

Biochemical and physiological responses of soybean [*Glycine max* (L.) Merrill] to nickel toxicity

Andersom Milech Einhardt¹ , Sandro Ferreira¹ , Fabrício Ávila Rodrigues^{1,*} 

1. Universidade Federal de Viçosa – Departamento de Fitopatologia – Viçosa (MG), Brazil.

Received: Apr. 10, 2020 | Accepted: Jan. 20, 2021

Section Editor: Robson Di Piero

*Corresponding author: fabricao@ufv.br

How to cite: Einhardt, A. M., Ferreira, S., Rodrigues, F. A. (2021). Biochemical and physiological responses of soybean (*Glycine max* (L.) Merrill) to nickel toxicity. *Bragantia*, 80, e1721. <https://doi.org/10.1590/1678-4499.20200152>

ABSTRACT: Despite the crucial role of nickel (Ni) in the plant metabolism, small increases in its concentration can cause leaf tissues injury. This study identified the highest dose of Ni foliar-applied that does not cause toxicity to soybean plants. Plants were sprayed with five Ni doses (0, 30, 60, 120, and 240 g·ha⁻¹). At 1, 3, and 5 days after spray (DAS), the malondialdehyde (MDA), superoxide (O₂⁻), hydrogen peroxide (H₂O₂), and photosynthetic pigments concentrations, antioxidant enzymes activities, and gas exchange and chlorophyll (Chl) *a* fluorescence parameters were determined. Symptoms of Ni toxicity started at 120 g·ha⁻¹ Ni and intense foliar necrosis occurred at 3 DAS. The concentrations of O₂⁻, H₂O₂, and MDA were significantly higher by 49% at 3 DAS, 47% at 3 DAS, and 19% at 5 DAS, respectively, for plants sprayed with 120 g·ha⁻¹ Ni and by 48% at 3 DAS, 48% at 3 DAS and 18% at 5 DAS, respectively, for plants sprayed with 240 g·ha⁻¹ Ni. Higher antioxidant enzymes activities and lower Chl *a* and Chl *b* concentrations occurred for plants sprayed with either 120 and 240 g·ha⁻¹ Ni compared to the other Ni doses. Decrease on energy destined to photochemical process [Y(II)] (8 and 8% at 5 DAS) and increase on the dissipation of energy by the nonregulated process [Y(NO)] (15 and 15% at 5 DAS) occurred for plants sprayed with 120 and 240 g·ha⁻¹ Ni, respectively. The Ni doses above 120 g·ha⁻¹ promoted oxidative stress to the plants and affected the functionality of their photosynthetic apparatus. Doses below 60 g·ha⁻¹ had a low risk of toxicity to plants without causing any biochemical or physiological damage.

Key words: antioxidant enzymes, phytotoxicity, photosynthesis, plant nutrition, ROS.

Nickel (Ni) was the latest element to have its nutritional essentiality recognized for plants (Brown et al. 1987). It is a component of various enzymes, including glyoxalases (family I), hydrogenases, superoxide dismutase and urease (Chen et al. 2009). Inadequate Ni supply promotes changes in the plant metabolism, including processes related to nitrogen metabolism, such as amino acids, urea and ureides metabolisms (Rodríguez-Jiménez et al. 2016; Bai et al. 2006). Legumes that are dependent on N₂ fixation (e.g., soybean) have their process impaired by Ni deficiency, because this element is an essential catalytic cofactor of [NiFe]-hydrogenase, an enzyme found in some symbiotic bacteria that recycles the H₂ produced by a side reaction of nitrogenase in root nodules formed by the plant-bacteria association (Cammack 1995; Bagyinka 2014). Moreover, Ni has shown the potential to control soybean diseases, such as powdery mildew (Barcelos et al. 2018) and Asian soybean rust (Einhardt et al. 2020a; 2020b).

The concentration of Ni required by the majority of plant species is very low (0.01 – 10 mg·kg⁻¹ dry weight), which is an extremely wide range compared to other elements (Gerendás et al. 1999). In soybeans, the critical concentration of Ni deficiency is between 0.02 and 0.04 mg·kg⁻¹ dry weight (Eskew et al. 1984). Even though low Ni concentration is usually sufficient to avoid visual symptoms of deficiency, soybean plants without visual symptoms may suffer from its deficiency (Freitas et al. 2019). On the opposite side, soil contamination with excess Ni can lead to a toxic effect on plants. According to Chen et al. (2009), the interference with other essential metal ions and induction of oxidative stress are two indirect pathways of Ni toxicity in plants. Plants under conditions of Ni-excess show various responses and toxicity symptoms, including retardation of germination, inhibition of growth, reduction of yield, induction of leaf chlorosis and wilting, disruption of photosynthesis, inhibition of CO₂ assimilation, as well as reductions in stomatal conductance



(Chen et al. 2009; Reis et al. 2017). High concentrations of Ni can promote an increase in the accumulation of reactive oxygen species (ROS), which can cause damage to cell membranes (Chen et al. 2009).

