ISABELA MARIA GROSSI LEAL

FOLIAR APPLICATION OF NUTRIENTS AND SILICON FOR INCREASING SOYBEAN RESISTANCE AGAINST *Phakopsora pachyrhizi* INFECTION

Dissertation submitted to the Plant Pathology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

Adviser: Fabrício de Ávila Rodrigues

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Fabrício de Ávila Rodrigues
Adviser

For my parents, Ronaldo and Cleuza, and
my sisters, Gabriela and Daniela.

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"O estudo é uma disposição do espírito, não um peso sobre o cérebro".

(São Tomás de Aquino)

ABSTRACT

LEAL, Isabela Maria Grossi, M.Sc., Universidade Federal de Viçosa, February, 2024. **Foliar application of nutrients and silicon for increasing soybean resistance against *Phakopsora pachyrhizi* infection**. Adviser: Fabrício de Ávila Rodrigues.

Rust, caused by the fungus *Phakopsora pachyrhizi*, is one of the most destructive diseases affecting soybean production. Foliar fertilization as a strategy to maintain higher amounts of nutrients besides promoting better plant growth may potentiate some biochemical pathways for defense reactions against pathogens. In this study, soybean plants were sprayed with water (control) or with Sikon Fert® [referred to as induced resistance (IR) stimulus] and non-inoculated or inoculated with *P. pachyrhizi*. The urediniospores germination was significantly reduced from 57 to 100% after being exposed to IR stimulus rates ranging from 0.5 to 10 mL/L compared to the control treatment. For inoculated plants, foliar concentrations of sulfur, copper, and zinc were significantly higher by 40, 490, and 13%, respectively, for IR stimulus treatment compared to the control treatment. The area under disease progress curve significantly decreased by 58% for IR stimulus-sprayed plants compared to plants from the control treatment. Infected and IR stimulus-sprayed plants had their photosynthetic apparatus preserved (significant increases for photochemical yield and electron transport rate values at 7 and 11 dai and significantly lower values for yield for non-regulated dissipation and yield for dissipation by down-regulation at 7 and 11 dai, respectively) along with great concentrations of chlorophyll *a+b* and carotenoids than infected plants from the control treatment. Lower concentrations of malondialdehyde and reactive oxygen species (hydrogen peroxide and anion superoxide) indicating less cellular damage imposed by fungal infection along with great activities of defense-related enzymes (chitinase, β-1,3-glucanase, phenylalanine ammonia-lyase, peroxidase, polyphenoloxidase, and lipoxygenase) and more lignin production allowed the IR-stimulus sprayed plants to hamp the infection by *P. pachyrhizi* more efficiently. These findings open the way to explore the possibility of using this IR stimulus in field conditions to reduce the yield losses caused by soybean rust outbreaks.

Keywords *Glycine max* · host defense reactions · induced resistance · mineral nutrition · photosynthesis.

RESUMO

LEAL, Isabela Maria Grossi, M.Sc., Universidade Federal de Viçosa, fevereiro, 2024. **Aplicação foliar de nutrientes e silício no aumento da resistência da soja contra a infecção por *Phakopsora pachyrhizi***. Orientador: Fabrício de Ávila Rodrigues.

A ferrugem, causada pelo fungo *Phakopsora pachyrhizi*, é uma das doenças mais destrutivas que afeta a produção da soja. A fertilização foliar, como estratégia para manter quantidades mais altas de nutrientes e promover melhor crescimento das plantas, pode potencializar algumas vias bioquímicas envolvidas nas reações de defesa. Neste estudo, as plantas foram pulverizadas com água (controle) ou com Sikon Fert® [referido como estimulador da resistência induzida (ERI)] e inoculadas ou não com *P. pachyrhizi*. A germinação dos urediniósporos foi significativamente reduzida de 57 a 100% em função das doses crescentes (0,5 a 10 mL/L) do ERI em comparação ao tratamento controle. Para as plantas inoculadas, as concentrações foliares de enxofre, cobre e zinco foram significativamente maiores em 40, 490 e 13%, respectivamente, para o tratamento ERI em comparação ao tratamento controle. A área abaixo da curva do progresso da doença diminuiu significativamente em 58% para as plantas pulverizadas com o ERI em comparação com as plantas do tratamento controle. As plantas infectadas e pulverizadas com o ERI apresentaram o aparato fotossintético preservado (aumentos significativos nos valores do rendimento fotoquímico e taxa de transporte de elétrons aos 7 e 11 dai e valores significativamente menores para o rendimento da dissipação não regulada e rendimento da dissipação por regulação negativa aos 7 e 11 dai, respectivamente), juntamente maiores concentrações de clorofila *a*+*b* e carotenoides do que as plantas infectadas do tratamento controle. Menores concentrações de aldeido malônico e espécies reativas de oxigênio (peróxido de hidrogênio e ânion superóxido) indicaram menor dano celular imposto pela infecção fúngica juntamente com maiores atividades de enzimas de defesa (quitinase, *β*-1,3-glucanase, fenilalanina amônia-liase, peroxidase, polifenoloxidase e lipoxigenase) e maior produção de lignina ajudaram as plantas pulverizadas com o ERI afetar o processo infeccioso de *P. pachyrhizi* de forma mais eficiente. Esses resultados mostram uma oportunidade de explorar esse ERI em condições de campo visando reduzir as perdas na produção em decorrência das epidemias severas da ferrugem.

Palavras-chave: *Glycine max* · resistência induzida · nutrição mineral · reações de defesa da planta · fotossíntese.

SUMMARY

1. INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] is one of the most profitable oilseed crops cultivated globally and has been used for biodiesel purposes, livestock, and human food production due to its higher protein content (20-25%) (Sun et al. 2018). Soybean plants are affected by several root and foliar diseases (Hartman et al. 2015). Among them, soybean rust (SR), caused by the obligate biotrophic fungus *Phakopsora pachyrhizi* H. Sydow & P. Sydow, has gained significant importance in South America, mainly in Brazil (Kelly et al. 2015; Paula et al. 2021). Intense SR severity (many necrotic lesions containing several uredinia on leaflets) in plants of the most susceptible cultivars facing favorable environmental conditions for disease epidemics results in premature defoliation, earlier pod maturation, and significant impairment on photosynthesis that end up in yield losses greater than 80% (Kumudini et al. 2008; Goellner et al. 2010; Hartman et al. 2015; Godoy et al. 2016). Using some cultural practices (e.g., early-maturing cultivars, checking for the first SR lesions on leaflets, avoiding sowing soybean in the off-season, and eliminating alternative hosts of *P. pachyrhizi*) and the spray of fungicides with different modes of action are the most effective strategies for SR management (Miles et al. 2007; Godoy et al. 2016). However, the increased sensitivity of *P. pachyrhizi* populations to the molecules in the available fungicides has been a concern of some chemical companies and growers (Miles et al. 2007; Godoy et al. 2016). Unfortunately, soybean cultivars exhibiting long-lasting resistance to SR are unavailable to growers mainly due to the difficulties in profoundly understanding the genetic structures of soybean-*P. pachyrhizi* interaction (Chicowski et al. 2023).

The availability of new and effective alternatives for SR management is the desire of soybean growers to become less dependent on using fungicides. The literature reports an array of products (e.g., *Bacillus subtilis*, acibenzolar-S-methyl, azelaic acid, harpin protein-derived peptides, hexanoic acid, inorganic salts, phosphites, saccharin, and salicylic acid) capable of boosting soybean resistance against SR (Paula et al. 2021; Ahmed et al. 2023; Reglinski et al. 2023; Rodrigues et al. 2023b; Sood et al. 2023). Nutrition of soybean plants with boron, nickel, and silicon (Si) was greatly efficient in increasing their resistance against infection by *P. pachyrhizi* (Cruz et al. 2020; Einhardt et al. 2020a; Picanço et al. 2021).

The foliar fertilization of macro and micronutrients, at small concentrations, as a strategy to deliver nutrients to the aerial parts of plants or to correct nutritional deficiencies, considering their rapid absorption via stomata and epidermis, is an interesting approach for to promote better plant growth and development besides great grain quality and better yield attributes mainly

because some physiological processes are positively regulated (Fernández and Brown 2013; Fernández and Eichert 2009; Bindraban et al. 2015; Ishfaq et al. 2022; Niu et al. 2021). One possible benefit arising from higher foliar concentration of nutrients in plant tissues is the potentiation of defense reactions against infection by pathogens considering their multiple roles in plant physiology such as being co-factors of some enzymes involved in biochemical pathways from which many antimicrobial compounds are produced (Dordas et al. 2008; Rengel et al. 2022; Tubana and Cruz 2023). The potassium (K) is involved in the synthesis of proteins, cellular osmotic (e.g., stomatal aperture and cell expansion), activation of several enzymes (e.g., starch synthetase), activation of cell plasma membrane-bound proton-pumping ATPases, and carbohydrate partitioning (Tubana and Cruz, 2023). The sulfur (S) influences the structure and enzymatic function of nonheme iron (Fe)-S proteins, notably the ferredoxin, that participates in the electron transport system in the thylakoid membranes during photosynthesis, is involved in glutamate synthetase activity as a Fe-S cofactor to maximize NH_3 assimilation, is a structural component of thiamine (vitamin B1) and biotin (vitamin H), and participates in the production of glutathione (a tripeptide of cysteine, glycine, and glutamate) and glucosinolates that play a pivotal role in plant defense response against diseases (Tubana and Cruz, 2023). Many different enzymes are catalyzed by zinc (Zn) because it establishes stable associations with polypeptide chains (Tubana and Cruz, 2023). This micronutrient is also involved in carbon fixation in C4 plants, influences carbohydrate metabolism, is a component of the CuZn-superoxide dismutase (SOD) that protects cell membranes, DNA, and proteins from oxidative damage caused by unbalanced electron transfer at chloroplasts and mitochondria and also from reactive oxygen species (ROS), is present in some plant resistance proteins forming the Zn finger domains, helps the conformational structure of RNA polymerase, in the stabilization of ribosome structure and integrity for an efficient synthesis of proteins (Broadley et al. 2007; Dimkpa and Elmer, 2023; Tubana and Cruz, 2023). Copper (Cu) is needed by enzymes involved in the alleviation of oxidative stress (e.g., CuZn-SOD and ascorbate oxidase), synthesis of aromatic metabolites (polyphenoloxidases), and hydrogen peroxide (diamine oxidase) for lignin production (Elmer et al. 2023; Tubana and Cruz, 2023). Plants supplied with Si display earlier and stronger defense reactions (e.g., greater activities of defense and antioxidative enzymes and higher production of phenolics, phytoalexins, and lignin) upon being infected by pathogens of different lifestyles (Debona et al. 2023).

The present study hypothesized that soybean plants sprayed with a foliar fertilizer containing Si and a mix of nutrients (K, S, Cu, and Zn) could raise their basal level of resistance to cope with infection by *P. pachyrhizi* more efficiently. Several analyses at the physiological

(photosynthetic parameters measurements and concentration of photosynthetic pigments) and biochemical (activities of defense enzymes and concentrations of soluble phenolics and lignin) levels were performed in plants that were non-sprayed or sprayed with the foliar fertilizer and non-inoculated or inoculated with *P. pachyrhizi* aiming to validate the hypothesis above-mentioned.

