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Impact of foliar nickel application on urease activity, antioxidant metabolism and control of powdery mildew (*Microsphaera diffusa*) in soybean plants

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Nickel (Ni) is a cofactor for urease, an enzyme that breaks down urea into ammonia and carbon dioxide. This study aimed to evaluate the physiological impact of Ni on urea, antioxidant metabolism and powdery mildew severity in soybean plants. Seven levels of Ni (0, 10, 20, 40, 60, 80 and 100 g ha^{-1}) alone or combined with the fungicides fluxapyroxad and pyraclostrobin were applied to soybean plants. The total Ni concentration ranged from 3.8 to 38.0 mg kg^{-1} in leaves and 3.0 to 18.0 mg kg^{-1} in seeds. A strong correlation was observed between Ni concentration in the leaves and seeds, indicating translocation of Ni from leaves to seeds. Application of Ni above 60 g ha^{-1} increased lipid peroxidation in the leaf tissues, indicative of oxidative stress. Application of 40 g ha^{-1} Ni combined with 300 mL ha^{-1} of fungicide reduced powdery mildew severity by up to 99%. Superoxide dismutase, catalase, peroxidase and urease enzyme activity were greatest under these conditions. Urea concentration decreased in response to Ni application. Urease activity in soybean leaves showed a negative correlation with powdery mildew severity. The leaf Ni concentration showed a positive correlation with the urease and a negative correlation with powdery mildew severity. The results of this study suggest that urease is a key enzyme regulated by Ni and has a role in host defence against powdery mildew by stimulating antioxidant metabolism in soybean plants.

Keywords: antioxidative metabolism, fungicides, nickel, soybean, urea, urease

Introduction

As a result of improved disease management and plant breeding programmes, soybean (*Glycine max*) has increased in production over the last few decades. Fertilizer application and effective pest and disease control are the main management practices adopted to ensure high soybean yield (Godoy *et al.*, 2016).

Disease occurrence is one of the limiting factors for higher soybean yields (Godoy *et al.*, 2016). Powdery mildew, caused by the fungus *Microsphaera diffusa*, develops on the leaf surface, forming a thin layer of mycelium and reducing the photosynthetic active area by up to 50%. This leads to leaf drying and premature fall in severe cases, thereby causing yield losses of 10–50% (McTaggart *et al.*, 2012).

The most efficient method of controlling powdery mildew is the use of resistant cultivars. However, some resistant cultivars become susceptible when sown in seasons most favourable to the occurrence of powdery mildew (Godoy *et al.*, 2016), and require fungicide application. The use of a fungicide mixture with at least two active ingredients with different actions, such as a strobilurin and a carboxamide fungicide, is recommended to avoid fungal resistance to chemicals. Both active ingredients block fungal mitochondrial respiration, but they act on distinct sites. In addition, these molecules are known to exert a positive physiological effect on plant antioxidant metabolism, fighting both biotic and abiotic stress (Godoy *et al.*, 2016).

An economically viable alternative for controlling powdery mildew is foliar fertilization with micronutrients. When applied at low concentrations, Ni can reduce fungal diseases when used alone or in combination with fungicides. The nutritional status of the plant determines susceptibility to the disease. The essential role of Ni in plants was demonstrated by Dixon *et al.* (1975), who found that this metal is a structural component of the metalloenzyme urease (urea amidohydrolase), which has two Ni ions in its active centre (Ciurli, 2001). This enzyme catalyses the hydrolysis of urea into ammonia and carbon dioxide (Dixon *et al.*, 1975). The importance of Ni has been shown in several plant species, as observed by Eskew *et al.* (1983) and Brown *et al.* (1987), who found low urease activity in soybean, bean and barley due to Ni deficiency,

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resulting in an accumulation of urea in the leaf tissue. Urease occurs in two isoenzymatic forms in soybean plants, one of high expression called embryo-specific urease, synthesized in the seed, and the other called ubiquitous urease, which is synthesized in the other plant tissues and plays an important role in nitrogen metabolism (Polacco *et al.*, 2013; Fabiano *et al.*, 2015).

In addition to the nutritional effects of Ni on nitrogen metabolism, this micronutrient can directly affect pathogenic microorganisms, as reported for bacteria and fungi (Wood & Reilly, 2007; Wiebke-Strohm *et al.*, 2012). Foliar spraying with Ni at low concentrations also increases tolerance to biotic and abiotic stress in plants because this micronutrient stimulates antioxidant metabolism, increasing the activity of enzymes of the ascorbate–glutathione cycle, such as catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD), which protect plant cells against reactive oxygen species (ROS) (Noctor & Foyer, 2016).

Foliar application of Ni alone or combined with chemical control with fungicides may be a viable alternative for the management of powdery mildew in soybean. This study aimed to evaluate the physiological and biochemical effect of Ni alone or combined with chemical control with fungicides on powdery mildew control and its relation with nutritional status, antioxidative stress, and nitrogen metabolism in soybean plants.

Materials and methods

Experimental site

The study was conducted from March to June 2015 at the Teaching and Research Farm of the Engineering School of Ilha Solteira, São Paulo State University (FEIS/UNESP) in Selvíria, Mato Grosso do Sul, Brazil (20° 22′ S, 51° 22′ W, 335 m a.s.l.). The experimental site is part of the Cerrado biome, and it has been cultivated for more than 25 years and has been under a no-tillage system for the last 10 years. The mean annual rainfall is 1232 mm, and the mean annual temperature is 24.5 °C. During the experiment, the mean daily temperature ranged from 27.2 to 15.3 °C, the mean daily rainfall was 3.0 mm, and the mean relative humidity was 86% (Fig. 1).

The soil of the site was classified as a typical dystrophic red latosol (DRL) that is very clayey and corresponds to the oxisol order. The chemical characteristics of the soil were 29 mg dm^{-3} phosphorus (resin), 21 g dm^{-3} organic matter, calcium chloride ($CaCl_2$) pH 5.3, 3.5 $mmol_c$ dm^{-3} potassium, 38 $mmol_c$ dm^{-3} calcium, 22 $mmol_c$ dm^{-3} magnesium, 8 $mmol_c$ dm^{-3} H^+ + Al, 0 $mmol_c$ dm^{-3} aluminium, 0.1 mg dm^{-3} nickel, 92.5 $mmol_c$ dm^{-3} cation exchange capacity, and 69% base saturation (V%).

Experimental design

The study was conducted using a randomized block experimental design with a 7 × 2 factorial scheme, including seven doses of Ni (0, 10, 20, 40, 60, 80 and 100 g ha^{-1}), using nickel sulphate as the source, alone or combined with 300 mL ha^{-1} of the fungicide Orkestra SC, applied during phenological stage R1 (beginning bloom), with four replicates, totalling 56 plots. The fungicide Orkestra SC was chosen because it is registered with the Brazilian Ministry of Agriculture and Livestock for the control of soybean diseases, including powdery mildew. In addition, this fungicide is of interest to this study because the physiological actions of the active ingredients (fluxapyroxad and pyraclostrobin) on the plant are known (Godoy *et al.*, 2016).

Experimental set-up and procedures

The experiment was conducted under a no-tillage system. Soil acidity was not corrected because the base saturation was adequate for the soybean requirements. The area was desiccated 20 days before sowing with Roundup Original (glyphosate, 4 L ha^{-1}), Aurora 400 EC (carfentrazone-ethyl, 200 mL ha^{-1}), and 0.5% mineral oil.

