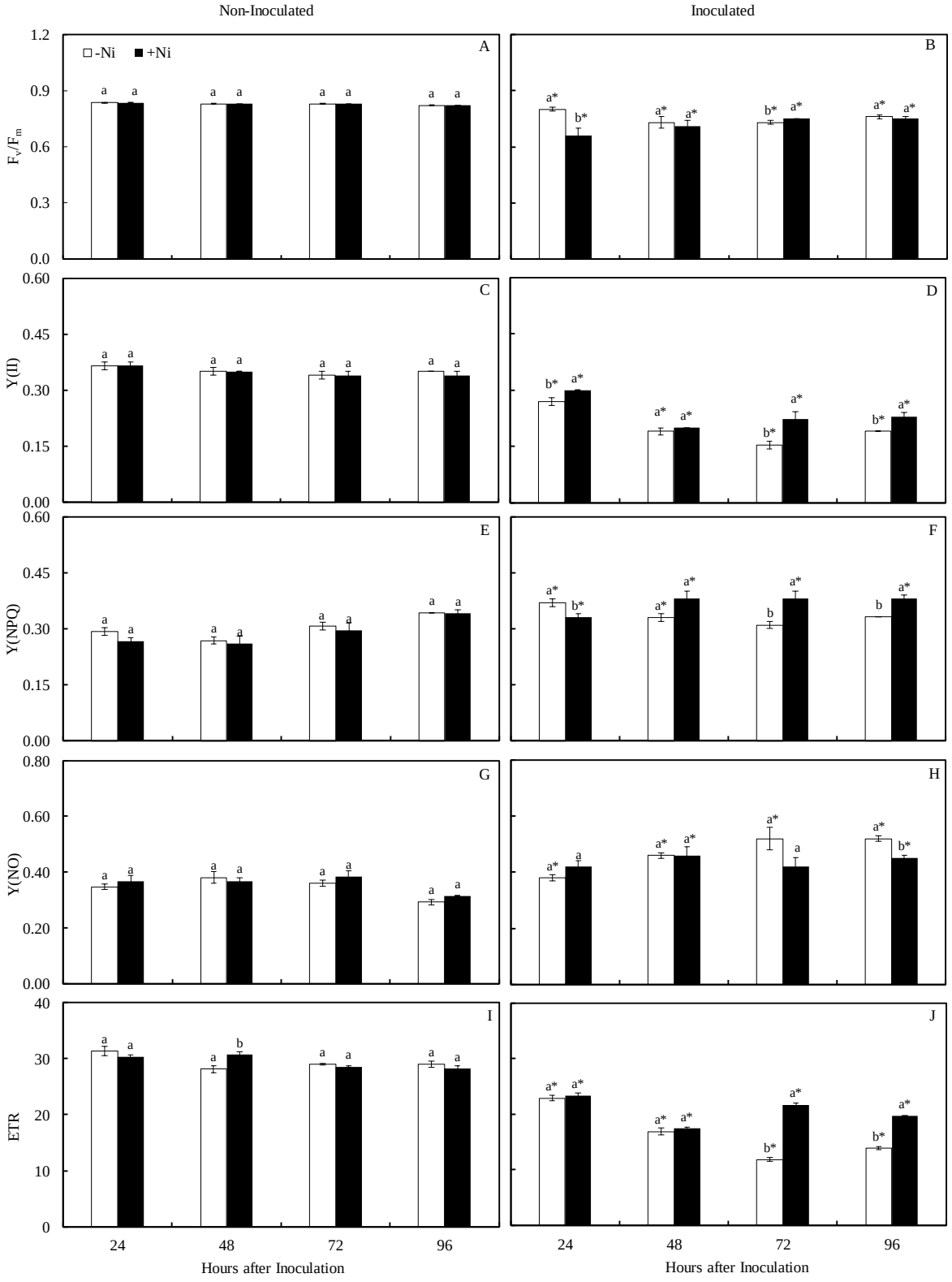

Non-Inoculated
Inoculated
□-Ni ■+Ni
A
B
C
D
E
F
G
H
I
J
F_v/F_m
Y(II)
Y(NPQ)
Y(NO)
ETR
Hours after Inoculation
Hours after Inoculation

Figure 6. Chlorophyll a parameters: maximum PSII quantum efficiency (F_v/F_m) (A and B), photochemical yield (Y(II)) (C and D), yield for dissipation by down-regulation (Y(NPQ)) (E and F), yield for other non-photochemical (non-regulated) losses (Y(NO)) (G and H), and electron transport rate (ETR) (I and J) determined on the leaflets of soybean plants non-inoculated (NI) (A, C, E, G, and I) or inoculated (I) (B, D, F, H, and J) with Sclerotinia sclerotiorum and non-sprayed (-Ni) or sprayed (+Ni) with nickel (Ni). For each evaluation time, means for the -Ni and +Ni treatments followed by different letters and for the NI and I 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 = 4.