Considering the pivotal role of Ni in the metabolism of soybean plants, including the increase of their resistance against diseases and the risk of its toxicity, this study aimed to identify the maximum Ni dose that could not cause toxicity to soybean leaf tissues by examining some alterations at the biochemical and physiological levels.

Soybean seeds from the cultivar TMG135, containing $0.54 \pm 0.03 \mu\text{g Ni}$ per seed (extracted by the nitric-perchloric digestion method [Zasoski and Burau 1977] and determined by inductive coupled plasma optical emission spectrometry [ICP-OES]) were sown in sand washed with HCl 1N. Seven days after sowing, five seedlings were transplanted to each 5 L plastic pots and cultivated in hydroponic system containing nutrient solution of Hoagland and Arnon (1950) with aeration. The nutrient solution was changed every four days and the pH adjusted to 6.0 daily. Plants were kept in a greenhouse (temperature of $28 \pm 3 \text{ }^\circ\text{C}$, relative humidity of $80 \pm 5\%$ and natural radiation). Plants at the V4 growth stage (three trifoliolate expanded leaves) were sprayed with solutions (9 mL per plant) of $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ at the concentrations of 0, 74.6, 149.2, 298.4 and $596.8 \text{ mg}\cdot\text{L}^{-1}$, equivalent to 0, 30, 60, 120 and $240 \text{ g}\cdot\text{ha}^{-1}$ Ni, considering a plant density of 200,000 plants per hectare. For biochemical analysis, the first three trifoliolate leaves of two plants from the replication of each treatment were collected at 1, 3 and 5 days after spray (DAS). Leaf samples were collected in liquid nitrogen and stored at $-80 \text{ }^\circ\text{C}$ until further analysis. Cellular oxidative damage was estimated based on the production of total 2-thiobarbituric acid reactive substances and expressed as equivalents of malondialdehyde (MDA) according to Hodges et al. (1999). The concentrations of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) were quantified according to Chaitanya and Naithani (1994) and Debona et al. (2012), respectively. The activities of superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), and ascorbate peroxidase (APX, EC 1.11.1.1) were determined according to the methodologies described by Debona et al. (2012). The gas exchange and chlorophyll (Chl) *a* fluorescence parameters, as well as the concentration of photosynthetic pigments, were evaluated on the second trifoliolate leaf of each plant at 1, 3, and 5 DAS. The photosynthetic pigments of six leaf-discs (0.8 cm^2 each) were extracted with dimethyl sulfoxide saturated with calcium carbonate (Santos et al. 2008). The concentrations of Chl *a*, Chl *b* and carotenoids were calculated based on the absorbance at 480, 649 and 665 nm (Sumanta et al. 2014). The Chl *a* fluorescence parameters maximum photosystem II quantum efficiency (F_v/F_m), photochemical yield [Y(II)], yield for dissipation by down-regulation [Y(NPQ)], yield for other nonphotochemical (nonregulated) losses [Y(NO)] and apparent electron transport rate (ETR) were obtained by using the Imaging-PAM image fluorometer and the Imaging Win software MAXI version (Heinz Walz GmbH, Effeltrich, Germany) following the procedures described by Fagundes-Nacarath et al. (2018) fixing in 5 min the time of actinic photon irradiance to obtain the steady-state fluorescence yield. The gas exchange parameters rate of net CO_2 assimilation (*A*), stomatal conductance to water vapor (g_s), transpiration rate (*E*) and internal CO_2 concentration (C_i) were determined in the lateral leaflet of the second trifoliolate leaf between 09:00 and 12:00 h, when *A* was at its maximum, under artificial and saturating photon irradiance ($1200 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and an external CO_2 concentration of $400 \mu\text{mol}\cdot\text{mol}^{-1}$ using a portable open-system infrared gas analyzer (LI-6400, LI-COR Inc., Lincoln, NE, USA). All measurements were performed by setting the block temperature at $25 \text{ }^\circ\text{C}$.

The experiment was arranged in a completely randomized design with five treatments (control [plants sprayed with water] and plants sprayed with 30, 60, 120 and $240 \text{ g}\cdot\text{ha}^{-1}$ Ni) with four replications. Each experimental unit consisted of a plastic pot containing five plants. Data from the variables and parameters evaluated were checked for normality and homogeneity of variance and then submitted to analysis of variance. Means of treatments were compared by Tukey's test ($p \leq 0.05$) by using the Minitab software v.18.