The soybean cultivar TMG 7062 IPRO (semideterminate growth, white flower, short cycle, susceptible to powdery mildew, INOX technology, and Intacta RR2 PRO), previously treated with Standak Top (2 mL kg^{-1} seed), was seeded at a density of approximately 267 000 plants per hectare. The experimental plots consisted of four 6-m rows spaced 0.45 m apart, and the useful area included the two central rows, excluding 0.5 m at the ends.

The seeds were inoculated simultaneously with 3 mL ha^{-1} of the liquid commercial product and the nitrogen-fixing bacterium *Bradyrhizobium japonicum* (strain SEMIA 6462, product registration number SP 00581-10030-1, 2×10^9 colony-forming units g^{-1}, BIOMAX). For fertilization with NPK, 350 kg ha^{-1} of 4-30-10 fertilizer was applied.

The soybean seeds were sown on 5 March 2015, and the seedlings emerged 7 days after seeding. The late sowings or winter interim-harvest and cultivation are periods more favourable to powdery mildew occurrence because the fungus requires temperatures of approximately 18–24 °C and low relative humidity for development (Godoy *et al.*, 2016).

When necessary, irrigation was performed by a centre-pivot sprinkler system, with a mean water depth of 14 mm and 72 h irrigation time. During the plant development cycle, phytosanitary control was performed with Roundup Original (glyphosate, 4 L ha^{-1}), Premio (chlorantraniliprole, 50 mL ha^{-1}), Belt (flubendiamide, 60 mL ha^{-1}) and Connect (imidacloprid + betacyfluthrin, 0.8 L ha^{-1}).

The use of a soybean variety susceptible to powdery mildew, along with a site favourable to the natural appearance of the disease and in the ideal season and climatic conditions for its development, was sufficient for the natural occurrence of powdery mildew, making inoculation unnecessary.

Physiological and biochemical analysis

Evaluation of growth and yield

The number of pods on the plants, excluding the empty pods, was evaluated at the R8 (full maturity) stage. The evaluation was performed in three plants from each plot. Yield was evaluated at the same stage, by harvesting the plants from the plots. Moisture was standardized at 13%.

Evaluation of disease severity

The severity of disease was evaluated using a scoring system based on percentage of the leaf area with visible disease symptoms, using a diagrammatic scale (Godoy *et al.*, 2016).

Scanning electron microscopy

At the R2 (full bloom) stage, 12 days after treatment application, leaf samples from the upper third of one plant of each plot

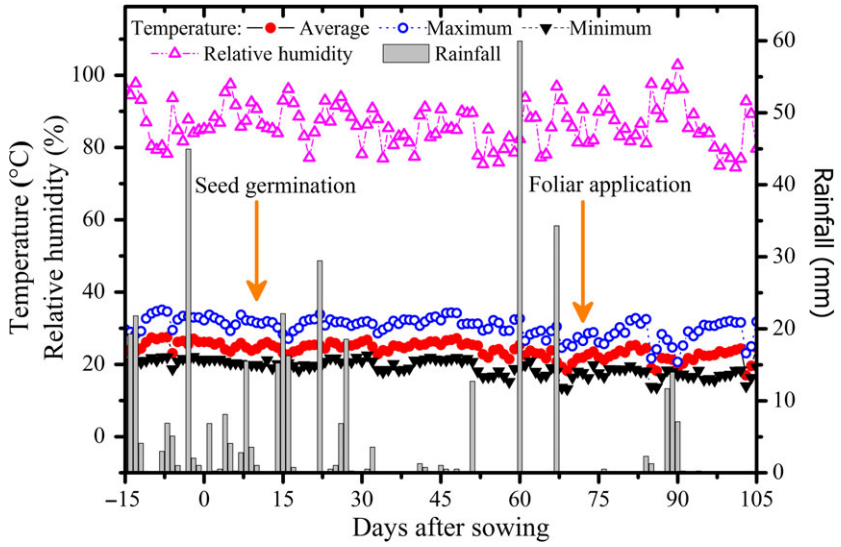

Figure 1 Rainfall (mm); relative air humidity (%); and minimum, mean, and maximum temperature (°C) during the experiment in the municipality of Selvíria, Mato Grosso do Sul, Brazil. [Colour figure can be viewed at wileyonlinelibrary.com]

were collected. Square leaf samples of approximately 0.5 mm^2 were subjected to a gold bath for approximately 3 min in a Quorum Q150T sputter coater. Next, the samples were placed under a Zeiss EVO LS-15 scanning electron microscope, where images were obtained using secondary electron detectors (to observe leaf relief and topography) and backscattered electron detectors (to observe the contrast in the leaves due to the presence of chemical elements of different atomic numbers).

Nutritional analysis

At the R2 stage, 12 days after the treatment application, the first newly expanded trefoil was collected from 20 plants in each plot. The material was dried and used to quantify the foliar macro- and micronutrient concentration. The seeds collected at the R8 stage were also analysed.

The nitrogen concentration was determined by the semi-micro-Kjeldahl method after sulphur digestion and nitric–perchloric acid digestion were used to quantify the other nutrients (Santos *et al.*, 2017). The determination was made using atomic absorption spectroscopy, except for phosphorus and sulphur, whose concentrations were determined using spectrophotometry as described by Reis *et al.* (2015).

Enzymatic analysis

Collection of material. The first fully expanded trefoil of the plants was collected in liquid nitrogen 10 days after the treatments for determination of the enzymatic activities. This material was macerated in liquid nitrogen and stored at −80 °C until analysis. Samples were removed from the leaves to quantify the concentration of urea, shikimic acid, hydrogen peroxide, lipid peroxidase, soluble protein, superoxide dismutase, catalase and peroxidase.

Urease activity. *In vivo* urease activity was determined as a function of $N\text{-}NH_4$ production, according to the method of McCullough (1967). The extract was obtained according to the method described by Hogan *et al.* (1983). After collection, 0.2 g of fresh leaf mass was added to 8 mL of sodium phosphate buffer with urea pH 7.4 and kept for 3 h in a 30 °C water bath with stirring for extraction. Then, 2.5 mL of reagent I, comprising phenol (0.1 M) and sodium nitroprusside (170 μM), was added to a 0.5 mL aliquot of the extract, followed by stirring, and then the addition of 2.5 mL of reagent II, comprising sodium hydroxide (0.125 M), disodium phosphate (0.15 M) and sodium hypochlorite (3% Cl_2). The mixture was incubated again in a 37 °C water bath for 35 min. $N\text{-}NH_4$ production was determined with a spectrophotometer at 625 nm, and the results are expressed in μmol $N\text{-}NH_4$ per g FW (fresh weight) h^{-1}.

Urea concentration. The urea concentration was determined as described by Kojima *et al.* (2007). One millilitre of formic acid (10 mM) was added to 0.5 g of plant material, followed by vortexing and centrifugation of the sample at 10 000 ***g*** at 4 °C for 15 min. An acid reagent was prepared that contained ferric chloride hexahydrate and orthophosphoric acid, which, when mixed with sulphuric acid, formed the mixed acid reagent. Then, two colourimetric reagents, one containing thiosemicarbazide and the other containing diacetyl monoxime, were combined. The final reagent, called colour-developing reagent, was prepared only on the day of the test, by mixing ultrapure water, the mixed acid reagent and the mixed colourimetric reagent at the ratio of 1:1:1. For urea determination, 1 mL of the prepared reagent was added to 100 μL of sample. The samples were incubated at 99 °C for 15 min, under stirring at 750 rpm, and then cooled for 5 min on ice and kept in the dark. The determination was performed in a spectrophotometer at 540 nm, and the results are expressed in μmol urea per g FW.