2. MATERIAL AND METHODS

2.1 *In vitro* assays

Different volumes of Sikon Fert® [phosphorus (145 g P_2O_5/L), potassium (90.4 g K_2O/L), sulfur (23.3 g S/L), copper (20.3 g Cu/L), zinc (31.6 g Zn/L), and silicon (99.2 g SiO_2/L); BIOEST S.A.S., Bogotá, Colômbia] were diluted in deionized water from a stock solution (50 mL/L) to obtain solutions with final concentrations of 0.5, 2, 5, and 10 mL/L. The control treatments corresponded to urediniospores suspension without Sikon Fert® or mixed with fungicide [Trifloxistrobina (375 g/L) + Ciproconazol (160 g/L), Sphere Max®, Bayer S.A., Brazil; 2 ml/L]. A total of 30 µl of urediniospore suspension from *P. pachyrhizi* (10^5 urediniospores/mL) were mixed with the different concentrations of Sikon Fert® and 60 µl were transferred to a glass slide and covered with a coverslip. The glass slides were transferred to a growth chamber (25°C and photoperiod of 12 h of light and 12 h of dark). Each glass slide received 40 µl of lactophenol after 12 h to stop urediniospores germination. One hundred urediniospores were randomly examined in each glass slide under a light microscope (Carl Zeiss AxioImager A1) at 40 × magnification. Urediniospores with germ tubes larger than their diameter were considered germinated. The percentage of urediniospores germination was calculated for the replication of each treatment. Details of urediniospores germination were acquired digitally (camera model AxioCam HR, Germany) and the images were further processed with the AXION VISION software v. 4.8.1.

2.2 Plant material, fertilization, and growth conditions

A total of six seeds from the soybean cultivar DS5916IPRO (https://www.brevant.com.br), susceptible to *P. pachyrhizi*, were sown in each plastic pot containing 2 kg of a 1:1 mixture of soil and substrate (1:1:1 mixture of pine bark, peat, and expanded vermiculite; Tropstrato®, Vida Verde, Mogi Mirim, SP, Brazil). After germination, a total of four seedlings were left per pot. Plants, in each pot, were fertilized weekly with 100 mL of nutrient solution (Clark 1975), with some modifications, as follows: 1.04 mM $Ca(NO_3)_2{\cdot}4H_2O$, 1 mM NH_4NO_3, 0.8 mM KNO_3, 0.6 mM $MgSO_4{\cdot}7H_2O$, 0.069 mM KH_2PO_4, 0.931 mM KCl, 19 µM H_3BO_3, 2 µM $ZnSO_4{\cdot}7H_2O$, 7 µM $MnCl_2{\cdot}4H_2O$, 0.6 µM $Na_2MoO_4{\cdot}4H_2O$, 0.5 µM $CuSO_4{\cdot}5H_2O$, 90 µM $FeSO_4{\cdot}7H_2O$, and 90 mM ethylenediaminetetraacetic acid disodium (EDTA). Plants were grown in a greenhouse [temperature of 25 ± 2°C, relative humidity of 70 ± 5%, and natural photosynthetically active radiation (PAR) of 915 ± 12 µmol photons m^{-2} s^{-1} measured at midday] until being inoculated with *P. pachyrhizi*.

2.3 Application of Sikon Fert®

Soybean plants (V4 growth stage; ≈ 30 days after seedlings emergence) were sprayed with Sikon Fert® (2 mL/L, 20 mL of solution per plant) using a VL Airbrush atomizer (Paasche Airbrush Co., Chicago, IL, USA). This treatment will be referred to as induced resistance (IR) stimulus thereafter according to the criteria proposed by Kesel et al. (2021). The IR stimulus solution was prepared using deionized water. Plants sprayed with deionized water served as the control treatment.

2.4 Inoculation of soybean plants with *P. pachyrhizi*

At 48 h after being sprayed with water or IR stimulus, plants were inoculated with a suspension of 10^5 urediniospores of *P. pachyrhizi*/mL prepared with gelatin (0.5% wt/vol) and Tween 80 (25 µl/L) by using a VL Airbrush atomizer. After inoculation, plants were kept in a mist chamber at 25°C for 16 h under darkness. After this period, plants were transferred to a greenhouse (temperature of 25 ± 2°C, relative humidity of 75 ± 5%, and natural PAR of 932 ± 20 µmol photons m^{-2} s^{-1} measured at midday) until the end of the experiment. Plants non-inoculated with *P. pachyrhizi* were kept in different mist chamber and greenhouse but with the same environmental conditions.

2.5 Experimental design

For the *in vitro* assay, the experiment was arranged in a completely randomized design with six treatments (control, four concentrations of IR stimulus, and fungicide) and eight replications. Each replication corresponded to one glass slide. The experiment was repeated. A 2 × 2 factorial experiment, consisting of plants sprayed with deionized water (control) or IR stimulus and non-inoculated or inoculated with *P. pachyrhizi*, was arranged in a completely randomized design with four replications per each evaluation time to assess rust severity, to obtain leaf samples for the scanning electron microscopy, histochemical assays and to determine the foliar concentrations of Si and nutrients [phosphorus (P), potassium (K), sulfur (S), copper (Cu), zinc (Zn), and silicon (Si)]. Another 2 × 2 factorial experiment with the same factors mentioned above and five replications was carried out to evaluate the parameters of chlorophyll (Chl) *a* fluorescence and to quantify the foliar concentration of pigments. Leaf samples for the biochemical assays were obtained from another 2 × 2 factorial experiment with the same factors described above and six replications. All experiments were repeated once.

2.6 Evaluation of SR severity

The leaflets of the second and third leaves (from base to top) of each plant per replication of each treatment (four replications, 16 plants, and 96 leaflets per experiment) were used to evaluate SR severity at 9, 11, 13, 15, and 17 days after inoculation (dai) according to the diagrammatic scale proposed by Franceschi et al. (2020). The area under disease progress curve (AUDPC) for each leaflet per leaf of each plant from the replications of each treatment was calculated using the trapezoidal integration of disease progress curves (Shaner and Finney 1977). At 17 dai, the second and third leaves of each plant per replication of each treatment were collected and scanned at 600 dpi resolution. The images were processed using the software QUANT (Fagundes-Nacarath et al. 2018) to obtain the values of final SR severity.

2.7 Determination of foliar concentrations of P, K, S, Cu, Zn, and Si

The leaflets of the second and third leaves (from base to top) of each plant per replication of each treatment (four replications, 8 plants, and 48 leaflets) were collected at 15 dai, washed in deionized water and dried in a drying oven with forced ventilation. The foliar concentrations of P, K, S, Cu, and Zn were determined by the method of nitric-perchloric digestion and inductively coupled plasma-optical emission spectrometry (ICP-OES). The foliar Si concentration was determined according to Korndörfer et al. (2004).

2.8 Processing leaf samples for scanning electron microscopy (SEM)

Leaf fragments (≈ 5 mm^2) were randomly obtained from the leaflets of the second and third leaves (from base to top) of each plant per replication of each treatment (four replications, 8 plants, and 48 leaflets) at 15 dai. The fragments were carefully transferred to glass vials containing 10 mL of fixative [3% (v/v) glutaraldehyde and 2% paraformaldehyde (v/v) in 0.1 M sodium cacodylate buffer (pH 7.2)] and storaged at 4°C for 10 days. The leaf fragments were washed with sodium cacodylate buffer (0.1 M), dehydrated in different ethanol concentrations, and subjected to critical point drying in CO_2 (model CPD 030) (Hatfield, PA, USA). Four specimens from each leaf fragment were mounted on aluminum stubs, sputter coated with gold (model FDU 010) (Hatfield, PA, USA) following examination in a LEO SEM model 1430VP (Jena, Thuringia, Germany) operating at 10 kV and working distance ranging from 10 to 20 mm to obtain the electron micrographs.

2.9 Imaging and quantification of Chl *a* fluorescence parameters

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 using the leaflets of the second leaf (from base to top) of each plant per replication of each treatment (five replications, 15 plants, and 45 leaflets) at 7, 11, and 15 dai. Leaflets of the second leaf from non-inoculated plants were also evaluated at these evaluation times. Plants were adapted to darkness for 30 min and then placed individually in support at a distance of 18.5 cm from the CCD ("charge-coupled device") camera to obtain images at 640 × 480 pixels resolution. The leaflets were exposed to a light pulse intensity of 0.5 μmol m^{-2} s^{-1}, 100 μs, 1 Hz to obtain the initial fluorescence (F_0). Next, 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 PS II photochemical efficiency of dark-adapted leaflets was estimated through the variable-to-maximum Chl *a* fluorescence ratio as follows: $F_v/F_m = [(F_m - F_0)/F_m)]$. Next, the leaflets were exposed to actinic photon irradiance (100 μmol m^{-2} s^{-1}) for 300 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 PS II 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) × PPFD × f × α according to Baker (2008). The parameters of Chl *a* fluorescence were determined on each leaflet (area of ≈ 0.5 cm^2) by selecting the circular option on the Imaging Win software.

2.10 Determining photosynthetic pigments concentration

Five leaf discs (1 cm^2 each) were obtained from the leaflets of the third leaves (from base to top) of each plant per replication of each treatment (five replications, 15 plants, and 45 leaflets) at 7, 11, and 15 dai. The discs were immersed in glass tubes containing 5 mL of saturated dimethyl sulfoxide solution and calcium carbonate (5 g/L), kept in the dark at room temperature for 24 h, and the absorbances of the extracts were read at 480, 649, and 665 nm to determine the concentrations of Chl *a*, Chl *b*, and carotenoids according to Picanço et al. (2021).

2.11 Histochemical detection of hydrogen peroxide (H_2O_2) and superoxide anion radical ($O_2^{\bullet-}$)

The leaflets of the second and third leaf (from base to top) of each plant per replication of each treatment (four replications, 4 plants, and 24 leaflets) were collected from both non-inoculated and inoculated plants at 15 dai. For H_2O_2 detection, five leaflets were randomly placed in each glass vial containing 25 mL of 3,3'-diaminobenzidine tetrahydrochloride solution (1 mg/mL) (Sigma-Aldrich, São Paulo, Brazil) and kept in the dark at 25ºC for 12 h. For $O_2^{\bullet-}$ detection, five leaflets were randomly placed in each glass vial containing 50 mL of nitro blue tetrazolium (0.1%) solution (Sigma-Aldrich, São Paulo, Brazil) prepared in potassium phosphate buffer (10 mM, pH 6.8) during 24 h. The leaflets were cleared in boiling aqueous ethanol (80%) for 80 min until brown and blue spots were noticed confirming, therefore, the presence of H_2O_2, and $O_2^{\bullet-}$, respectively.

2.12 Biochemical assays

The second and third leaves (from base to top) of each plant per replication of each treatment (six replications, 30 plants, and 180 leaves) were collected at 1, 3, 5, 10, and 15 dai from both non-inoculated and inoculated plants. Leaf samples were kept in liquid nitrogen during sampling and stored at -80ºC until further analysis.