No symptoms of Ni toxicity were reported from plants sprayed with doses up to $60 \text{ g}\cdot\text{ha}^{-1}$ Ni (Fig. 1a-c). Irrespective of the evaluation time, soybean plants exhibited symptoms of Ni toxicity starting at $120 \text{ g}\cdot\text{ha}^{-1}$ Ni. Necrotic areas were observed on the leaflets of plants sprayed with 120 and $240 \text{ g}\cdot\text{ha}^{-1}$ Ni, mostly at 5 DAS (Fig. 1d-e). The O_2^- concentration was significantly higher by 49 and 48%, respectively, for plants sprayed with 120 and $240 \text{ g}\cdot\text{ha}^{-1}$ Ni at 3 DAS and by 42% for plants sprayed with $240 \text{ g}\cdot\text{ha}^{-1}$ Ni in comparison to that plants sprayed with water (Fig. 2a). At 3 DAS, H_2O_2 concentration was higher by 47 and 48%, respectively, for plants sprayed with 120 and $240 \text{ g}\cdot\text{ha}^{-1}$ Ni in comparison to plants sprayed with water (Fig. 2b). The MDA concentration at 5 DAS was higher by 19 and 18%, respectively, for plants sprayed with 120 and $240 \text{ g}\cdot\text{ha}^{-1}$ Ni in comparison to plants sprayed with water (Fig. 2c). Superoxide dismutase activity significantly increased at 3 DAS by 57 and 54% for plants sprayed with 120 and $240 \text{ g}\cdot\text{ha}^{-1}$ Ni, respectively, and at 5 DAS by 37% for plants sprayed with $120 \text{ g}\cdot\text{ha}^{-1}$ Ni in comparison to

plants sprayed with water (Fig. 2d). Catalase activity significantly increased at 1 DAS by 26 and 25% for plants sprayed with 120 and 240 g·ha⁻¹ Ni, respectively, and at 3 DAS by 38% for plants sprayed with 240 g·ha⁻¹ Ni in comparison to plants sprayed with water (Fig. 2e). Ascorbate peroxidase activity significantly increased at 3 DAS by 30 and 32% and at 5 DAS by 32 and 31% for plants sprayed with 120 and 240 g·ha⁻¹ Ni, respectively, in comparison to that plants sprayed with water (Fig. 2f). Irrespective of the evaluation time, the Chl *a*, Chl *b*, and Chl *a* + *b* concentrations decreased for plants sprayed with 120 and 240 g·ha⁻¹ Ni, except for Chl *b* at 1 DAS for the dose of 120 g·ha⁻¹ Ni (Fig. 3a-c). Carotenoid concentration decreased by 19 and 14% at 3 and 5 DAS, respectively, for plants sprayed with 120 g·ha⁻¹ Ni in comparison to plants sprayed with water (Fig. 3d).

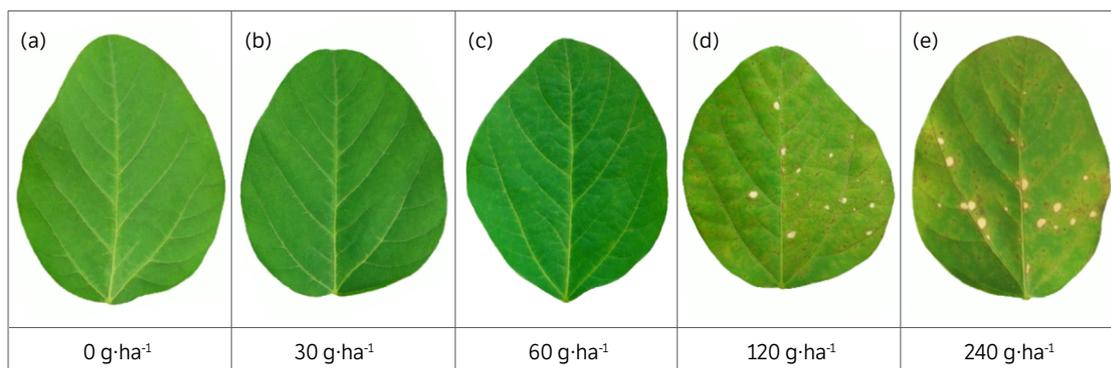


Figure 1. Leaflets from soybean plants at 5 days after being sprayed with water (0) or with solutions containing 30, 60, 120 and 240 g·ha⁻¹ Ni. Doses above 120 g·ha⁻¹ resulted in necrotic areas in the sprayed leaflets.