Shikimic acid concentration. For determination of shikimic acid concentration, the method described by Singh & Shaner (1998) was used. A total of 750 μL of hydrochloric acid (0.25 M) was added to 250 mg plant material at a ratio of 1:3. The material was homogenized by vortexing and was then centrifuged at 10 606 ***g*** for 15 min at 4 °C. Twenty microlitres of the sample was mixed with 0.5 mL of periodic acid solution (1%). The solution was allowed to stand for 3 h. Next, 0.5 mL

of sodium hydroxide (1 M) and 0.3 mL of glycine (0.1 M) were added. The readings were performed immediately in a spectrophotometer at 380 nm, and the results are expressed in μmol of shikimic acid per g FW.

Hydrogen peroxide concentration. The hydrogen peroxide (H_2O_2) concentration was determined by reaction of the plant sample with potassium iodide (KI), according to Alexieva *et al.* (2001). One hundred milligrams of frozen plant material was mixed with 1 mL of 0.1% trichloroacetic acid (TCA) in an Eppendorf tube, homogenized by vortexing, and then centrifuged at 7780 ***g*** for 15 min at 4 °C. Then, 200 μL of potassium phosphate buffer pH 7.5 (100 mM) and 800 μL of KI solution (1 M) were added to 200 μL of the supernatant. The samples were kept on ice for 1 h. The samples were then allowed to return to room temperature, and the absorbance was read at 390 nm. The hydrogen peroxide concentration in the leaf tissue was calculated based on a standard curve, and the results are expressed in nmol per g FW.

Lipid peroxidation. Lipid peroxidation was determined by the production of metabolites reactive to 2-thiobarbituric acid (TBA), mainly malondialdehyde (MDA), according to Heath & Packer (1968). The extraction was performed by homogenizing 200 mg of plant material in 2 mL of 0.1% TCA (w/v) + 20% polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 7780 ***g*** for 5 min at 4 °C. Then, 1 mL of a solution containing 20% TCA + 0.5% TBA was added to 250 μL of supernatant. The samples were kept in a dry bath at 95 °C for 30 min and then transferred to ice, where they remained for another 10 min. Next, the material was centrifuged again at 7780 ***g*** for 10 min. Samples were read at two wavelengths: 532 and 600 nm. The results are expressed in nmol MDA per g FW.

Enzyme extraction and activity. The frozen plant material was macerated in a mortar with liquid nitrogen. Subsequently, the extract obtained from approximately 1 g of processed plant material was transferred to a 15 mL Falcon tube, where 5 mL of 100 mM potassium phosphate buffer (pH 6.8, containing 1 mM ethylenediaminetetraacetic acid (EDTA), 3 mM dithiothreitol (DTT), and 4% (w/v) PVPP) was added, according to the method described by Santos *et al.* (2017). The homogenate was centrifuged at 7780 ***g*** for 30 min, and the supernatant was stored at −80 °C for further determination of the SOD, CAT and POD activities. The protein concentration was determined following the method of Bradford (1976), with bovine serum albumin being used as a standard.

SOD activity was determined according to Giannopolitis & Ries (1977). The reaction was performed in a reaction chamber (box), under a 15 W fluorescent lamp at 25 °C. Fifty microlitres of the sample was added to 5 mL of the following solution: sodium phosphate buffer pH 7.8 (50 mM), methionine (13 mM), nitroblue tetrazolium (NBT, 75 mM), EDTA (0.1 mM) and riboflavin (2 μM). The samples were placed in the box while covered and unexposed to any exterior light, and kept under box illumination for 15 min. In parallel, the same solution was kept in the dark in test tubes capped with aluminium foil, constituting the blank for each sample. Subsequently, the samples were homogenized by vortexing. The determination was carried out in a spectrophotometer at 560 nm, and the results are expressed in U SOD per mg protein.

CAT activity was determined by monitoring the H_2O_2 degradation according to the method described by Reis *et al.* (2015). One millilitre of potassium phosphate buffer pH 7.5 (100 mM) and 2.5 μL of H_2O_2 (30%) were added to 25 μL of the protein extract, and the samples were immediately vortexed. The determination of the H_2O_2 decomposition was performed at 2 min intervals at 25 °C in a spectrophotometer at 240 nm, and the results are expressed in nmol per mg protein.

POD activity was determined according to the method described by Reis *et al.* (2014), with small changes. Phosphate buffer solution pH 6.7, H_2O_2 (30%), amino-antipyrine and phenol were added to 150 μL of protein extract, with subsequent incubation in a water bath at 30 °C for 5 min. Next, 2 mL of ethanol was added, and the samples were vortexed. The determination was performed in a spectrophotometer at 505 nm, and the results are expressed in μmol per min per mg protein.

Statistical analysis

The normality of all data was tested using the Anderson–Darling test, and the homoscedasticity was tested using the equality of variances test (or Levene's test). The data were subjected to analysis of variance at a significance level of 0.05 probability by the *F* test. When significant, the means were subjected to the Tukey test at the 0.05 probability level using the statistical programs MINITAB and SIGMAPLOT.

Results

The number of pods of soybean increased in response to Ni application (Fig. 2a), and the soybean yield increased in response to fungicide application (Fig. 2b). However,

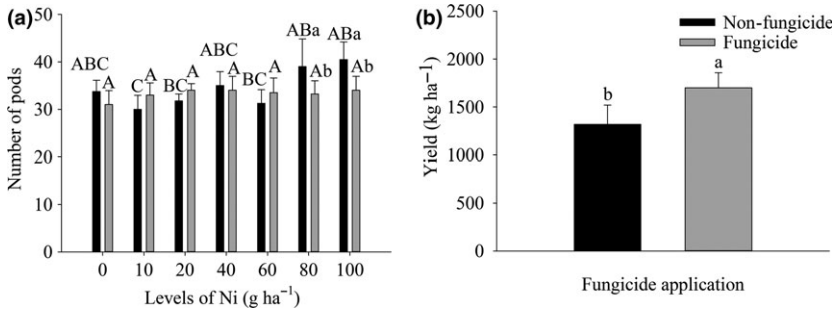

Figure 2 Number of pods and soybean yield in response to Ni application alone (black bars) or combined with fungicide (fluxapyroxad + pyraclostrobin; grey bars). Means followed by the same uppercase and lowercase letters are not significantly different according to the *t*-test ($P \leq 0.05$), following a horizontal comparison of bars from the different Ni doses and presence or absence of fungicide, respectively. The error bars represent the standard error of the mean ($n = 4$).