2.12.1 Determining the activities of defense-related enzymes: leaf tissue (0.2 g) was ground into a fine powder with liquid nitrogen using a vibration ball mill (Retsch, Haan, Germany) and homogenized in 2 mL of a solution containing 50 mM of potassium phosphate buffer (pH 6.8), 0.1 mM of EDTA, 1 mM of phenylmethylsulfonyl fluoride, and 2% (w/v) of polyvinylpyrrolidone. The homogenized solution was centrifuged at 13,000 *g* for 15 min at 4°C and the supernatant was used to determine the activities of chitinase [CHI; Enzyme Commission (EC) number 3.2.1.14], *β*-1,3-glucanase (GLU; EC number 3.2.1.39), phenylalanine ammonia-lyase (PAL; EC number 4.3.1.5), peroxidase (POX; EC number 1.11.1.7), polyphenoloxidase (PPO; EC number 1.10.3.1), and lipoxygenase (LOX; EC number 1.13.11.12) according to Fortunato et al. (2015).

2.12.2 Malondialdehyde (MDA) concentration: leaf tissue (0.1 g) was ground into a fine powder as described above and homogenized in 2 mL of 0.1% (w/v) trichloroacetic acid (TCA) solution in an ice bath. The homogenate was centrifuged at 12,000 *g* for 15 min at 4ºC. After centrifugation, a total of 250 µL of the supernatant was reacted with 750 µl of 2-thiobarbituric

acid solution (0.5% in 20% TCA) for 60 min in a boiling water bath at 95°C. After this period, the reaction was stopped in an ice bath. The samples were centrifuged at 10,000 *g* for 10 min and the specific absorbance was determined at 532 nm. The non-specific absorbance was estimated at 600 nm and subtracted from the specific absorbance value. The extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer 1968) was used to calculate the MDA concentration.

2.12.3 Concentrations of total soluble phenols (TSP) and lignin-thioglycolic acid (LTGA) derivatives: leaf tissue (0.1 g) was ground into a fine powder as described above and homogenized in 1 mL of 80% (v/v) methanol solution. The crude extract was shaken at 300 rpm at 25°C for 2 h and the mixture was centrifuged at 17,000 *g* for 30 min. The TSP concentration was determined in the methanolic extract and the pellet was used to determine the LTGA derivatives concentration according to Tatagiba et al. (2014).

2.13 Data analysis

Data from urediniospores germination were subjected to analysis of variance (ANOVA) and treatment means were compared using Tukey's test ($P \leq 0.05$). For other variables and parameters, data were subjected to ANOVA and comparisons between control and IR stimulus treatments as well as between non-inoculated and inoculated plants were made using the *F* test ($P \leq 0.05$). Data were checked for normality and homogeneity of variance before ANOVA. The procedures described by Moore and Dixon (2015) were followed to combine the data from the variables and parameters evaluated from the repeated experiments. The Minitab Statistical software was used for the statistical analysis mentioned above (Minitab, Inc., 2024).

3. RESULTS

3.1 Analysis of variance

The effect of IR stimulus and control (water) treatments [named as products (P)] on urediniospores germination was analyzed by one-way ANOVA. The factor P was significant for urediniospores germination. The response of all variables and parameters for the factors P, plant inoculation (PI), and sampling time (ST) as well as for their interactions was analyzed by a two-way ANOVA. For most of the variables and parameters studied, the factors P, PI, and ST as well as the interactions P × PI, P × ST, PI × ST, and P × PI × ST were significant (Table 1).

3.2 Germination of urediniospores *in vit*ro

In comparison to control and IR stimulus (0.5 mL/L) treatments (Fig. 1A-B), the germinated urediniospores of *P. pachyrhizi* exposed to IR stimulus at 2 mL/L showed shorter germ tubes (Fig. 1C). The germination of *P. pachyrhizi* urediniospores was dramatically inhibited by the IR stimulus (5 and 10 mL/L) and fungicide (Fig. 1D, E, and F) compared to the control treatment (Fig. 1A). Urediniospores germination was significantly reduced by 57, 82, 94, and 100%, respectively, for 0.5, 2, 5, and 10 mL IR stimulus/L compared to control treatment (Fig. 1G). Significant reductions of 189, 216, and 230% occurred for 2, 5, and 10 mL IR stimulus/L compared to 0.5 mL IR stimulus/L (Fig. 1G).

3.3 Foliar concentrations of P, K, S, Cu, Zn, and Si

For non-inoculated plants, foliar concentrations of K, Cu, and Zn significantly increased by 13, 591, and 14%, respectively, for IR stimulus treatment compared to control treatment (Fig. 2A and C). For inoculated plants, the foliar concentrations of S, Cu, and Zn were significantly higher by 40, 490, and 13%, respectively, for IR stimulus treatment and the K concentration was significantly lower by 34% compared to the control treatment (Fig. 2B and D). For control treatment, only K concentration was significantly higher by 10% for inoculated plants compared to non-inoculated plants (Fig. 2A-B). For IR stimulus treatment, K concentration was significantly lower by 36% for inoculated plants, while S and Si concentrations were significantly higher by 37 and 22%, respectively, compared to non-inoculated plants (Fig 2A-B and E-F).

3.4 Rust symptoms, rust severity, AUDPC, and SEM observations

Necrotic lesions containing uredinia were abundant in the leaflets of plants from control treatment in contrast to the leaflets of IR stimulus-sprayed plants (Fig. 3A-B). Rust severity was significantly reduced by 59, 67, 59, 56, and 54% at 9, 11, 13, 15, and 17 dai, respectively, for IR stimulus-sprayed plants compared to plants from the control treatment (Fig. 3C). The AUDPC significantly decreased by 58% for IR stimulus-sprayed plants compared to plants from the control treatment (Fig. 3D). The uredinia formed in the leaflets of IR-stimulus sprayed plants were smaller and more compact than those observed on the leaflets of plants from the control treatment, which showed abundant production of urediniospores inside of them (Fig. 4A-B).

3.5 Concentration of MDA

For non-inoculated plants, there was no significant difference between control and IR stimulus treatments regardless of the evaluation time (Fig. 5A). For inoculated plants, MDA concentration was significantly lower by 30, 15, and 12% at 1, 3, and 15 dai, respectively, and significantly higher by 30% at 5 dai for IR stimulus treatment compared to control treatment (Fig 5B). For control treatment, MDA concentration was significantly higher by 70 and 31% at 1 and 15 dai, respectively, and significantly lower by 16 and 35% at 3 and 5 dai, respectively, for inoculated plants compared to non-inoculated plants (Fig. 5A-B). For IR stimulus treatment, MDA concentration was significantly lower by 20% at 3 dai for inoculated plants compared to non-inoculated ones (Fig. 5A-B).

3.6 Histochemical detection of H_2O_2 and $O_2^{\bullet-}$

The spray of IR stimulus did not cause any physiological perturbation to the leaflets of non-inoculated plants considering the absence of staining for H_2O_2 and $O_2^{\bullet-}$ compared to leaflets from plants of the control treatment (Fig. 6A-B). Depositions of H_2O_2 and $O_2^{\bullet-}$ (brown and blue colors, respectively) were less intense in the leaflets of IR stimulus-sprayed plants than on the leaflets from plants of the control treatment at 15 dai (Fig. 6A-B).

3.7 Imaging and quantification of Chl *a* fluorescence parameters

Based on the darker areas in the images corresponding to F_v/F_m, Y(II), Y(NPQ), and Y(NO) parameters, the photosynthetic apparatus from inoculated plants of the control treatment compared to inoculated plants sprayed with IR stimulus was slightly damaged (Fig. 7). For F_v/F_m, there was no significant difference between control and IR stimulus treatments

regardless of plant inoculation and evaluation time (Fig 8A-B). For non-inoculated plants, values for Y(II) (17, 15, and 6% at 7, 11, and 15 dai), Y(NPQ) (40% at 7 dai), and ETR (14, 14, and 6% at 7, 11, and 15 dai) were significantly higher for IR stimulus treatment compared to control treatment (Fig. 8C, E, and I). Significant decreases for Y(NO) of 14 and 10% at 7 and 11 dai, respectively, occurred for non-inoculated and IR stimulus-sprayed plants compared to non-inoculated plants from the control treatment (Fig. 8G). For inoculated plants, values of Y(II) and ETR significantly increased by 17 and 12% and by 17 and 13% at 7 and 11 dai, respectively, for IR stimulus treatment compared to control treatment (Fig. 8D and J). For Y(NPQ) and Y(NO), significant decreases of 2 and 11% at 11 and 7 dai, respectively, occurred for inoculated and IR stimulus-sprayed plants compared to inoculated plants from the control treatment (Fig. 8F and H).

For the control treatment, there were significant increases for Y(II) (7 and 6% at 7 and 11 dai), Y(NPQ) (6 and 9% at 7 and 11 dai), and ETR (5% at 11 dai) for inoculated plants compared to non-inoculated plants (Fig. 8C, D, E, F, I, and J). For control treatment, Y(NO) significantly decreased by 10% at 11 dai for inoculated plants compared to non-inoculated plants (Fig. 8G-H). For IR stimulus treatment, significant increase of 7% at 7 dai for both Y(II) and ETR occurred for inoculated plants compared to non-inoculated plants from the IR stimulus treatment (Fig. 8C-D and I-J). The Y(NPQ) was significantly lower by 18% at 7 dai for inoculated plants compared to non-inoculated plants from the IR stimulus treatment (Fig. 8E-F).

3.8 Concentration of photosynthetic pigments

For non-inoculated plants, Chl *a*+*b* concentration was significantly higher by 34, 7, and 14% at 7, 11, and 15 dai, respectively, for IR stimulus treatment compared to control treatment (Fig. 9A). Carotenoids concentration was significantly higher by 43, 10, and 16% at 7, 11, and 15 dai, respectively, for IR stimulus treatment compared to control treatment (Fig. 9C). For inoculated plants, Chl *a*+*b* concentration significantly increased by 22 and 9% at 7 and 11 dai, respectively, for IR stimulus treatment compared to control treatment (Fig. 9B). The concentration of carotenoids was significantly higher by 21 and 5% at 7 and 11 dai, respectively, for IR stimulus treatment compared to control treatment (Fig. 9D).

Significant increases of 16 and 5% for Chl *a*+*b* concentration as well as of 15 and 11% for carotenoids concentration at 7 and 11 dai, respectively, occurred for inoculated plants compared to non-inoculated plants from the control treatment (Fig 9A-D). For IR stimulus treatment, there was a significant increase of 7% for Chl *a*+*b* concentration and of 8% for

carotenoids concentration both at 11 dai as well as a significant reduction of 10% in carotenoids concentration at 15 dai for inoculated plants compared to non-inoculated plants (Fig. A-D).

3.9 Activities of defense-related enzymes

For non-inoculated plants, CHI (21% at 10 dai), PAL (60, 61, and 16% at 1, 5, and 15 dai, respectively), POX (26% at 3 dai), PPO (15, 54, and 22% at 1, 3, and 15 dai, respectively), and LOX (51% at 3 dai) activities were significantly lower while GLU (12 and 9% at 5 and 10 dai, respectively), PAL (26% at 10 dai), POX (32, 174, and 12% at 1, 5, and 15 dai, respectively), PPO (32 and 20% at 5 and 10 dai, respectively), and LOX (61% at 10 dai) activities significantly increased for IR stimulus treatment compared to control treatment (Fig. 10A, C, E, G, I, and K).