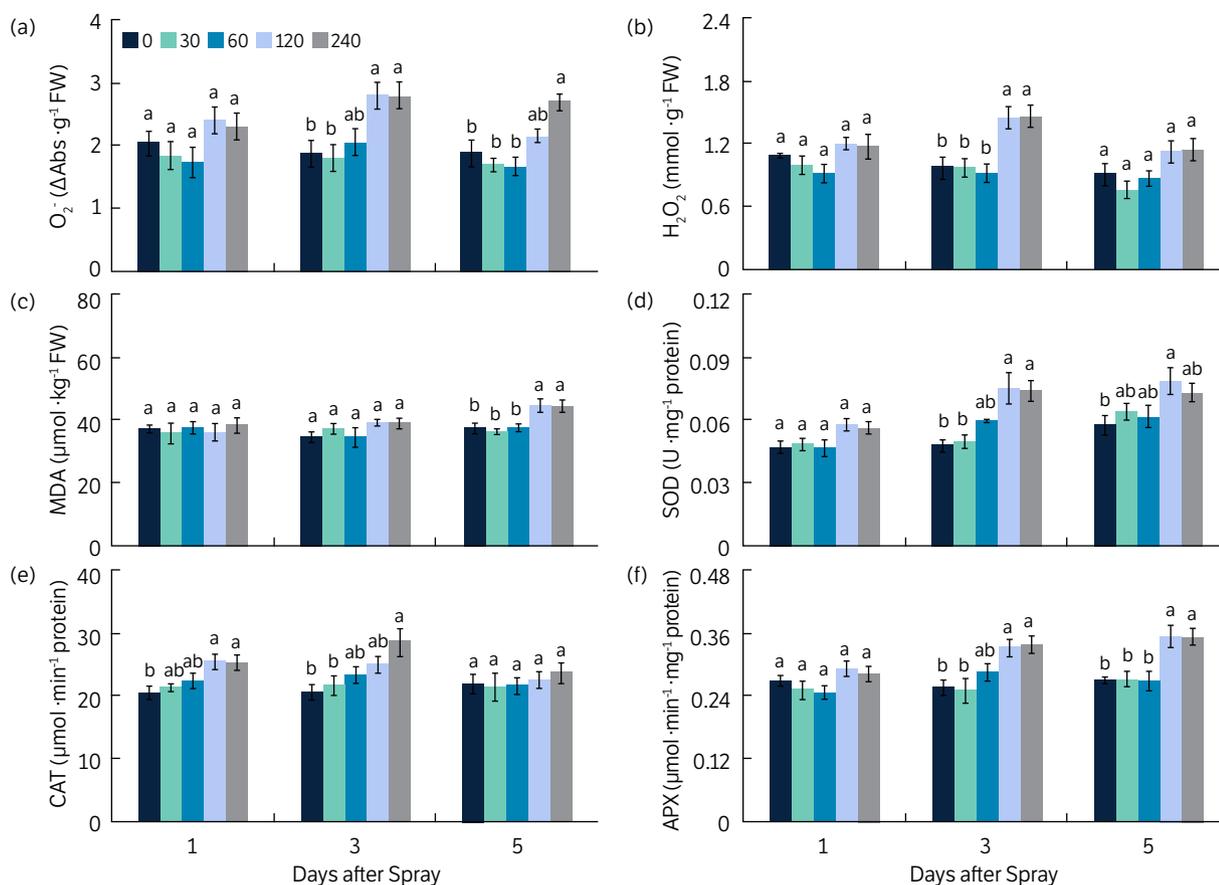


Figure 2. Concentrations of superoxide radical (O₂⁻) (a), H₂O₂ (b) and MDA (c), as well as activities of SOD (d), CAT (e) and APX (f) determined on the leaves of soybean plants sprayed with water (0) or with solutions of 30, 60, 120 and 240 g·ha⁻¹ Ni. Means for each treatment followed by different letters are significantly different (p ≤ 0.05) according to Tukey's test. FW = fresh weight. Bars represent the standard error of the means. n = 4.