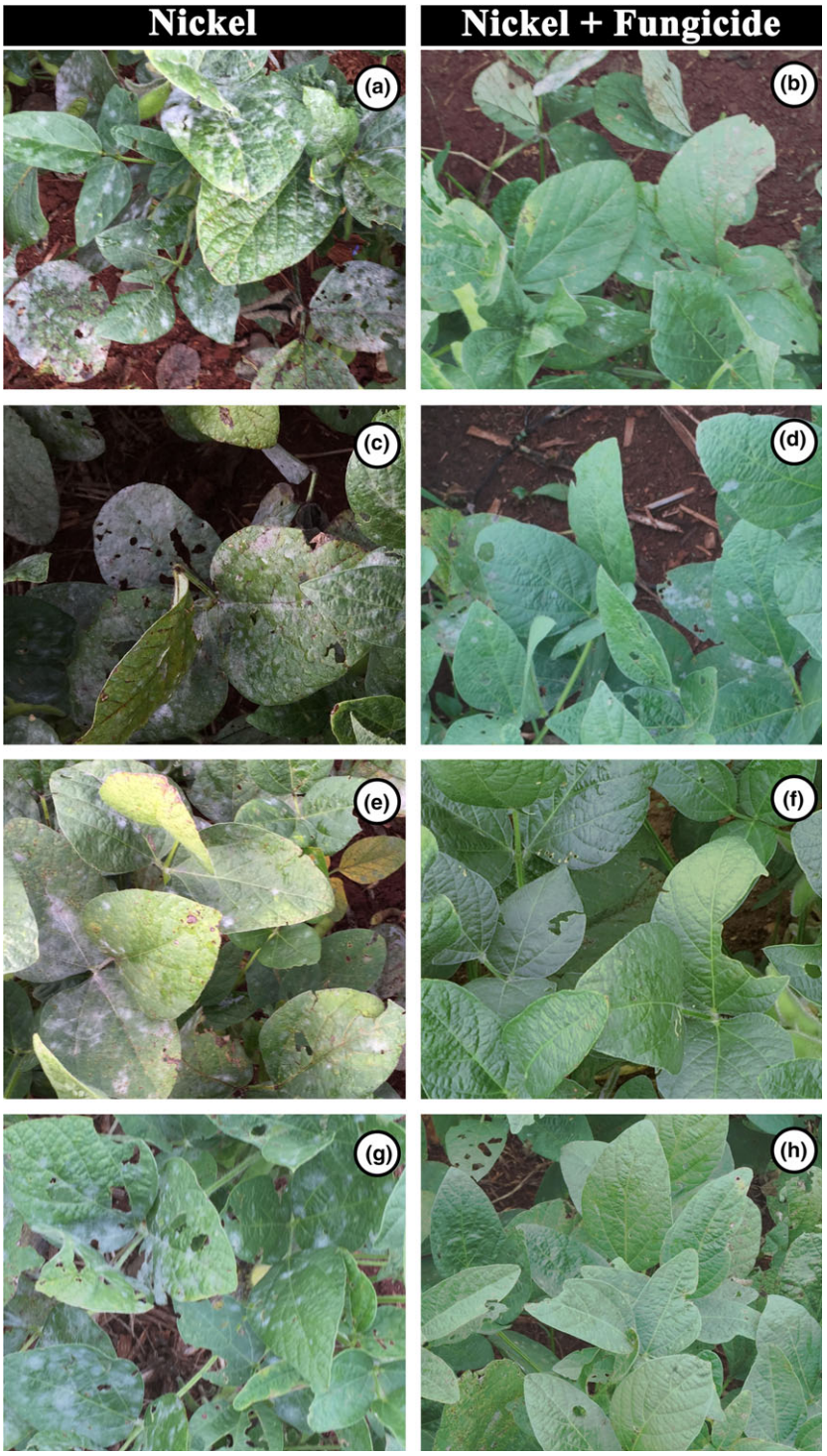

Figure 3 Soybean powdery mildew severity in response to the application of Ni doses alone or combined with fungicide. (a) Control (no application of Ni or fungicide); (b) application of 300 mL ha^{-1} fungicide (fluxapyroxad + pyraclostrobin) only; (c) application of 10 g ha^{-1} of Ni; (d) application of 10 g ha^{-1} Ni + 300 mL ha^{-1} fungicide; (e) application of 60 g ha^{-1} Ni; (f) application of 60 g ha^{-1} Ni + 300 mL ha^{-1} fungicide; (g) application of 100 g ha^{-1} Ni; (h) application of 100 g ha^{-1} Ni + 300 mL ha^{-1} fungicide. [Colour figure can be viewed at wileyonlinelibrary.com]

soybean yield did not increase in response to Ni foliar application.

Figures 3 and 4 clearly show the antifungal effect of the foliar application of Ni, with or without fungicide, with a reduction in powdery mildew severity. Treatments with 60 g ha^{-1} of Ni combined with 300 mL ha^{-1} fungicide decreased fungal severity by 99%, leaving the leaves intense green (Fig. 3). The electron micrographic images (Fig. 4) show the effect of Ni on the fungal reproductive structures (conidia) and on the hyphae. In the control treatment the conidia and hyphae were visible (Fig. 4a). With the application of Ni, these structures were either damaged (Fig. 4c) or did not develop. Notably, even the higher

Ni doses did not cause Ni toxicity in the leaf or ultrastructural alterations.

Ni applications rates from 20 g Ni ha^{-1} led to lower disease severity (Fig. 5). Control of the powdery mildew was more effective when nickel was combined with the fungicide. Applications starting at 40 g ha^{-1} of Ni combined with 300 mL ha^{-1} of fungicide significantly decreased the disease severity in soybean. When applied alone, Ni was more effective in controlling the disease at applications of 80 and 100 g ha^{-1} (Fig. 5).

Foliar Ni application combined with the fungicide altered the leaf nitrogen concentration in the soybean plants (Fig. 6a). Among the other nutrients, manganese and iron concentration in the soybean seeds varied according to the Ni dose, alone or combined with the fungicide (Fig. 6b,c). Foliar Ni concentration increased in response to Ni application rates (Fig. 6d). The manganese concentration did not differ significantly until the dose of 80 g ha^{-1}. Similar to the foliar tissue, the dose of 20 g ha^{-1} led to slightly higher manganese concentration in seeds (Fig. 7a,b).

The application of Ni at doses between 10 and 40 g ha^{-1}, alone or combined with fungicide, increased the urease activity (Fig. 8a). The urea concentration in soybean leaves decreased (Fig. 8b) with foliar Ni application.

Nickel acts on the shikimic acid pathway that is responsible for biosynthesis of aromatic amino acids such as phenylalanine, tryptophan and tyrosine (Kutman *et al.*, 2013). The shikimic acid concentration remained constant with foliar Ni application, except for the 40 g ha^{-1} application rate, which showed a peak in