For inoculated plants, CHI (62% at 10 dai), PAL (84 and 76% at 1 and 5 dai, respectively), POX (31, 35, 252, and 53% at 1, 3, 5, and 15 dai, respectively), PPO (19% at 10 dai), and LOX (48 and 182% at 3 and 5 dai, respectively) activities were significantly higher while CHI (23% at both 3 and 15 dai, respectively), GLU (17% at 5 dai), PAL (55 and 15% at 3 and 15 dai, respectively), POX (52% at 10 dai), and PPO (34% at 3 dai) activities were significantly lower for IR stimulus treatment compared to control treatment (Fig. 10B, D, F, H, J, and L).

For control treatment, CHI (36 and 52% at 3 and 5 dai, respectively), GLU (13% at 5 dai), POX (182 and 11% at 10 and 15 dai, respectively), and PPO (20% at 10 dai) activities were significantly higher while CHI (37% at 10 dai), PAL (36, 51, and 8% at 1, 5, and 10 dai, respectively), PPO (14% at 15 dai), and LOX (31 and 67% at 3 and 5 dai, respectively) activities were significantly lower for inoculated plants compared to non-inoculated ones (Fig. 10A-L).

For IR stimulus treatment, CHI (25 and 30% at 5 and 10 dai, respectively), GLU (26% at 3 dai), PAL (190 and 123% at 1 and 5 dai, respectively), POX (69, 29, and 52% at 3, 5, and 15 dai, respectively), PPO (37 and 8% at 3 and 15 dai, respectively), and LOX (108% at 3 dai) activities were significantly higher while GLU (16% at 5 dai), PAL (40, 26, and 11% at 3, 10, and 15 dai, respectively), PPO (15% at 5 dai), and LOX (26% at 10 dai) activities were significantly lower for inoculated plants compared to non-inoculated plants (Fig. 10A-L).

3.10 Concentrations of TSP and LTGA derivatives

For non-inoculated plants, TSP concentration was significantly lower by 23 and 19% at 5 and 15 dai, respectively, for IR stimulus treatment compared to control treatment (Fig 11A). The concentration of LTGA derivatives was significantly higher by 35, 60, 51, and 46% at 1, 3, 5, and 10 dai, respectively, for IR stimulus treatment compared to control treatment (Fig.

11C). For inoculated plants, concentration of LTGA derivatives was significantly higher by 17, 49, and 54% at 3, 10, and 15 dai, respectively, for IR stimulus treatment compared to control treatment (Fig. 11D).

For control treatment, TSP concentration was significantly lower by 27% at 5 dai for inoculated plants compared to non-inoculated ones (Fig 11A-B). The concentration of LTGA derivatives was significantly higher by 50, 81, 20, and 30% at 1, 3, 5, and 10 dai, respectively, and significantly lower by 28% at 15 dai for inoculated plants compared to non-inoculated ones (Fig. 11C and D). For IR stimulus treatment, TSP concentration was significantly higher by 32% at 10 dai for inoculated plants compared to non-inoculated ones (Fig. 11A-B). The concentration of LTGA derivatives was significantly higher by 20, 33, and 32% at 1, 3, and 10 dai, respectively, for inoculated plants compared to non-inoculated plants (Fig. 11C-D).

4. DISCUSSION

Ensuring optimal plant nutrition (equilibrium among nutrients in the soil solution for their prompt uptake by the root system of plants following the right balance in the tissues of different organs) is of pivotal importance for the resilience of profitable crops such as soybean to the different types of stress. Interestingly, nutrients will ensure adequate plant growth and development, providing a competitive advantage against infection by pathogens of different lifestyles due to earlier, stronger, and steady defense reactions (Rengel et al. 2022; Tubana and Cruz 2023). In line with these aspects, the present study brings physiological and biochemical pieces of evidence to explain the increased resistance of soybean plants exposed to the IR stimulus against infection by *P. pachyrhizi*. In this scenario, reduction of SR symptoms and, consequently, less leaf tissues colonization by fungal hyphae (less developed uredinia in the necrotic lesions) were biochemically supported by the lower pool of MDA, H_2O_2, and $O_2^{\bullet-}$ indicating, therefore, less cellular oxidative stress. On top of that, the *in vitro* aspect of germinated urediniospores from *P. pachyrhizi* was profoundly affected by the IR stimulus. It is tempting to assume that the foliar spray of this IR stimulus may affect the germination of *P. pachyrhizi* urediniospores deposited over the soybean leaves resulting in lower disease severity due to a reduction in the number of infection sites (necrotic lesions) established per unit of leaf area. A large body of evidence has shown a direct antifungal activity of different types of IR stimuli (e.g., azelaic acid, acibenzolar-S-methyl, hexanoic acid, compounds containing Ca, and a copper-polyphenolic compound) against pathogens *in vitro* (Paula et al. 2021; Rodrigues et al. 2023a; 2023b). Besides displaying an antifungal activity during the pathogen infection process, the IR stimuli provide soybean plants with more efficient strategies to defend themselves against infection by *P. pachyrhizi* (Langenbach et al. 2016; Siah et al. 2018; Paula et al. 2021; Rodrigues et al. 2023a; 2023b).

The use of commercial products containing different forms and concentrations of nutrients for growing plants in substrates (e.g., peat moss, soil-based mixes, and rockwool) or foliar spray will enhance the content of nutrients in the root system or at different organs of the plant (Fernández and Brown 2013; Cruz et al. 2020; Einhardt et al. 2020a; Picanço et al. 2021; Hawerroth et al. 2023; Otolakoski et al. 2023;). In the present study, the spray of IR stimulus significantly enhanced the foliar concentrations of Cu, Zn, S, and Si for plants infected by *P. pachyrhizi*. It is plausible to postulate that soybean plants benefited from the increased availability of these nutrients in leaf tissues to mount robust defense reactions against *P. pachyrhizi* infection considering their intrinsic role in different aspects of plant metabolism

(Rengel et al. 2022). For instance, Zn is needed as a co-factor for a diverse range of enzymes, including metalloenzymes and zinc finger proteins, besides being involved in photosynthesis, auxin production, membrane integrity, inhibitor of ROS by generating NADPH oxidase and favoring superoxide dismutase activity, and increasing the resistance of some plant species against pathogens (Cabot et al. 2019; Kalia et al. 2020; Tubana and Cruz 2023). The S is involved in the synthesis of some amino acids, such as cysteine and methionine, that are integral components of many enzymes, as well as in the redox reactions as part of coenzymes such as coenzyme A and glutathione (Narayan et al. 2023; Tubana and Cruz 2023). Moreover, S is also a key component of various secondary metabolites (e.g., glucosinolates and phytoalexins) involved in host defense reactions (Künstler et al. 2020; Kaur et al. 2022). The role played by Si in reducing the intensities of many diseases, including soybean rust in soybean, and in the potentiation of an array of defense reactions is indisputable (Debona et al. 2023).

Growth, development, and productivity are dramatically affected due to impairments in the photosynthetic capacity of plants infected by pathogens (Selvaraj and Fofana 2012; Sowden et al. 2018). Disruption of chloroplast structure and function by non-host selective toxins and other pathogens-related metabolites, as well as ROS generated during the oxidative stress, reduction in the pool of photosynthetic pigments, damage to the photosynthetic machinery (e.g., profound changes in the light-dependent reactions of photosynthesis that includes the electron transport chain and ATP synthesis), alteration of metabolic pathways, and redistribution of resources, operate collectively to increase the susceptibility of plants against pathogens of different lifestyles (Selvaraj and Fofana 2012; Debona et al. 2014; Rios et al. 2014; Silveira et al. 2015; Zabala et al. 2015; Lu and Yao 2018; Dias et al. 2020; Sterling and Melgarejo 2021). During the infection process of *P. pachyrhizi* on soybean leaves, changes in leaf gas exchange (lower A, g_s, C_i, and E) and Chl *a* fluorescence [lower F_v/F_m, Y(II), and Y(NPQ) followed by increases in Y(NO)] parameters along with reduced Chl $a+b$ and carotenoid concentrations indicate impairments on photosynthesis (Rios et al. 2018). In the present study, the IR stimulus-sprayed plants displayed better photosynthetic performance during the infection process of *P. pachyrhizi*. It is noteworthy in this context that IR stimulus-sprayed plants suffered less photodamage as indicated by higher Y(II) values and reductions in Y(NPQ) and Y(NO). Notably, heat dissipation to mitigate any level of damage occurring at the PSII level was minimal based on higher and lower values for ETR and Y(NPQ), respectively. Based on ETR values, it is plausible to postulate that a balance between the electron flow and CO_2 assimilation during photosynthesis occurred for diseased leaves of IR stimulus-sprayed plants that favored the preservation of the photosynthetic apparatus. Surprisingly, the F_v/F_m values were similar

between plants from control and IR stimulus treatments regardless of *P. pachyrhizi* infection, indicating that the damage to the reaction centers associated with the photosystems was less perceptible. As a result of reduced disease symptoms, the pool of photosynthetic pigments (Chl *a+b* and carotenoids at 7 and 11 dai) was preserved in the leaves of IR stimulus-sprayed plants. Both Chl *a+b* and carotenoids are involved in light absorption and the successive energy transport to the centers of reaction where the photochemical phase of photosynthesis takes place (Taiz and Zeiger 2006). Interestingly, Chl *a+b* and carotenoid concentrations were higher for IR-stimulus plants from 7 to 15 dai in contrast to plants from the control treatment that were non-inoculated with *P. pachyrhizi*. Considering the importance of photosynthesis as a source of energy for plants to mount their different strategies of defense against foliar pathogens, the stress they impose on this pivotal physiological process, including *P. pachyrhizi* on soybean leaves, can be alleviated by different IR stimuli (Rios et al. 2014; Fagundes-Nacarath et al. 2018; Aucique-Pérez et al. 2019; Dias et al. 2020; Einhardt et al. 2020b; Picanço et al. 2022).

During the interactions of pathogens with their hosts, a series of either structural (e.g., callose deposition, papillae formation, and cell wall strengthening) and or biochemical [e.g., production of antimicrobial compounds (phenolics, flavonoids, and phytoalexins) and expression of genes coding for different pathogenesis-related proteins (glucanases, chitinases, phenylalanine ammonia-lyase, peroxidase, polyphenoloxidase, and lipoxygenase)] defense reactions take place to hamper their infection process (Durrant and Dong 2004; Sharma et al. 2011; Kaur et al. 2022). The PAL is pivotal in the phenylpropanoid pathway of plants facing infection by pathogens of different lifestyles (Kumar et al. 2020). In this scenario, great PAL activity at the infection sites of pathogens will result in a great diversity of phenolics being the phenolic monomers (e.g., coniferyl alcohol, sinapyl alcohol, and *p*-coumaryl alcohol) undergoing polymerization to form lignin in the plant cell wall in an attempt to restrict pathogen colonization of host tissues (Fortunato et al. 2015; Hossain et al. 2018; Kumar et al. 2020; Kaur et al. 2022). Earlier increases in PAL activity (3, 5, and 10 dai) in infected leaflet tissues of IR stimulus-sprayed plants was a great piece of biochemical evidence for the potentiation of soybean resistance. In the present study, plants sprayed with IR stimulus and infected by *P. pachyrhizi* showed higher POX (from 1 to 15 dai) and PPO (at 10 dai) activities, which can be directly linked to the increased concentration of LTGA derivatives (at 3, 10, and 15 dai) originated from the pool of phenolics. Higher foliar Cu concentration in leaflets of IR-stimulus sprayed plants may have contributed to great POX and PPO activities. The Cu is an important co-factor of POX and PPO by directly interacting with their active site or indirectly by stabilizing their structure or facilitating substrate binding (Tubana and Cruz 2023).