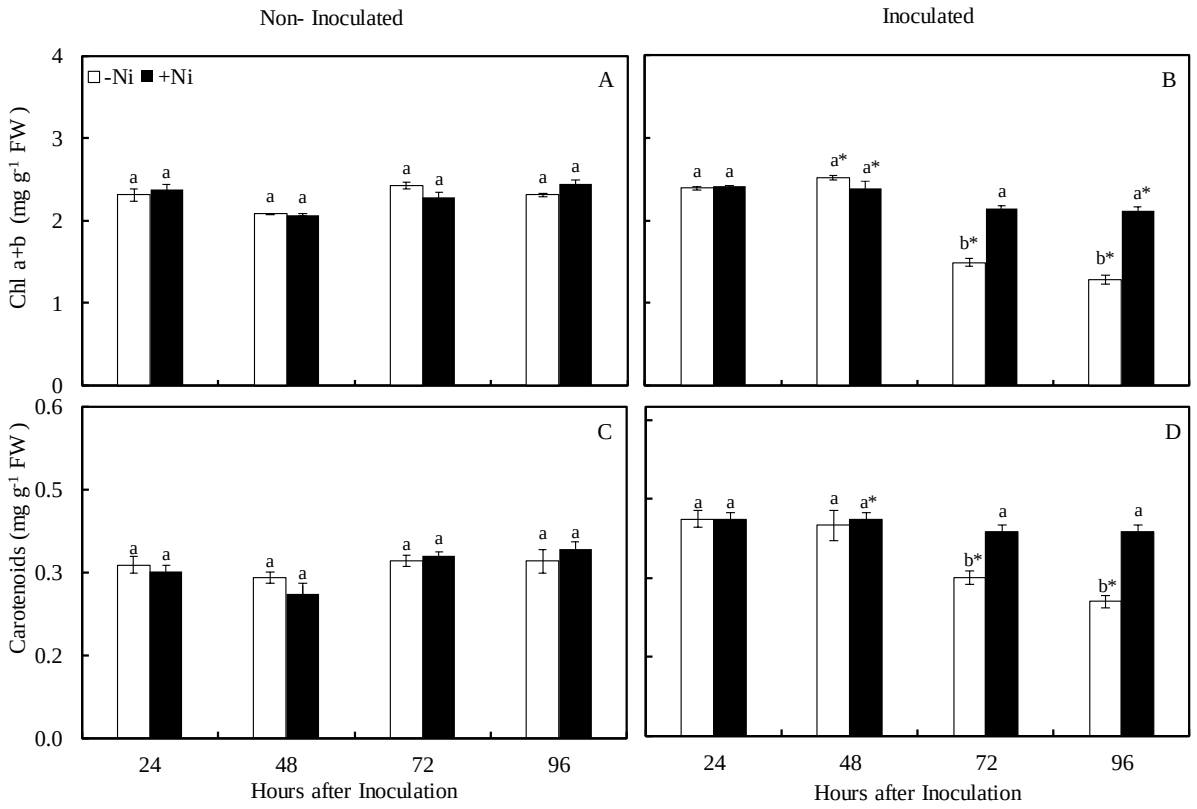


Figure 7. Concentrations of total chlorophylls a+b (Chl a+b) (A and B) and carotenoids (C and D) determined on the leaflets of soybean plants non-inoculated (NI) (A and C) or inoculated (I) (B and D) with Sclerotinia sclerotiorum and non-sprayed (-Ni) or sprayed (+Ni) with nickel (Ni). For each evaluation time, means for the -Ni and +Ni treatments followed by different letters and for the NI and I treatments followed by an asterisk (*) are significantly different ($P \leq 0.05$) according to F test. Bars represent the standard error of the means. FW = fresh weight. n = 4.