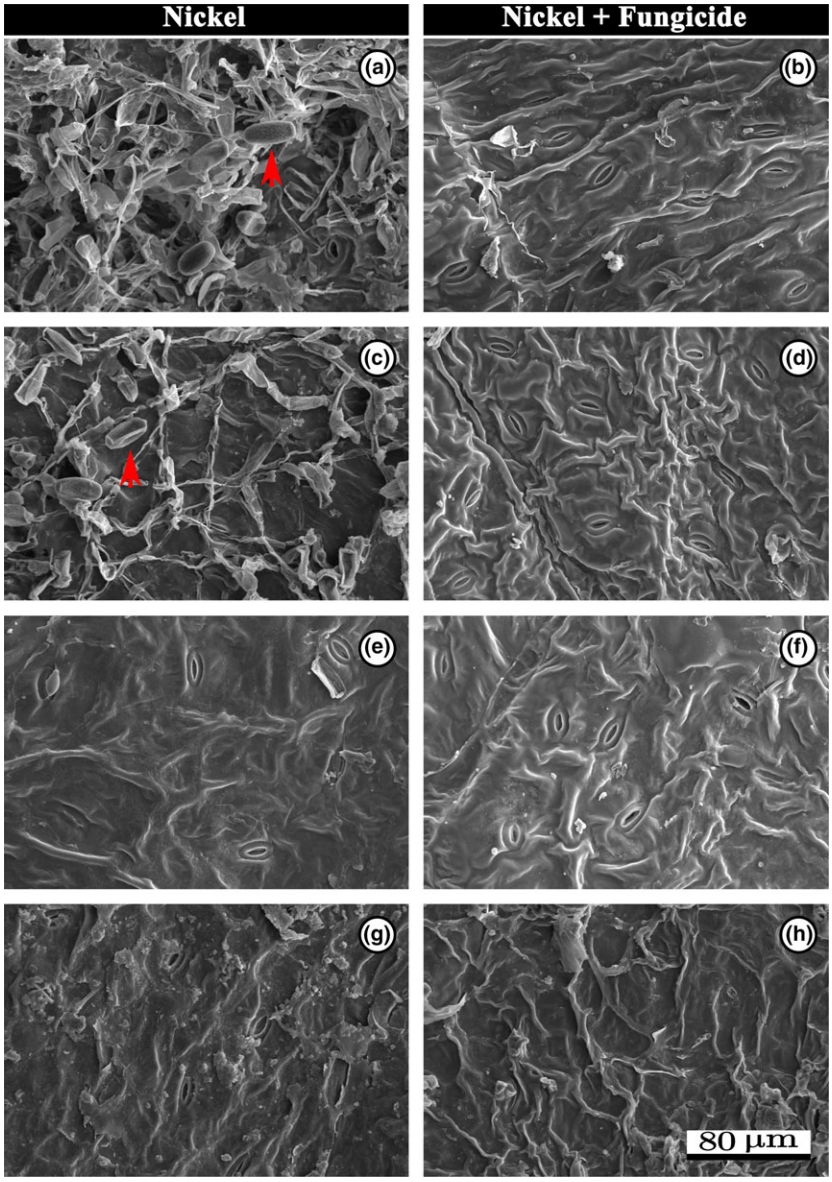

Figure 4 Electron micrograph of conidium and hypha of *Microsphaera diffusa* in soybean leaves in response to the application of Ni doses alone or combined with fungicide. (a) Control (no application of Ni or fungicide); (b) application of 300 mL ha^{-1} fungicide (fluxapyroxad + pyraclostrobin) only; (c) application of 10 g ha^{-1} Ni; (d) application of 10 g ha^{-1} Ni + 300 mL ha^{-1} fungicide; (e) application of 60 g ha^{-1} Ni; (f) application of 60 g ha^{-1} Ni + 300 mL ha^{-1} fungicide; (g) application of 100 g ha^{-1} Ni; (h) application of 100 g ha^{-1} Ni + 300 mL ha^{-1} fungicide. Arrows indicate details of the hypha and conidium of *M. diffusa*. [Colour figure can be viewed at wileyonlinelibrary.com]

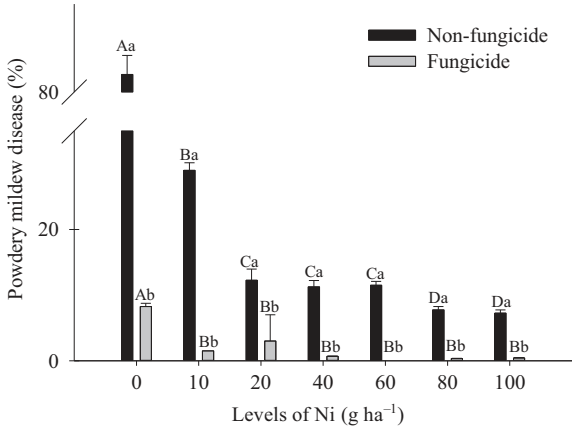

Figure 5 Percentage of powdery mildew on soybean trefoils as a function of the application of Ni doses alone (black bars) or combined with fungicide (fluxapyroxad + pyraclostrobin; grey bars). Means followed by the same lowercase letter comparing the fungicide application and by the same uppercase letter comparing the Ni doses do not differ significantly ($P \leq 0.05$) according to the Tukey test. Error bars indicate the standard deviation of the mean of four replicates ($n = 4$).

shikimic acid concentration (Fig. 9a). However, for the treatments with fungicide, a reduction in the shikimic acid concentration was observed at the higher doses.

SOD is a key enzyme that acts in the first line of defence against ROS, being responsible for the conversion of superoxide ($O_2^{\bullet -}$) into the less toxic forms H_2O_2 and O_2 (Thakur & Sohal, 2013). In the present study, the application of Ni up to the dose of 60 g ha^{-1} promoted a linear increase in SOD activity (Fig. 9b), and for the same Ni doses combined with the fungicide, the SOD activity was significantly higher compared to the application of Ni alone. The CAT activity also increased in response to foliar Ni application up to 60 g ha^{-1} relative to the control. The POD activity (Fig. 9d) increased up to 40 g ha^{-1} of Ni.

The H_2O_2 concentration was lower in the treatments that combined Ni and fungicide application when compared to treatments in which Ni was applied alone. Foliar application of Ni up to 60 g ha^{-1} increased the H_2O_2 concentration (Fig. 9e). By contrast, the lipid peroxidation rate increased in response to Ni doses when applied in combination with fungicide (Fig. 9f). In the absence of fungicide, no change was observed in the lipid