Interestingly, POX has an important role in the scavenging of ROS in plant tissues infected by pathogens (Kumar et al. 2020). The polymerization of phenolics towards lignin production as well as more toxic compounds (e.g., quinones) are greatly dependent on higher POX and PPO activities (Lattanzio et al. 2006; Gajewska and Sklodowska 2007) and these enzymes were very important for the resistance of soybean plants against infection by *P. pachyrhizi* after being exposed to different IR stimuli (Paula et al. 2021; Picanço et al. 2021, 2022; Rodrigues et al. 2023a, 2023b). Bedin et al. (2020) reported a reduction in soybean rust severity on plants sprayed with Cu (rates ranging from 30 to 120 g/ha using cuprous oxide and copper carbonate as Cu source) linked to increased lignin production. Surprisingly, Gabardo et al. (2020) did not obtain reduction in rust severity on soybean plants sprayed with different commercial products that contained calcium, macronutrients (N +K) or a mixture of micronutrients (Cu + Mn + Zn or Mn + Zn + Mo).

The colonization of plant tissues by fungi is restricted when chitin and β-1,3-glucan are greatly hydrolyzed, respectively, by an abundant pool of CHI and GLU at the infection sites (Shetty et al. 2009). Although CHI and GLU are the most important pathogenesis-related proteins in plants exposed to IR stimuli (Durrant and Dong 2004), in the present study, their level of activities in infected leaves of IR stimulus-sprayed plants was not directly involved with the reduction of SR symptoms. The oxidative action of LOX against the polyunsaturated fatty acids found in the cell membrane originates oxylipins from which jasmonic acid is formed to initiate the induced systemic resistance pathway (Vlot et al. 2021; Kaur et al. 2022; Porta and Rocha-Sosa 2002). The IR stimulus-sprayed plants showed higher LOX activity at earlier stages of *P. pachyrhizi* infection (at 3 and 5 dai). According to Rodrigues et al. (2023a), the expression of *LOX* was higher on leaflets of soybean plants sprayed with a copper-polyphenolic compound in response to *P. pachyrhizi* infection at 5 dai.

Considering the physiological and biochemical outcomes, assuming a possible biostimulant effect of the IR stimulus on soybean plants non-infected by *P. pachyrhizi* is tempting. In this scenario, these plants displayed increased foliar concentrations of K, Zn, and Cu, better photosynthetic performance [great values for Y(II) and ETR and lower values for Y(NO)] associated with greater pool of Chl *a*+*b* and carotenoids, increased activities of enzymes related indirectly or directly to host defense (GLU at 5 and 10 dai; PAL at 10 dai; POX at 1, 5, and 15 dai, and LOX at 10 dai) as well as more production of lignin (at 1, 3, 5, and 10 dai). Facing future infections by *P. pachyrhizi*, soybean plants were primed for an earlier and stronger activation of defense response that will hamper the fungal infection process with great efficiency. Growth stimulation and higher yields for profitable crops can be achieved by

either spraying plants with different types of biostimulants (especially those ranked in the category of products placed between fertilizers and plant growth regulators) or by adding them to the rhizosphere (Bulgari et al. 2015). In this case, the efficiency of nutrient uptake by plants will be increased along with their great tolerance against abiotic stresses due to positive modulation of both primary and secondary metabolisms favoring the photosynthetic apparatus and activating specific biochemical pathways, resulting, therefore, in products of better quality for human consumption (Bulgari et al. 2015; Franzoni et al. 2022).

The outcomes of the variables and parameters evaluated in the present study clearly indicate the efficiency of the IR stimulus to provide the soybean plants with a more defense capacity against infection by *P. pachyrhizi*. The reduced SR symptoms gained by spraying the plants with the IR stimulus was a collective contribution of a better nutritional status (higher foliar concentrations of Cu, Zn, S, and Si), less cellular damage, and the interplay of PAL, POX, and PPO activities for more lignin production. Taken together, these findings open the way to explore the possibility of using this IR stimulus in field conditions to reduce the yield losses caused by SR outbreaks and, at the same time, reduce the use of fungicides that, besides increasing production costs, impose serious risks to both environment and human health.

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TABLE AND FIGURES

Table 1. Analysis of variance for the effects of products (P), plant inoculation (PI), sampling time (ST), and the interactions P × PI, P × ST, PI × ST, and P × PI × ST for urediniospores germination (UG), soybean rust severity (Sev), area under disease progress curve (AUDPC), foliar concentrations of silicon (Si) and nutrients [phosphorus (P), potassium (K), sulfur (S), copper (Cu), zinc (Zn)], chlorophyll *a* fluorescence parameters [variable-to-maximum chlorophyll *a* fluorescence ratio (F_v/F_m), photochemical yield (Y(II)), yield for dissipation by down-regulation (Y(NPQ), yield for non-regulated dissipation (Y(NO), and electron transport rate (ETR)], concentration of photosynthetic pigments [chlorophyll *a*+*b* (Chl *a*+*b*) and carotenoids (Car)], activities of defense-related enzymes [chitinase (CHI), *β*-1,3-glucanase (GLU), phenylalanine ammonia-lyase (PAL), polyphenoloxidase (PPO), peroxidase (POX), and lipoxygenase (LOX)], and metabolites [malondialdehyde (MDA), total soluble phenolics (TSP), and lignin-thioglycolic acid (LTGA) derivatives].

Variables/Parameters		**P**	**PI**	**ST**	**P × PI**	**P × ST**	**PI × ST**	**P × PI × ST**
In vitro assay	UG	**< 0.001**	-	-	-	-	-	-
Soybean rust	Sev	**< 0.001**	-	**< 0.001**	-	**< 0.001**	-	-
	AUDPC	**< 0.001**	-	**< 0.001**	-	**< 0.001**	-	-
Foliar Si concentration	Si	0.589	0.051	**-**	0.094	**-**	-	-
Foliar concentrations of nutrients	P	**< 0.001**	**< 0.001**	**-**	0.094	**-**	-	-
	K	**< 0.001**	**< 0.001**	**-**	**< 0.001**	**-**	-	-
	S	0.286	0.415	**-**	**< 0.001**	**-**	-	-
	Cu	**< 0.001**	0.515	**-**	0.509	**-**	-	-
	Zn	0.003	0.429	**-**	0.938	**-**	-	-
Parameters of chlorophyll *a* fluorescence	F_v/F_m	0.003	0.093	**< 0.001**	0.401	0.588	0.764	0.354
	Y(II)	**< 0.001**	**< 0.001**	**< 0.001**	0.275	**< 0.001**	**< 0.001**	0.425
	Y(NPQ)	**< 0.001**	0.554	**< 0.001**	0.027	**< 0.001**	**< 0.001**	**< 0.001**
	Y(NO)	**< 0.001**	0.054	**< 0.001**	0.017	**< 0.001**	0.045	0.343

	ETR	**< 0.001**	0.008	**< 0.001**	0.858	**< 0.001**	0.024	0.236
Photosynthetic pigments	Chl *a*+*b*	**< 0.001**	**< 0.001**	**< 0.001**	0.638	**< 0.001**	0.037	0.531
	Car	**< 0.001**	**< 0.001**	**< 0.001**	0.891	**< 0.001**	0.012	0.512
Defense-related enzymes	CHI	0.034	0.003	**< 0.001**	0.044	0.139	**< 0.001**	**< 0.001**
	GLU	0.034	0.088	**< 0.001**	0.483	0.201	**< 0.001**	**< 0.001**
	POX	**< 0.001**	**< 0.001**	**< 0.001**	0.037	**< 0.001**	**< 0.001**	**< 0.001**
	PAL	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
	PPO	**< 0.001**	0.376	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
	LOX	0.010	0.033	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
Metabolites	MDA	0.196	0.121	**< 0.001**	0.083	**< 0.001**	**< 0.001**	**< 0.001**
	TSP	0.076	0.796	**< 0.001**	0.021	0.312	0.002	0.254
	LTGA derivatives	**< 0.001**	**< 0.001**	**< 0.001**	0.968	0.444	**< 0.001**	**< 0.001**

Bold values are significant at $P \leq 0.05$.

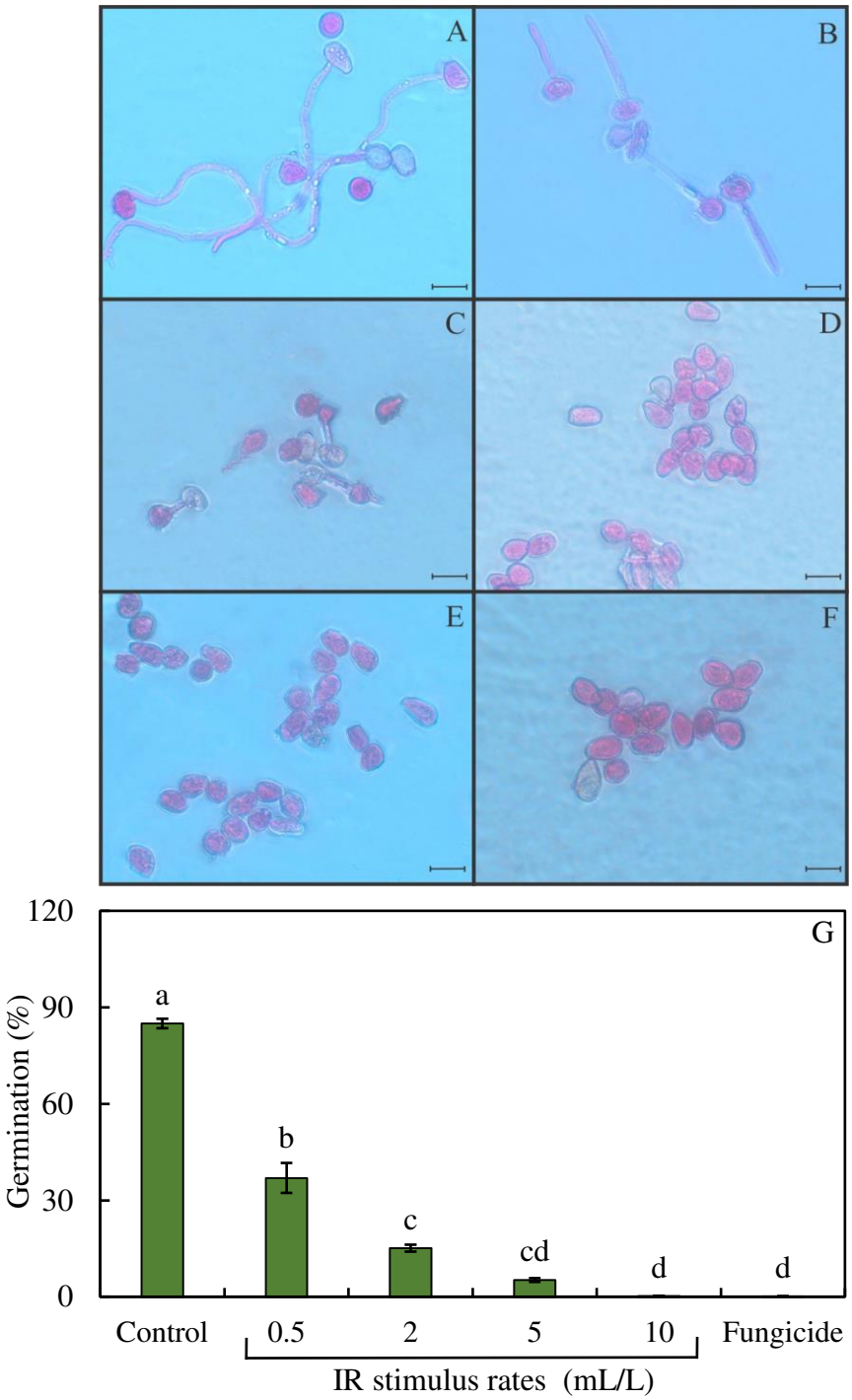

Figure 1. Aspects of urediniospores germination from *Phakopsora pachyrhizi* in glass slides containing different rates of induced resistance (IR) stimulus (0.5, 2, 5, and 10 mL/L, respectively, to B, C, D, and E). Control treatments corresponded to urediniospores suspension which were not exposed to either IR stimulus (A) or fungicide (F). Scale bars = 10 μm. Percentage of urediniospores germination as affected by different treatments. Treatment means followed by different letters are significantly different ($P \leq 0.05$) according to Tukey's test (graph G). Bars represent the standard error of the means.