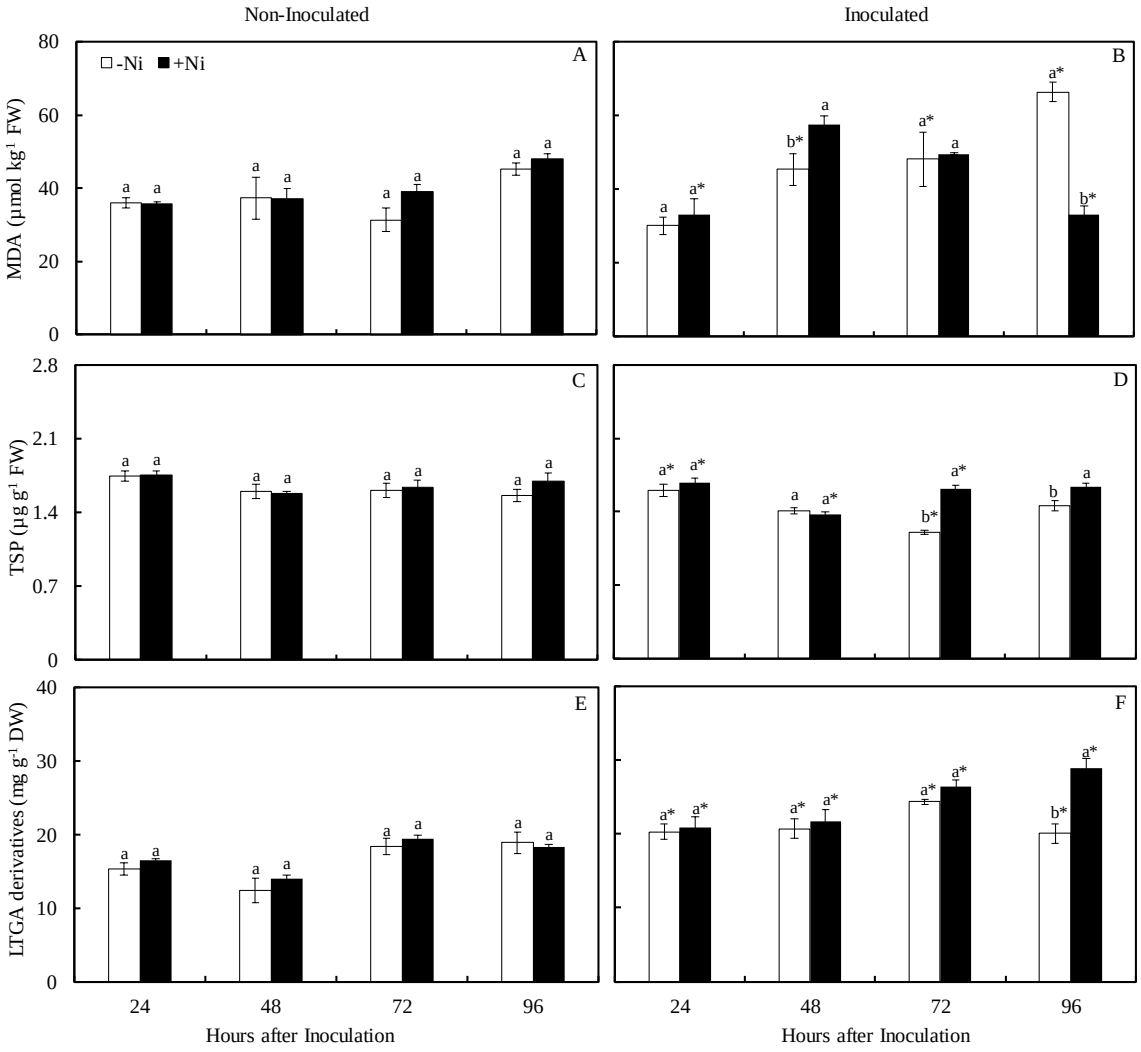


Figure 8. Concentrations of malondialdehyde (MDA) (A and B), total soluble phenolics (TSP) (C and D), and lignin-thioglycolic acid (LTGA) derivatives (E and F) determined on the leaflets of soybean plants non-inoculated (NI) (A, C, and E) or inoculated (I) (B, D, and F) with Sclerotinia sclerotiorum and non-sprayed (-Ni) or sprayed (+Ni) with nickel (Ni). For each evaluation time, means for the -Ni and +Ni treatments followed by different letters and for the NI and I treatments followed by an asterisk (*) are significantly different ($P \leq 0.05$) according to F test. Bars represent the standard error of the means. FW = fresh weight and DW = dry weight. n = 4.