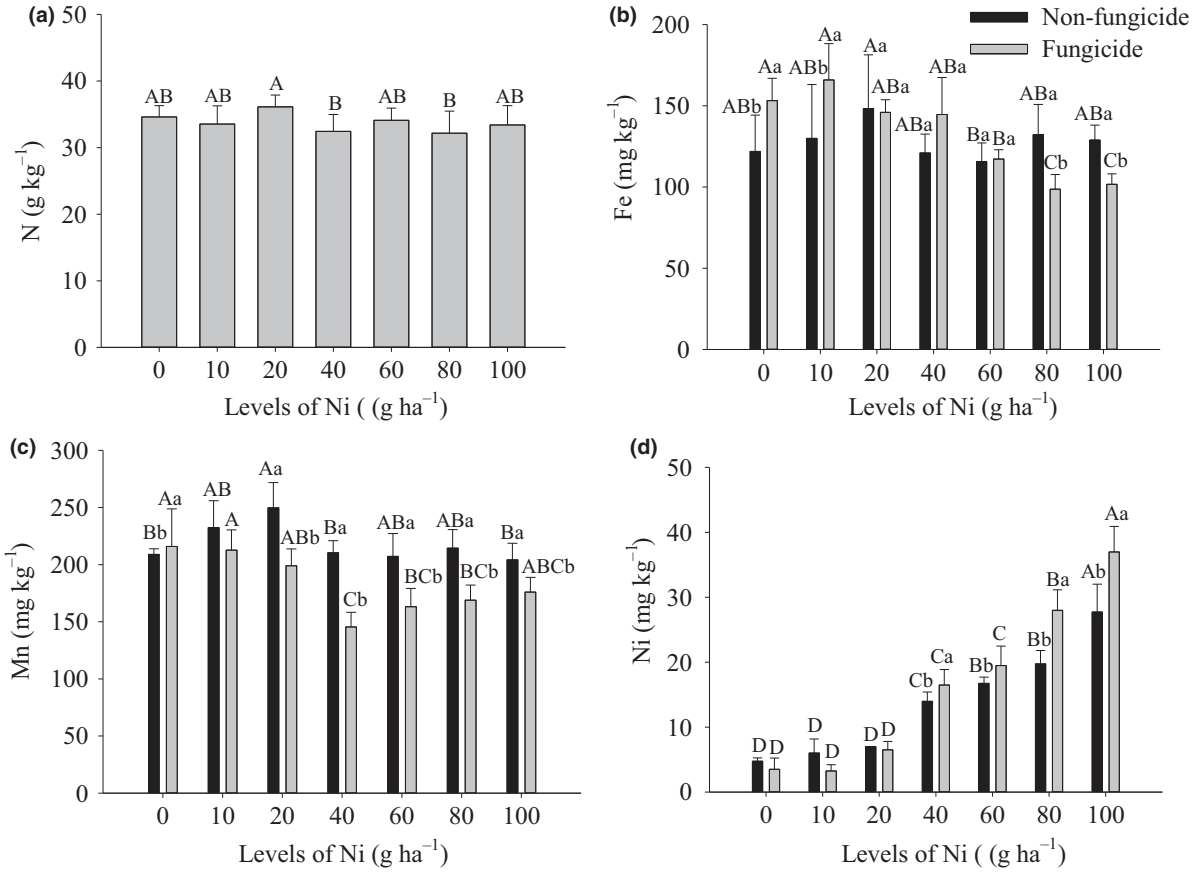

Figure 6 Nitrogen and micronutrient leaf contents in soybean in response to Ni application alone (black bars) or combined with fungicide (fluxapyroxad + pyraclostrobin; grey bars). (a) Nitrogen, (b) iron, (c) manganese, (d) nickel. Means followed by the same lowercase letter comparing the fungicide application and by the same uppercase letter comparing the Ni doses do not differ significantly ($P \leq 0.05$) according to the Tukey test. The error bars represent the standard error of the mean ($n = 4$).

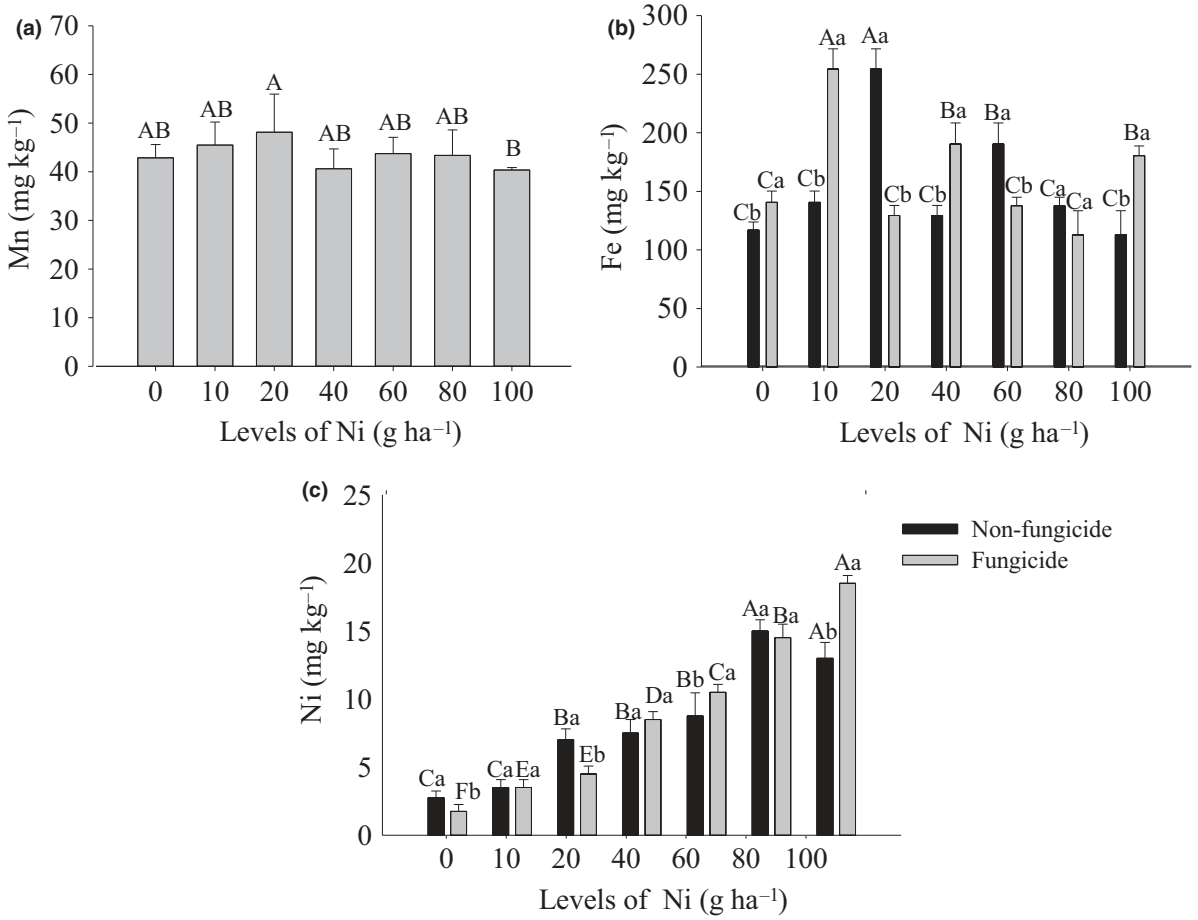

Figure 7 Nutrient concentration in soybean seeds in response to Ni application alone (black bars) or combined with fungicide (fluxapyroxad + pyraclostrobin; grey bars). (a) Manganese, (b) iron, (c) nickel. Means followed by the same lowercase letter comparing the fungicide application and by the same uppercase letter comparing the Ni doses do not differ significantly ($P \leq 0.05$) according to the Tukey test. The error bars represent the standard error of the mean ($n = 4$).

peroxidation up to the dose of 60 g ha^{-1}. The application of higher doses significantly increased the lipid peroxidation rate in the soybean leaves.

The application of Ni doses above 60 g ha^{-1}, alone or combined with fungicide, promoted a decrease in the SOD, CAT and POD activity and in the H_2O_2 concentration (Fig. 9). A significant increase in the lipid peroxidation rate was observed in response to doses above 60 g ha^{-1} (Fig. 9f), indicating that the cells underwent oxidative damage.

Discussion

The number of pods showed a positive correlation with the Ni concentration in leaves and seeds, but the effect of the treatments was variable. Rodak (2014) applied different Ni doses to soybean under greenhouse conditions and did not observe a significant Ni effect on the number of pods. The yield showed a positive correlation with the Ni concentration in the leaves (Fig. 10), although no significant effect of Ni doses was observed, as had been reported in several studies on the application of Ni in soybean (Kutman *et al.*, 2014; Rodak, 2014).

Powdery mildew control in soybean can be achieved by the use of fungicides. Among the fungicides used are the quinone outside inhibitors (QoI) and succinate dehydrogenase inhibitors (SDHI), which act on fungal mitochondrial respiration. QoIs inhibit the electron transport in the mitochondrial complex 3, thus inhibiting fungal respiration and, consequently, ATP formation. The SDHIs act on complex 2 of the electron transport chain, called the succinate dehydrogenase complex, resulting in the blockade of ATP production and the formation of intermediary molecules that are harmful to the cell (Yorinori *et al.*, 2004; Attanayake *et al.*, 2010).

Nickel is a constituent of urease, which is the enzyme responsible for the breakdown of urea into ammonia and carbon dioxide (Dixon *et al.*, 1975) and therefore responsible for the recycling of nitrogen in the plant (Polacco *et al.*, 2013; Fabiano *et al.*, 2015). Nickel, as well as urease, showed no consistent trend in the effect

of Ni on nitrogen concentrations in leaves and seeds (Fig. 10). This result corroborates some studies conducted with soybean (Rodak, 2014) in which no changes were observed in the nitrogen concentration in the leaf tissue with the application of Ni.