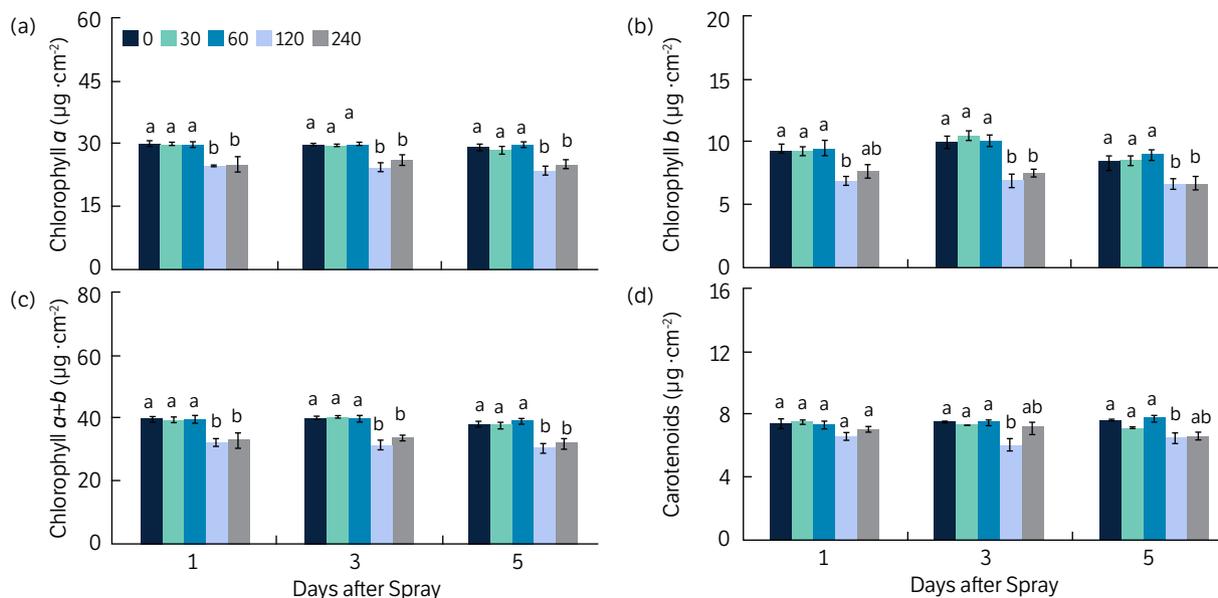


Figure 3. Concentrations of Chl *a* (a), Chl *b* (b), total Chl (Chl *a* + *b*) (c) and carotenoids (d) determined in the leaves of soybean plants sprayed with water (0) or with solutions of 30, 60, 120 and 240 g·ha⁻¹ Ni. Means for each treatment followed by different letters are significantly different ($p \leq 0.05$) according to Tukey's test. Bars represent the standard error of the means. $n = 4$.

Images of Chl *a* fluorescence on the leaflets obtained from plants sprayed with water or sprayed with 30 and 60 g·ha⁻¹ Ni did not show any difference between them regarding color patterns for the parameters maximum photosystem II F_v/F_m , Y(II), Y(NPQ) and Y(NO) (Fig. 4). Alterations in the images of Chl *a* fluorescence parameters were observed on the leaflets of plants sprayed with 120 and 240 g·ha⁻¹ Ni in comparison to leaflets of plants sprayed with water (Fig. 4). The F_v/F_m was significantly lower by 8 and 9% at 1 DAS, by 8 and 9% at 3 DAS and by 8 and 8% at 5 DAS for plants sprayed with 120 and 240 g·ha⁻¹ Ni, respectively, in comparison to plants sprayed with water (Fig. 5a). Similarly to F_v/F_m , Y(II) significantly decreased by 6 and 7% at 1 DAS, 8 and 8% at 3 DAS and 8 and 8% at 5 DAS, respectively, for plants sprayed with 120 and 240 g·ha⁻¹ Ni in comparison to plants sprayed with water (Fig. 5b). No significant difference was observed for Y(NPQ) among the treatments (Fig. 5c). Regarding Y(NO), there were significant increases of 8 and 7% at 1 DAS, 12 and 14% at 3 DAS and 15 and 15% at 5 DAS, respectively, for plants sprayed with 120 and 240 g·ha⁻¹ Ni in comparison to plants sprayed with water (Fig. 5d). Electron transport rate was significantly lower by 10 and 11% at 1 DAS, 10 and 11% at 3 DAS and 9 and 10% at 5 DAS, respectively, for plants sprayed with 120 and 240 g·ha⁻¹ Ni in comparison to plants sprayed with water (Fig. 5e).

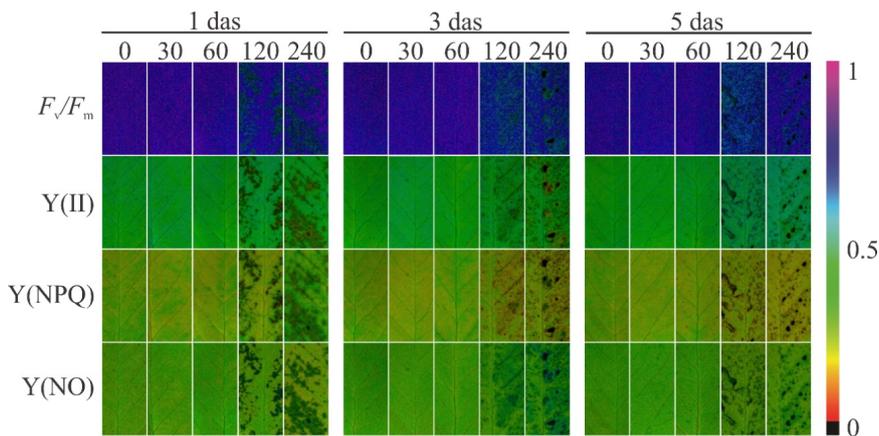


Figure 4. Images of the Chl *a* fluorescence parameters: maximum photochemical efficiency of photosystem II (PSII) (F_v/F_m), effective yield of PSII [Y(II)], yield for dissipation by down-regulation energy [Y(NPQ)] and yield for other nonphotochemical (nonregulated) losses [Y(NO)] from leaflets of soybean plants sprayed with water (0) or with solutions of 30, 60, 120 and 240 g·ha⁻¹ Ni. das = days after spray.