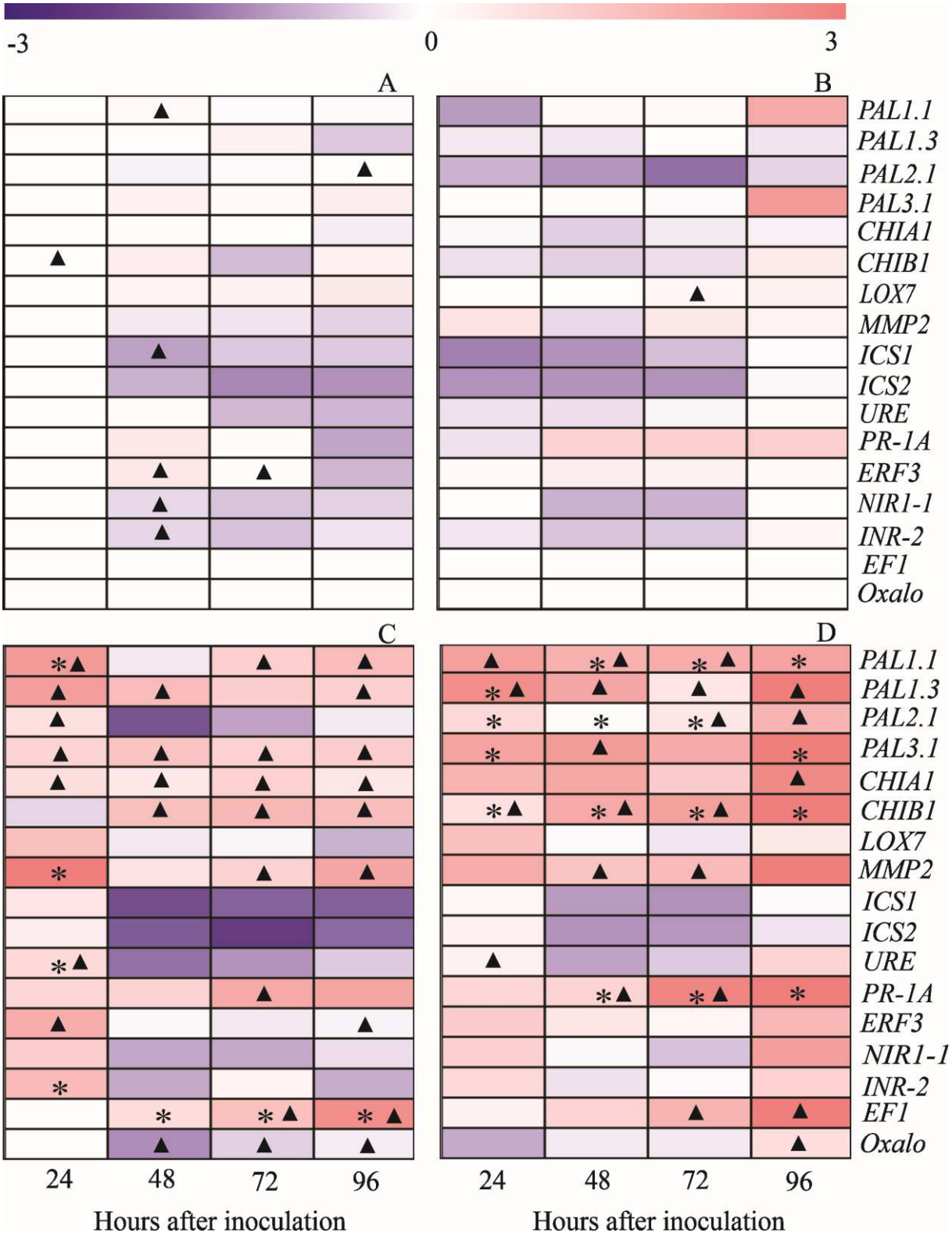

-3
0
3
A
B
C
D
PAL1.1
PAL1.3
PAL2.1
PAL3.1
CHIA1
CHIB1
LOX7
MMP2
ICS1
ICS2
URE
PR-1A
ERF3
NIR1-1
INR-2
EF1
Oxalo
24
48
72
96
Hours after inoculation
Hours after inoculation

Figure 9. Expression profile of defense genes determined in the leaflets of soybean plants from the following treatments: (A) non-sprayed with nickel (-Ni) and non-inoculated (NI) with Sclerotinia sclerotiorum, (B) sprayed with nickel (+Ni) and NI with S. sclerotiorum, (C) -Ni and inoculated (I) with S. sclerotiorum, and (D) +Ni and I with S. sclerotiorum. Colour cells represents the relative transcript levels ranging from purple (-3) to red (3). 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 obtained from samples of the -Ni NI treatment at 24 hours after inoculation (hai), except for the EF1 gene whose transcript level originated from samples of the -Ni I treatment at 24 hai. For each leaflet sample, four biological replications were used with their respective two technical replicates. For each evaluation time, means for the -Ni and +Ni treatments followed by an asterisk (*) and for the NI and I treatments followed by a triangle (▲) are significantly different ($P \leq 0.05$) according to F test. $n = 4$.