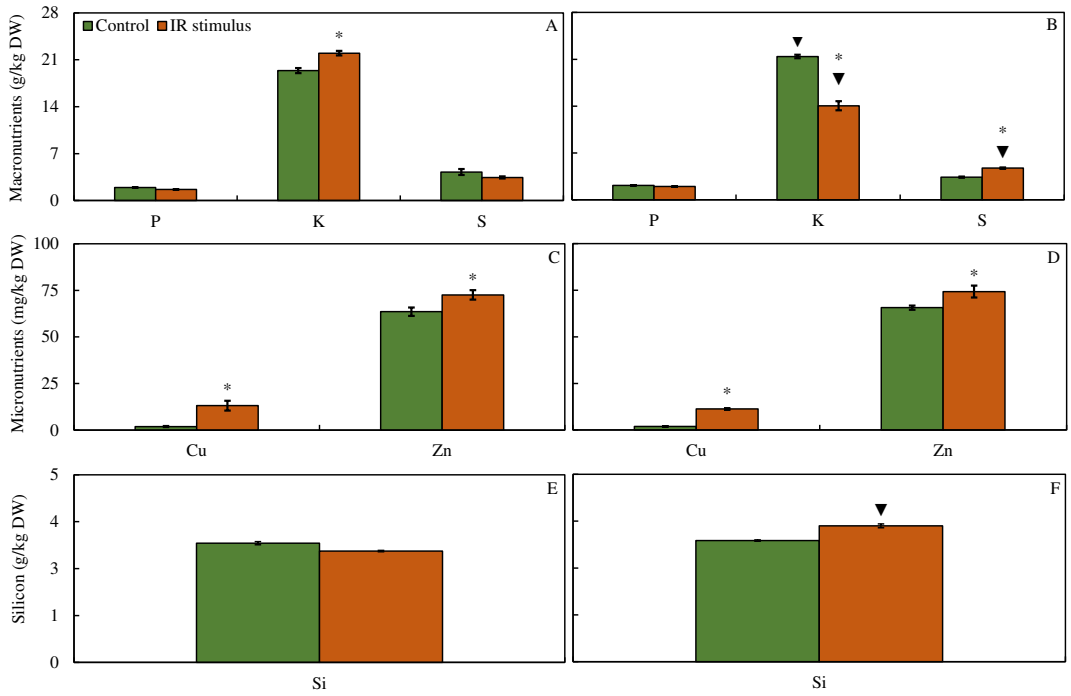

Figure 2. Foliar concentrations of phosphorus (P) (A and B), potassium (K) (A and B), sulfur (S) (A and B), copper (Cu) (C and D), zinc (Zn) (C and D), and silicon (E and F) for soybean plants sprayed with either water (control) or induced resistance (IR) stimulus and non-inoculated (NI) (A, C, and E) or inoculated (I) (B, D, and F) with *Phakopsora pachyrhizi*. For each nutrient and Si, means for control and IR stimulus treatments followed by an asterisk (*) and for NI and I plants followed by an inverted triangle (▼) are significantly different ($P \leq 0.05$) according to *F* test. Bars represent the standard error of the means. DW = dried weight.

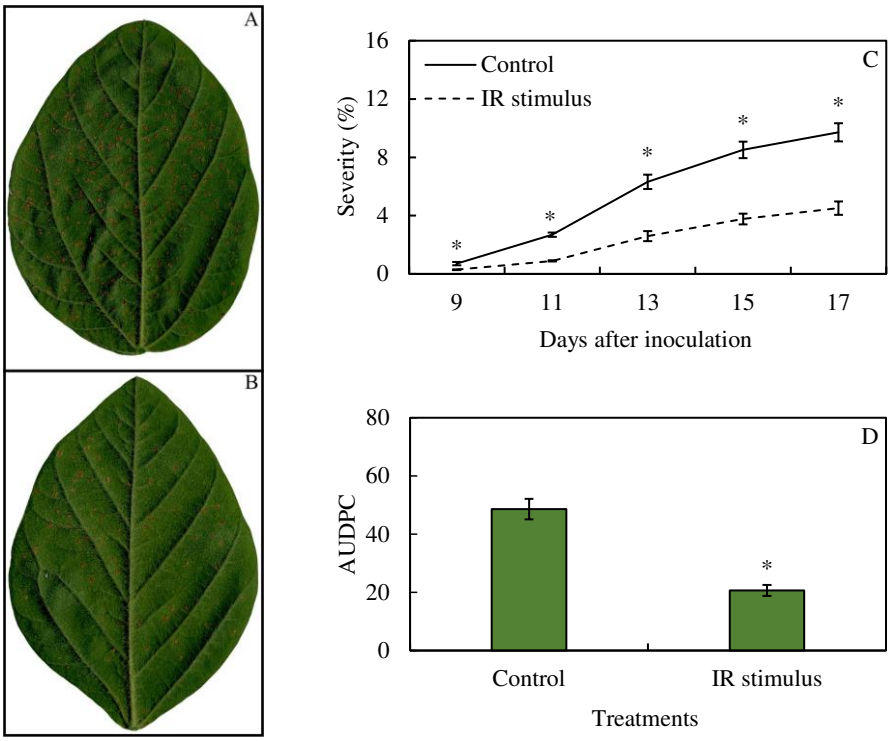

Figure 3. Rust symptoms (chlorosis and necrosis) and sporulation of *Phakopsora pachyrhizi* (A and B), severity of soybean rust (C), and area under disease progress curve (AUDPC) (D) for soybean plants sprayed with either water (control) or induced resistance (IR) stimulus. Means for control and IR stimulus treatments followed by an asterisk (*) (C), at each evaluation time, or between these treatments followed by * for AUDPC (D) are significantly different ($P \leq 0.05$) according to F test. Bars represent the standard error of the means.

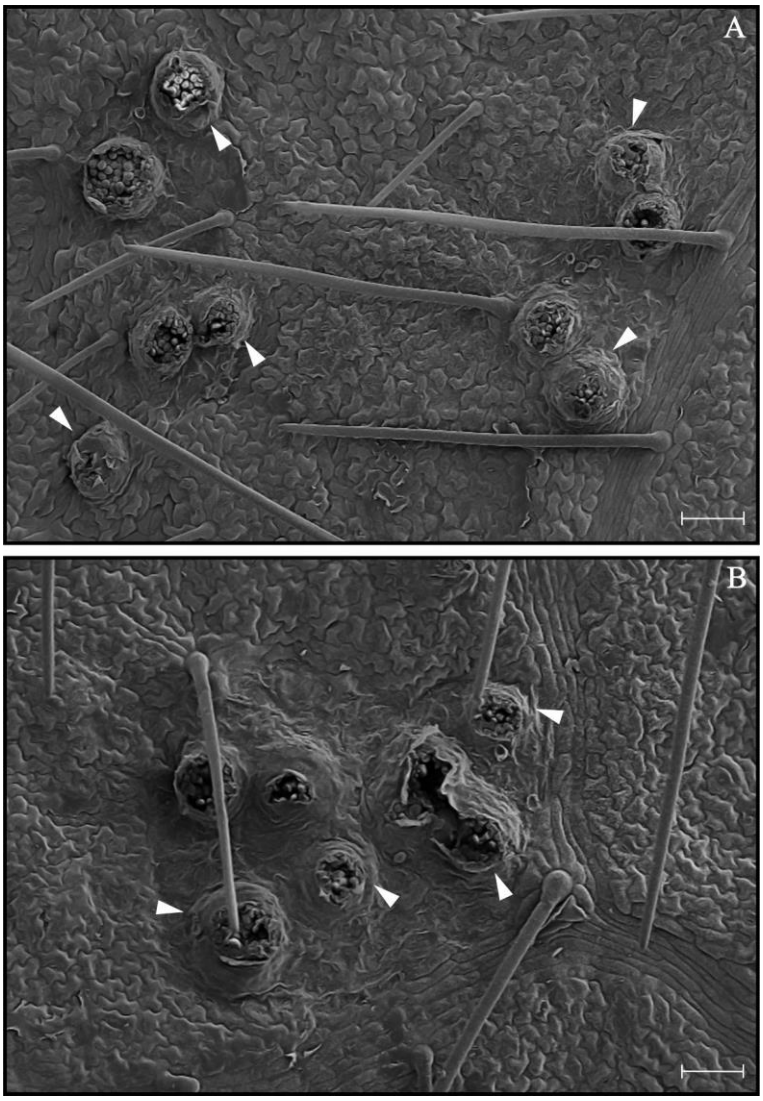

Figure 4. Scanning electron micrographs of the abaxial surface of leaflets obtained from soybean plants that were previously sprayed with either water (control) (A) or induced resistance (IR) stimulus (B) and infected by *Phakopsora pachyrhizi*. Uredia are indicated by arrowheads. The leaflets were collected at 15 days after plant inoculation with *P. pachyrhizi*. Scale bars = 200 μm.