Regarding the interaction with the other nutrients, in general, the absorption of cationic macro- and micronutrients is reduced at high Ni concentrations in plant organs. This reduced absorption occurs because Ni uses nonspecific cation transporters, such as YSL (yellow stripe 1-like protein), ZIP (ZRT/IRT-like protein), NRAMP (natural resistance-associated macrophage protein), and nicotianamine synthase. Thus, it causes competition between the divalent cations, including Cu^{2+}, Fe^{2+}, Mn^{2+} and Zn^{2+} (Mizuno *et al.*, 2005).

A reduction in iron uptake by Ni-fertilized plants has been reported (Nishida *et al.*, 2015), and therefore, excess Ni causes leaf chlorosis symptoms similar to iron deficiency symptoms in plants. Seeking to elucidate the underlying mechanism of Ni phytotoxicity, Nishida *et al.* (2015) demonstrated that Ni can use the iron transporter IRT1 (iron-regulated transporter 1), which is homologous to the transporters of the ZIP family, and also stimulates its expression, further accelerating Ni accumulation in leaf tissue.

Moreover, according to Kazemi *et al.* (2010), some antioxidant enzymes, such as CAT and POD, contain iron in their structure, which explains the positive correlation between the leaf concentration of iron and CAT and POD activity. Given that high Ni doses decrease the iron concentration in plant tissue, the reduction of the activity of these enzymes may be due to the lack of iron for their biosynthesis (Kazemi *et al.*, 2010). This may be related to the negative correlation between the leaf Ni concentration and the activity of CAT and POD enzymes, as well as to the MDA and H_2O_2 concentration (Fig. 10).

The leaf Ni concentration ranged from 3.8 to 38.0 mg kg^{-1}, increasing as a function of its foliar application and independently of the fungicide application. When Ni was applied with the fungicide, higher Ni concentration was observed starting at the dose 40 g ha^{-1}. The Ni concentration in seeds ranged from 3 to 18 mg kg^{-1}. A pattern similar to the leaf Ni concentration was observed, thus indicating a high translocation rate.

Nickel is a component of the urease prosthetic group (Dixon *et al.*, 1975), and its supply increases the enzyme activity. The increase in urease activity has also been observed in other crops, such as cereals (Brown *et al.*, 1987) and pecan (*Carya illinoinensis*; Bai *et al.*, 2006). However, at high Ni doses, the urease activity decreases. In the present study, foliar Ni applications above 60 g ha^{-1} alone or combined with fungicide decreased the urease activity in the leaves. Rodak (2014) also obtained a peak of urease activity (0.5 and 0.6 mg dm^{-3} of Ni), followed by a decrease in enzymatic activity at the higher doses and observed symptoms of Ni toxicity in the leaves.

Under conditions of low Ni concentration in the leaf tissue, urea accumulates in the tissues (Eskew *et al.*, 1983). Similar effects were also observed in tomato (Eskew *et al.*, 1983) and cowpea (*Vigna unguiculata*; Walker *et al.*, 1985). The addition of Ni to the nutrient solution was shown to relieve the symptoms of toxicity caused by urea accumulation (Tan *et al.*, 2000). The same occurs with foliar Ni application (Kutman *et al.*, 2014).

In this study, the fungicide used in the absence of Ni did not reduce the urea concentration. Conversely, application of Ni in combination with the fungicide treatments resulted in greater urease activity. The urea concentration was higher than that observed for the treatments without fungicide. At the dose of 100 g ha^{-1} of Ni combined with the fungicide, the urea concentration was higher than the concentration observed in the control treatment without Ni, showing that the fungicide effect when combined with Ni doses, despite contributing to the increase in urease activity, did not contribute to a reduction in the urea concentration.

Ni indirectly affects plant pathogens via the action of urease. This enzyme in plants has an antifungal

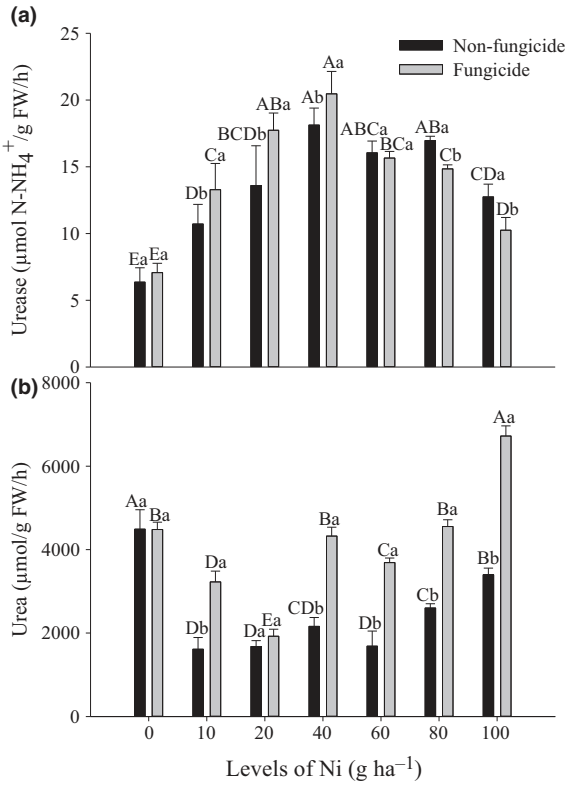

Figure 8 Urease activity (a) and urea concentration (b) in soybean leaf tissue in response to Ni application alone (black bars) or combined with fungicide (fluxapyroxad + pyraclostrobin; grey bars). Means followed by the same lowercase letter comparing fungicide application and by the same uppercase letter comparing Ni doses do not differ significantly ($P \leq 0.05$) according to the Tukey test. Error bars indicate the standard deviation of the mean of four replicates ($n = 4$).