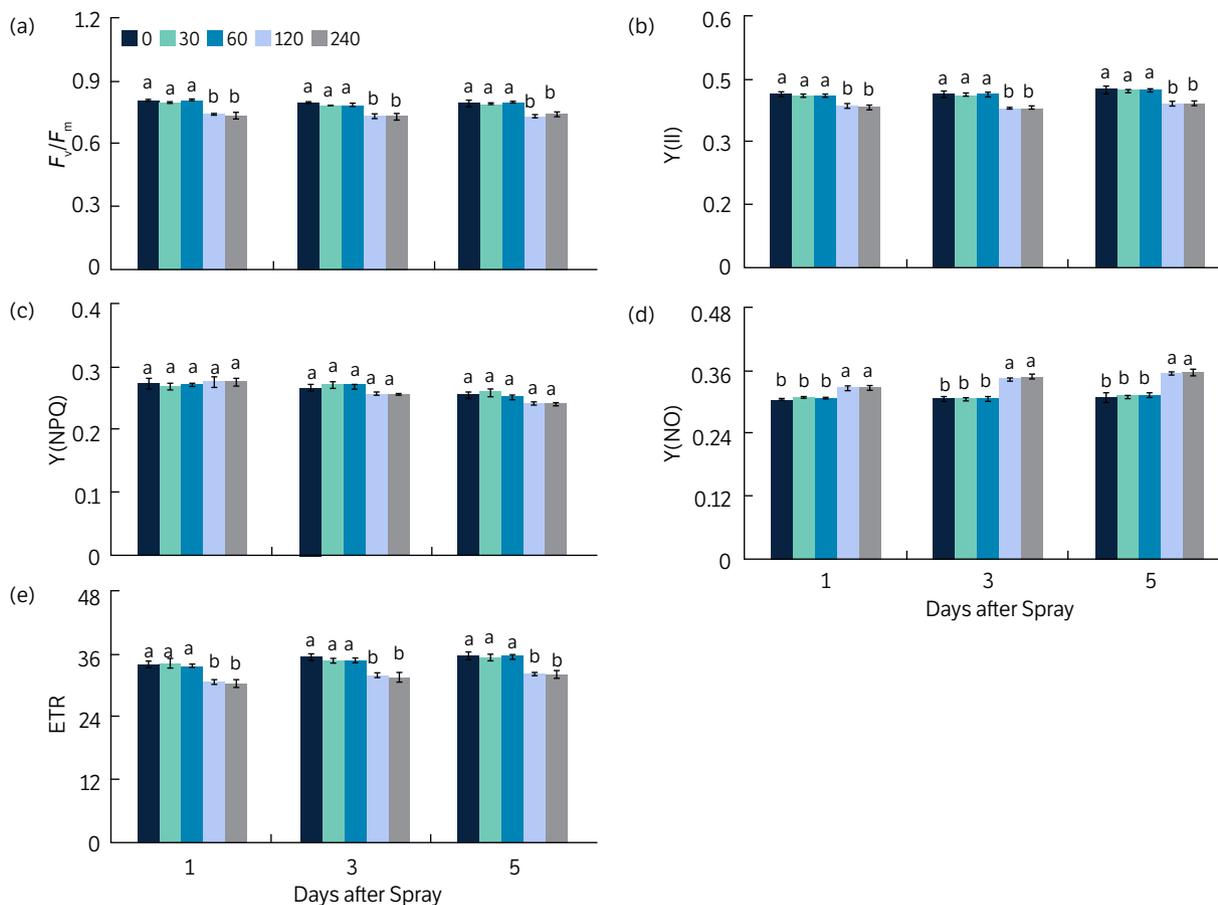


Figure 5. Chlorophyll a parameters: maximum photosystem II quantum efficiency (F_v/F_m) (a), photochemical yield [Y(II)] (b), yield for dissipation by down-regulation [Y(NPQ)] (c), yield for other nonphotochemical (nonregulated) losses [Y(NO)] (d) and apparent ETR (e) determined in the leaflets of soybean plants sprayed with water (0) or with solutions of 30, 60, 120 and 240 g·ha⁻¹ Ni. Means for each treatment followed by different letters are significantly different ($p \leq 0.05$) according to Tukey's test. The bars represent the standard error of the means. $n = 4$.