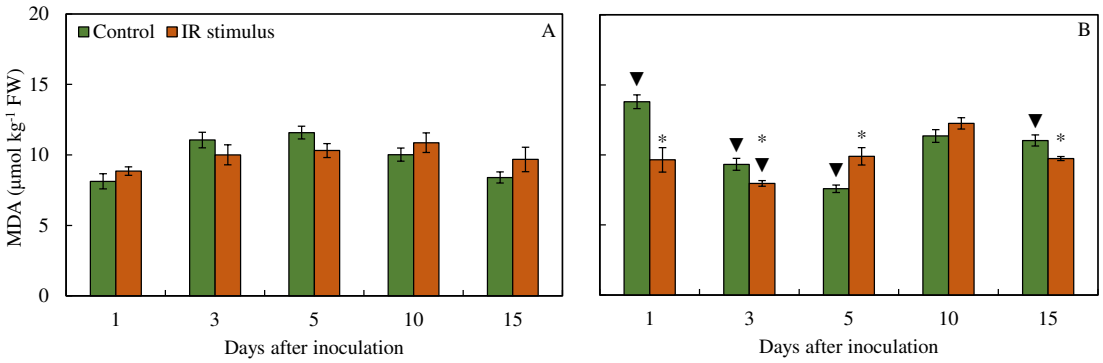

Figure 5. Concentration of malondialdehyde (MDA) determined on the leaflets of soybean plants non-inoculated (A) or inoculated (B) with *Phakopsora pachyrhizi* and sprayed with either water (control) or induced resistance (IR) stimulus. Means for control and IR stimulus treatments followed by an asterisk (*) and for NI and I plants followed by an inverted triangle (▼), at each sampling time, are significantly different according to *F* test ($P \leq 0.05$). Bars represent the standard deviation of the means. FW = fresh weight.

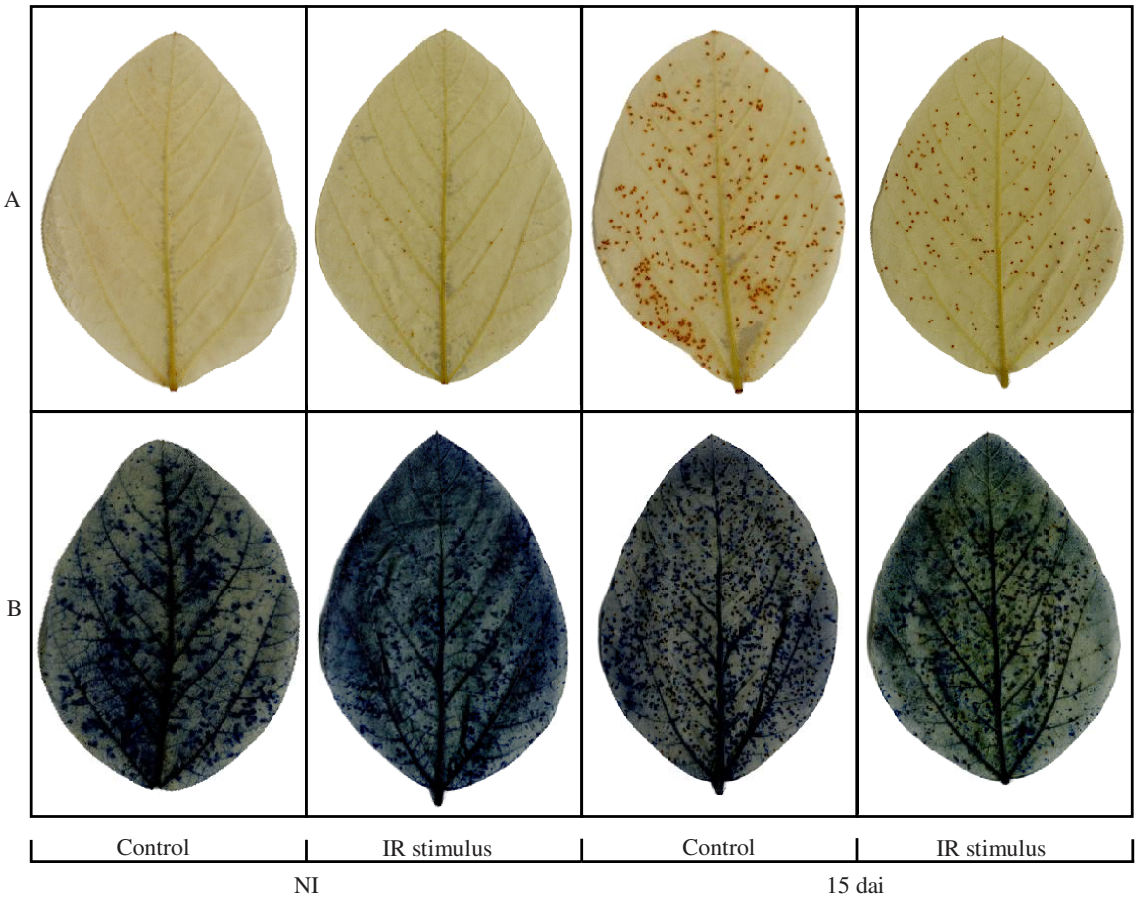

Figure 6. Histochemical detection of hydrogen peroxide (A) and superoxide anion radical (B) on the leaflets of soybean plants that were sprayed with either water (control) or induced resistance (IR) stimulus and non-inoculated (NI) or at 15 days after inoculation (dai) with *Phakopsora pachyrhizi*.

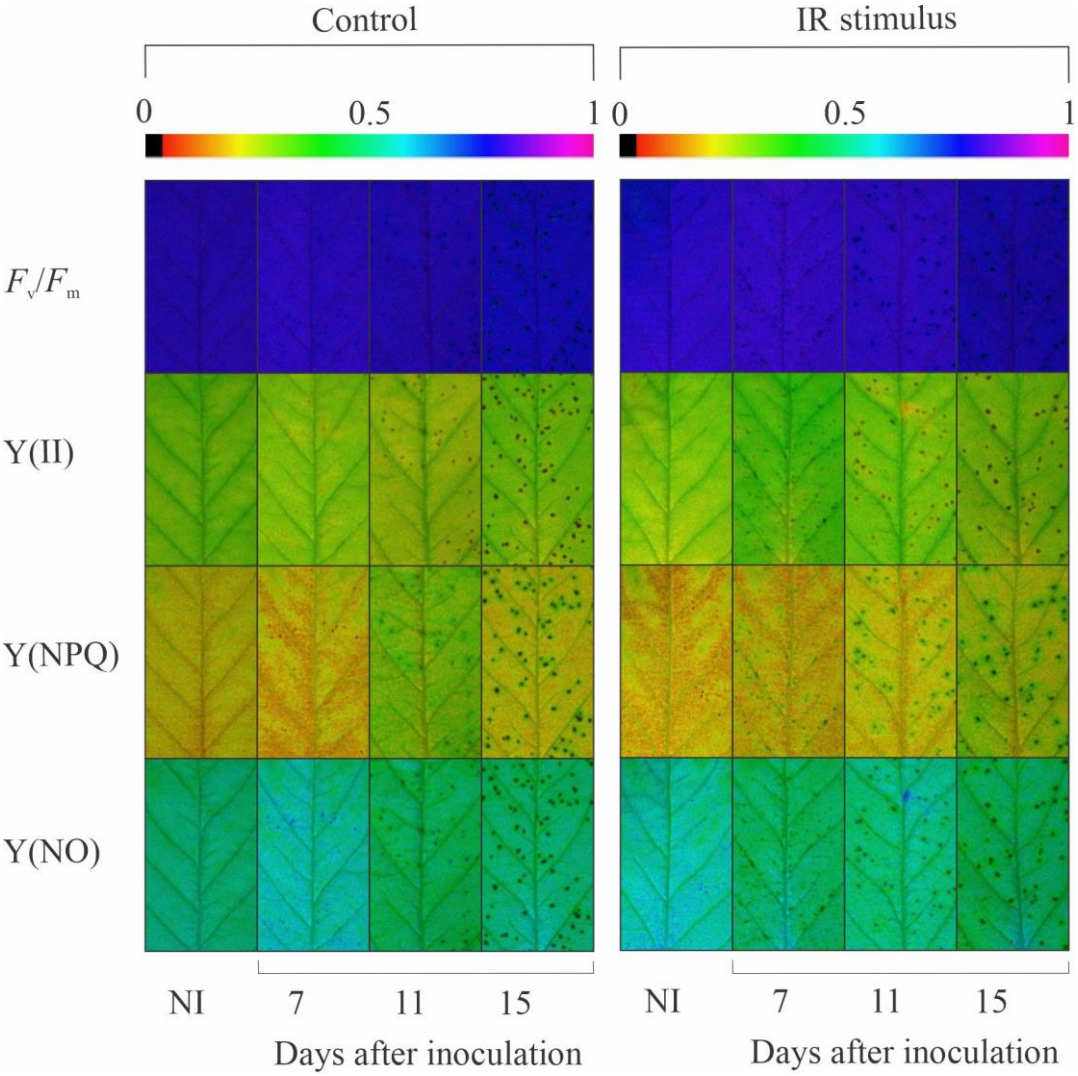

Figure 7. Images of chlorophyll *a* fluorescence parameters maximum PSII quantum efficiency (F_v/F_m), photochemical yield [Y(II)], yield for dissipation by down-regulation [Y(NPQ)], and yield for non-regulated dissipation [Y(NO)] obtained from leaflets of soybean plants sprayed with either water (control) or induced resistance (IR) stimulus and non-inoculated (NI) or at different time-points after inoculation with *Phakopsora pachyrhizi*.