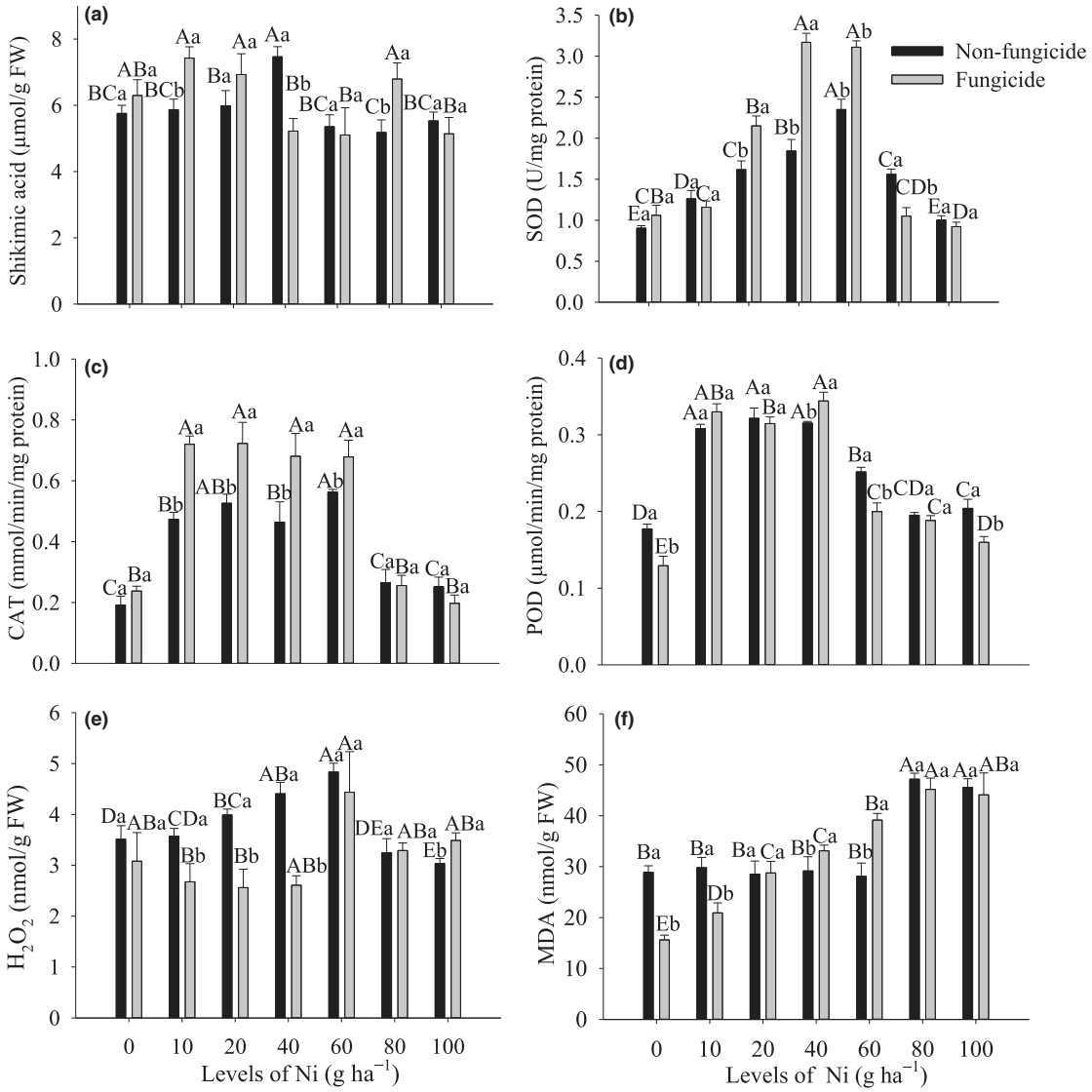

Figure 9 Shikimic acid concentration and antioxidant enzyme activity in soybean leaf tissue as a function of Ni application alone (black bars) or combined with fungicide (fluxapyroxad + pyraclostrobin; grey bars). (a) Shikimic acid concentration, (b) superoxide dismutase activity, (c) catalase activity, (d) peroxidase activity, (e) hydrogen peroxide concentration, (f) lipid peroxidation (measured as MDA concentration). Means followed by the same lowercase letter comparing fungicide application and by the same uppercase letter comparing Ni doses do not differ significantly ($P \leq 0.05$) according to the Tukey test. Error bars indicate the standard deviation of the mean of four replicates ($n = 4$).

effect (Carlini & Ligabue-Braun, 2016). Wiebke-Strohm *et al.* (2012) studied transgenic soybean with suppression of the urease gene and observed that *Rhizoctonia solani*, *Phomopsis* sp. and *Penicillium herguei* presented higher incidence when the urease activity was low under greenhouse conditions. They also observed more evident *Phakopsora pachyrhizi* foliar lesions in the genotype with the suppressed urease gene. These results corroborate the negative correlation observed in this study between the urease activity and powdery mildew severity.

During the photosynthetic metabolism of plants, ROS are commonly produced, and production is increased under conditions of stress, as in the case of attack by pathogens. Thus, the activation of enzymatic and nonenzymatic antioxidant mechanisms is important for the maintenance of the physiological state of the plant (Thakur & Sohal, 2013).

Some of the nonenzymatic metabolites produced by plants, such as flavonoids, anthocyanins and phenols, originate in the shikimic acid pathway. These compounds are important in plant defence metabolism, including nonenzymatic defence against oxidative stress (Carlini & Ligabue-Braun, 2016). Such a defence was observed in this study in the correlation between the shikimic acid and lipid peroxidation.

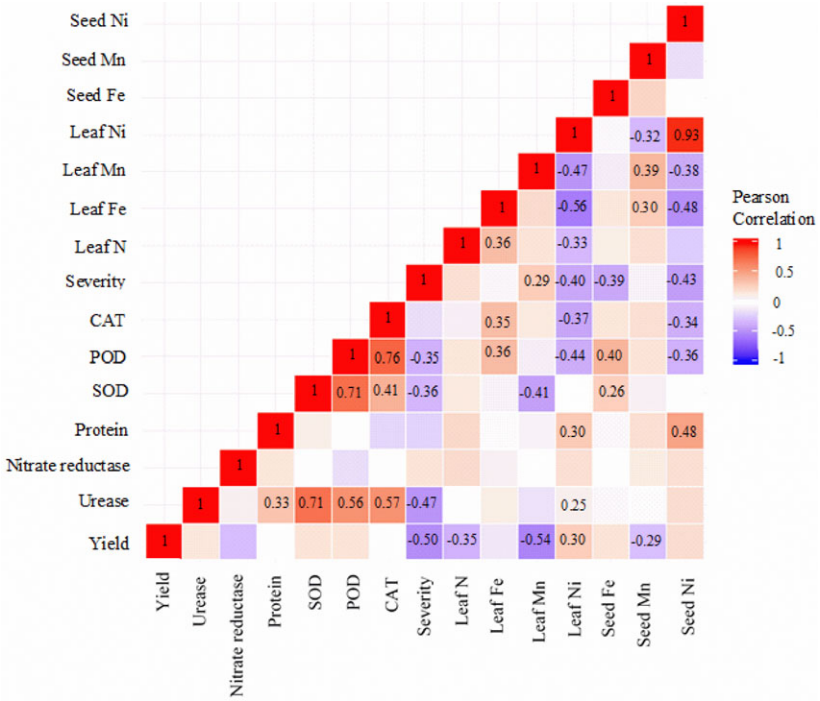

Figure 10 Pearson correlation of analysed parameters in response to Ni and fungicide application. [Colour figure can be viewed at wileyonlinelibrary.com]

The responses of antioxidant enzymes due to Ni fertilization may vary among species. Low Ni doses (<0.2 mM) promote increases in the SOD, CAT and POD activity in soybean (<2 mM), and therefore Ni is considered to stimulate antioxidant metabolism, helping to regulate biotic and abiotic stress responses, and contributing to plant defence (Thakur & Sohal, 2013; Santos *et al.*, 2017).

The Ni doses used did not cause ultrastructural changes in the soybean leaves, and when combined with fungicide, Ni drastically reduced the powdery mildew severity in the soybean leaves. The maximum recommended dose of Ni is 40 g ha^{-1} combined with 300 mL ha^{-1} fungicide to reduce powdery mildew severity and to promote maximal urease, SOD, CAT and POD enzymatic activity.

With increased urease activity, the urea concentration in the leaf decreased, contributing to nitrogen metabolism in the soybean plants. Urease is regulated by Ni and has an important physiological role in reducing the powdery mildew severity in soybean. In the presence of the fungicide, an increase in yield was observed, but the Ni dose applied had no effect on the soybean yield.

This study clearly shows the effectiveness of Ni as a plant protectant. In addition, the distinctive contribution of these findings is that urease is a key enzyme regulated by Ni and is responsible for control of powdery mildew, either with or without fungicide treatment, in soybean plants.

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