Mainly, after 3 DAS, there were decreases for A , g_s and E values and increase of C_i values for plants sprayed with 120 and 240 g·ha⁻¹ Ni in comparison to plants sprayed with water or sprayed with 30 and 60 g·ha⁻¹ Ni (Fig. 6A-D). Net CO₂ assimilation values were lower by 21 and 18% at 3 DAS and by 19 and 17% at 5 DAS, respectively, for plants sprayed with 120 and 240 g·ha⁻¹ Ni in comparison to plants sprayed with water (Fig. 6a). Regarding g_s , there were significant decreases of 18 and 20% at 3 DAS and 27 and 26% at 5 DAS, respectively, for plants sprayed with 120 and 240 g·ha⁻¹ Ni in comparison to plants sprayed with water (Fig. 6b). Internal CO₂ concentration values were higher by 8 and 8% at 3 DAS and by 8 and 9% at 5 DAS, respectively, for plants sprayed with 120 and 240 g·ha⁻¹ Ni in comparison to plants sprayed with water (Fig. 6c). The E values were lower by 11 and 11% at 3 DAS and by 21 and 19% at 5 DAS, respectively, for plants sprayed with 120 and 240 g·ha⁻¹ Ni in comparison to plants sprayed with water (Fig. 6d).

The results of the present study demonstrated that the foliar spray of Ni at doses up to 60 g·ha⁻¹ was no toxic while doses above 120 g·ha⁻¹ caused oxidative stress and damage to the photosynthetic apparatus of soybean leaf tissues. Plants sprayed with doses above 120 g·ha⁻¹ Ni presented chlorosis of leaves followed by tissue necrosis corroborating with the symptoms of Ni toxicity reported by Gajewska et al. (2006) and Antonkiewicz et al. (2016). The ROS are continuously produced in plant tissues as byproducts of the metabolic process (Dat et al. 2000). However, abiotic and biotic stresses in plants promote overproduction and accumulation of ROS. The O₂⁻ and H₂O₂ are two moderate ROS and may be converted to hydroxyl (OH) and hydroperoxyl radicals (HO₂⁻) causing cellular damage by lipid peroxidation (Demidchik 2015; Zhang et al. 2010). The increase on O₂⁻ and H₂O₂ concentrations associated with the highest MDA concentration in plants supplied with 120 and

240 g·ha⁻¹ Ni indicated that these doses caused oxidative stress in the leaves resulting in lipid peroxidation. Moreover, the increased antioxidant enzyme activities were not sufficient to detoxify the ROS on the cells of plant tissues sprayed with 120 and 240 g·ha⁻¹ Ni. Similarly, the major cause of Ni toxicity in the roots of grapevine (Pavlovkin et al. 2016) and in the leaves of wheat (Gajewska and Skłodowska 2007) was the oxidative stress resulting from an imbalance in the generation and or removal of ROS.