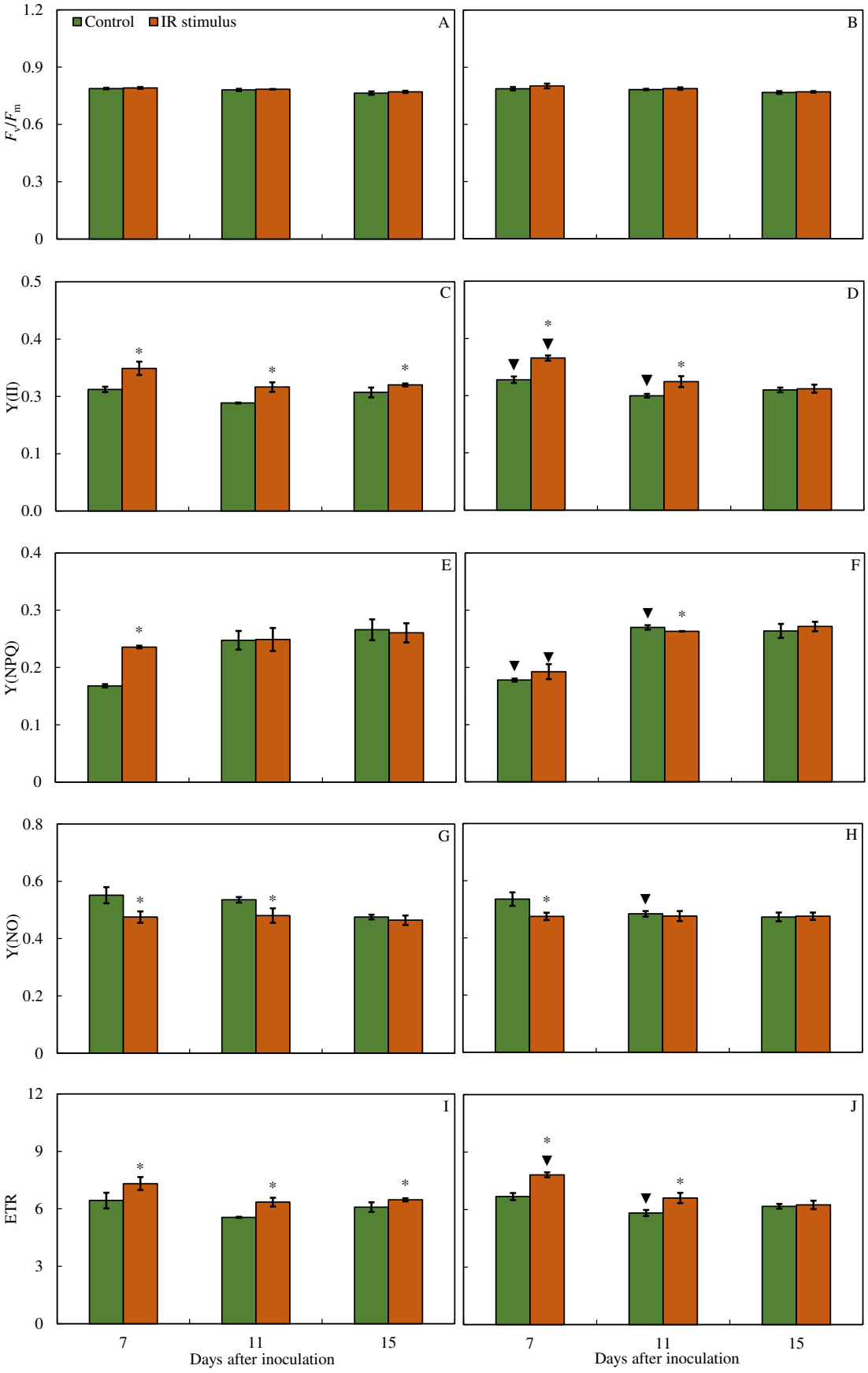

Figure 8. Quantification of chlorophyll *a* fluorescence parameters maximum PSII

quantum efficiency (F_v/F_m) (A and B), photochemical yield [Y(II)] (B and C), yield for dissipation by down-regulation [Y(NPQ)] (D and E), yield for non-regulated dissipation [Y(NO)] (G and H), and electron transport rate (ERT) (I and J) 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 *Phakopsora pachyrhizi* and sprayed with either water (control) or induced resistance (IR) stimulus. Means for control and IR stimulus treatments followed by an asterisk (*) and means for NI and I plants followed by an inverted triangle (▼), at each evaluation time, are significantly different ($P \leq 0.05$) according to *F* test. Bars represent the standard error of the means. The parameters were quantified on the images of leaflets obtained from non-inoculated or inoculated plants with *P. pachyrhizi* at 15 days.

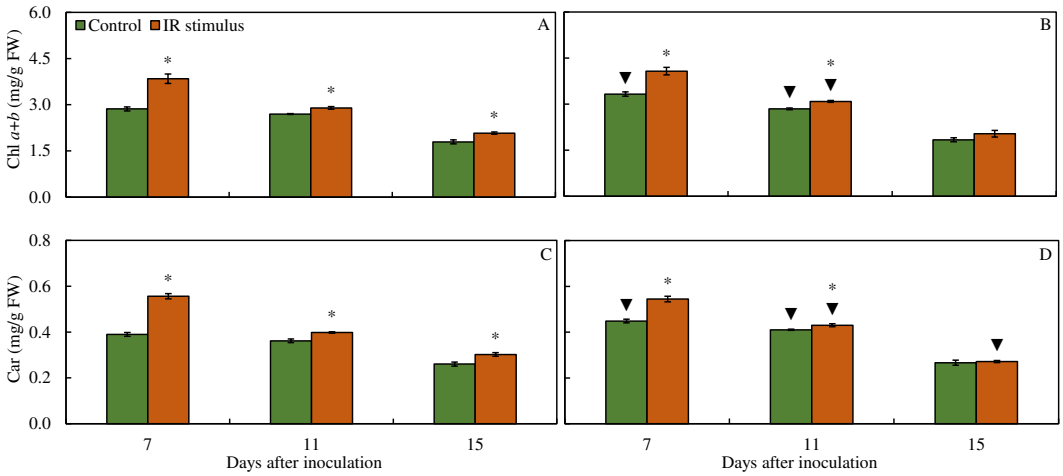

Figure 9. Concentrations of chlorophyll *a+b* (Chl *a+b*) (A and B) and carotenoids (Car) (C and D) determined on the leaflets of soybean plants non-inoculated (A and C) or inoculated (B and D) with *Phakopsora pachyrhizi* and sprayed with either water (control) or induced resistance (IR) stimulus. Means for control and IR stimulus treatments followed by an asterisk (*) and means for NI and I plants followed by an inverted triangle (▼), at each evaluation time, are significantly different ($P \leq 0.05$) according to *F* test. Bars represent the standard error of the means. FW = fresh weight.

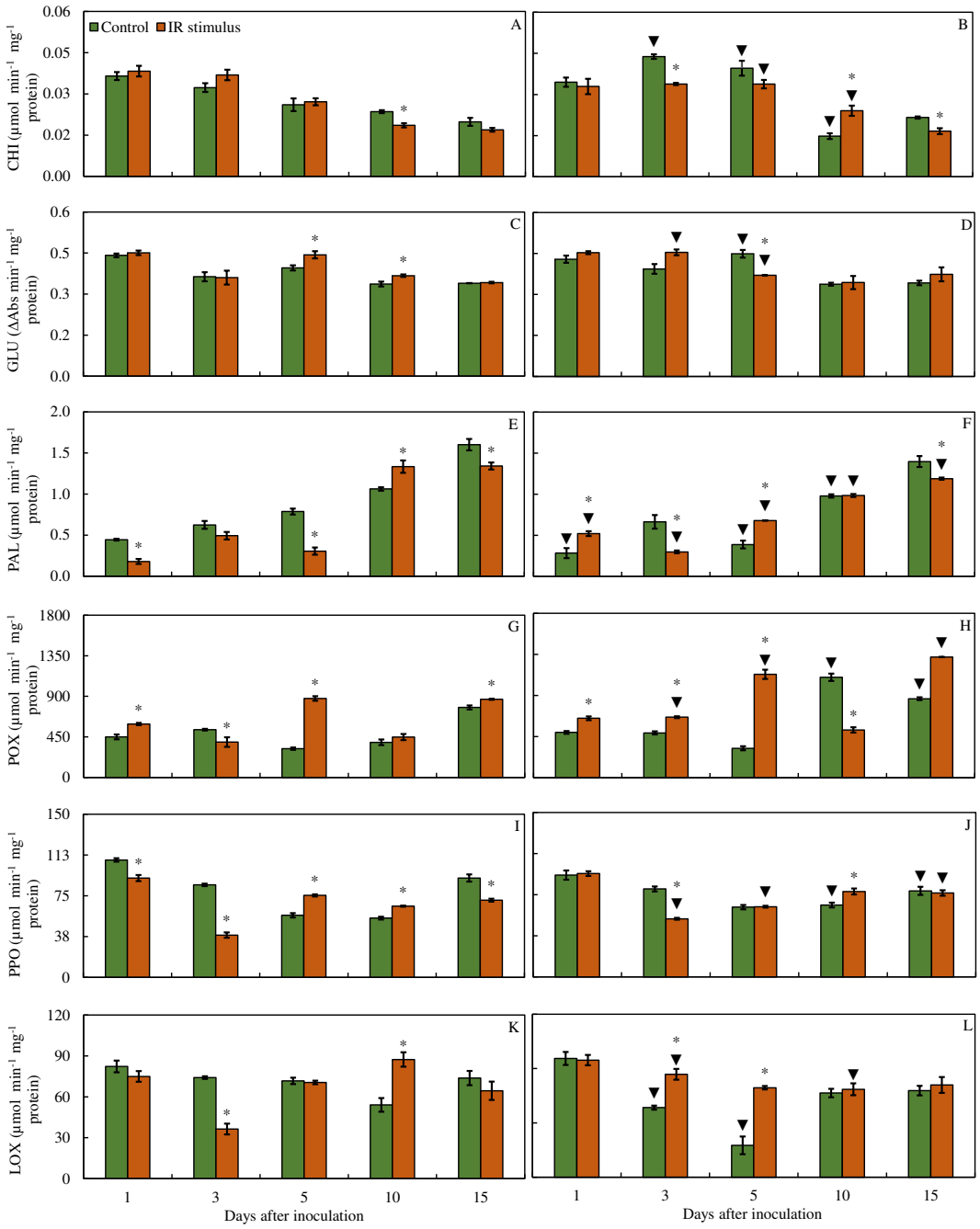

Figure 10. Activities of chitinase (CHI) (A and B), *β*-1,3-glucanase (GLU) (C and D), phenylalanine ammonia-lyase (PAL) (E and F), peroxidase (POX) (G and H), polyphenoloxidase (PPO) (I and J), and lipoxygenase (LOX) (K and L) determined on the leaflets of soybean plants non-inoculated (NI) (A, C, E, G, I, and K) or inoculated (I) (B, D, F, H, J, and L) with *Phakopsora pachyrhizi* and sprayed with either water (control) or induced resistance (IR) stimulus. Means for control and IR stimulus treatments followed by an asterisk (*) and means for NI and I plants followed by an inverted triangle (▼), at each evaluation time, are significantly different ($P \leq 0.05$) according to F test. Bars represent the standard error of the means.

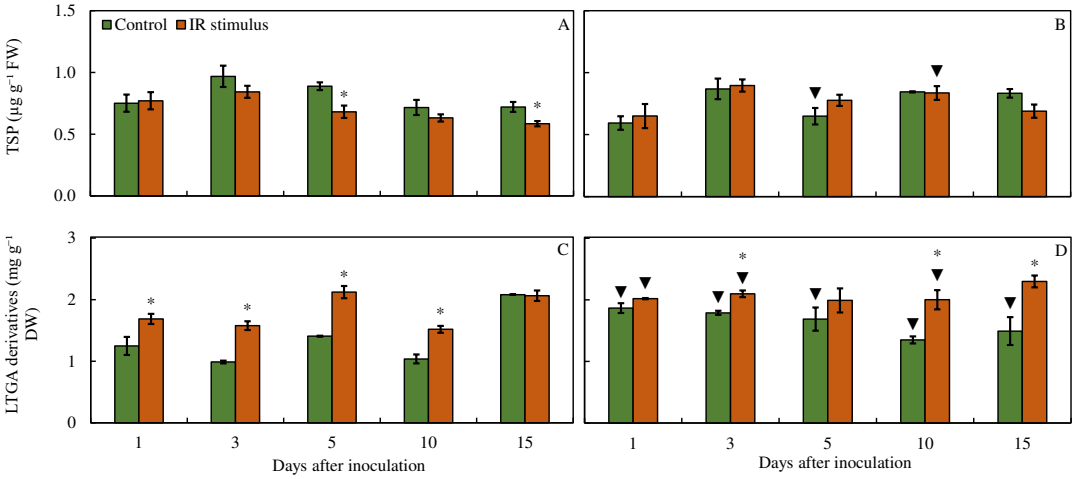

Figure 11. Concentrations of total soluble phenolics (TSP) (A and B) and lignin-thioglycolic acid (LTGA) derivatives (C and D) determined on the leaflets of soybean plants non-inoculated (A and C) or inoculated (B and D) with *Phakopsora pachyrhizi* and sprayed with either water (control) or induced resistance (IR) stimulus. Means for control and IR stimulus treatments followed by an asterisk (*) and means for NI and I plants followed by an inverted triangle (▼), at each evaluation time, are significantly different ($P \leq 0.05$) according to F test. Bars represent the standard error of the means. FW and DW = fresh weight and dry weight, respectively.