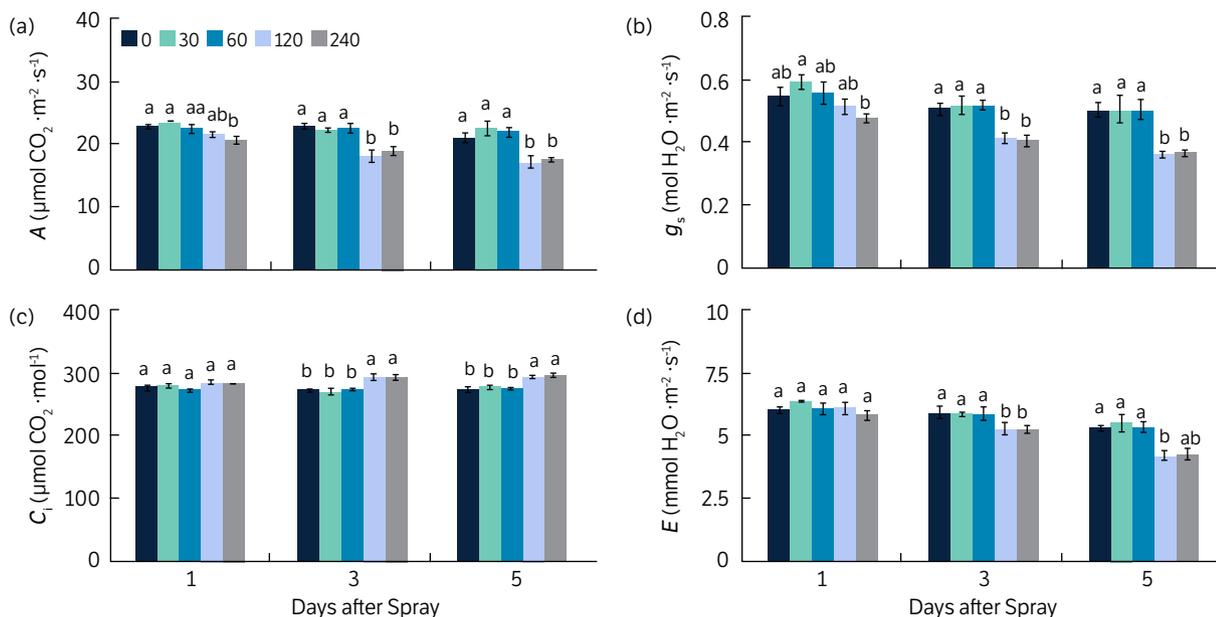


Figure 6. Leaf gas exchange parameters: rate of A (a), g_s (b), C_i (c) and transpiration rate (E) (d) determined in the leaflets of soybean plants sprayed with water (0) or with solutions of 30, 60, 120 and 240 g·ha⁻¹ Ni. Means for each treatment followed by different letters are significantly different ($p \leq 0.05$) according to Tukey's test. The bars represent the standard error of the means. $n = 4$.

The Ni does not generate ROS directly because it is not a redox-active metal (Chen et al. 2009). However, Ni interferes indirectly with several antioxidant enzymes, increasing or decreasing their activities (Baccouch et al. 1998; Gajewska and Skłodowska 2005). In the present study, the effect of high doses of Ni, increasing the accumulation of ROS, cannot be associated with a decrease in the activity of antioxidant enzymes. However, it is reasonable to relate the increase in ROS with the toxic effect of Ni on photosynthesis. Several indirect and direct paths are known, which lead to nonspecific metal inhibition of photosynthesis (Yusuf et al. 2011). The Ni excess damages the structure of thylakoid membranes and the structure of grana (Szalontai et al. 1999; Molas 2002), reducing the size of grana and increasing the number of nonappressed lamellae (Molas 1997). In fact, in the present study, high doses of Ni (120 and 240 g·ha⁻¹) caused damage to the photosynthetic apparatus based on the low values of F_v/F_m , $Y(II)$ and ETR and high $Y(NO)$ values. An increase on the dissipation of energy by nonregulated process [$Y(NO)$] generally leads to an increase in ROS production that may have damaged the photosystems and other cellular constituents (Huang et al. 2018).

Distinctly from what was reported by Barcelos et al. (2017), we did not observe an increase on the concentration of photosynthetic pigments in the leaves of plants sprayed with nontoxic Ni concentrations in comparison to plants sprayed with water. These results were distinct probably due to the early plant growth stage evaluated in this study and the advanced plant growth stage reported by Barcelos et al. (2017). Considering the low Ni concentration demanded by plants, it is plausible to take into account that the Ni content in the seeds was sufficient to supply the plants demand at their growth stage evaluated with no effect on the synthesis of photosynthetic pigments. The decreases on Chl concentrations in the leaves of plants sprayed with toxic Ni doses (above 120 g·ha⁻¹ Ni) are in agreement with the findings of Gopal et al. (2002), Gajewska et al. (2006) and Freitas et al. (2019) and can be attributed to disturbances in the synthesis of pigments (Stobart et al. 1985), as well as an increase on their degradation (Somashkaraiah et al. 1992).

The limitations in the capacity of the photosynthetic apparatus to capture light and direct energy to photochemical process associated with limitation at stomatal conductance level on leaves of plants sprayed with 120 and 240 g·ha⁻¹ Ni resulted in a decrease on the capacity to process CO₂. Plant leaf tissues exposed to heavy metals frequently decrease the stomatal aperture (Rucińska-Sobkowiak 2016). Reduction in stomatal conductance in leaves of soybean plants under Ni stress was observed by Reis et al. (2017). Rauser and Dumbroff (1981) reported that leaf tissues of common bean plants treated with Ni showed an increased level of abscisic acid, which is known to cause stomatal closure. Decreased stomatal conductance, associated with the limitations of light capture and use of its energy, already mentioned, may explain the increased C_i values observed for plants sprayed with 120 and 240 g·ha⁻¹ Ni and probably not associated with biochemical limitations.

In conclusion, the spray of Ni doses above 120 g·ha⁻¹ promoted oxidative stress on the leaf tissues of soybean plants and affected the functionality of their photosynthetic apparatus. The Ni doses below 60 g·ha⁻¹ had a low risk to cause toxicity to soybean plants considering the absence of biochemical or physiological damage. It is imperative to consider that Ni concentration supplied by the soil when plants are grown in the field may decrease the threshold line of toxicity at lower levels than what is reported in the present study.

AUTHORS' CONTRIBUTION

Conceptualization: Einhardt A. M., Ferreira S., and Rodrigues F. A.; **Methodology:** Einhardt A. M., Ferreira S., and Rodrigues F. A.; **Investigation:** Einhardt A. M. and Ferreira S.; **Writing – Original Draft:** Einhardt A. M. and Ferreira S.; **Writing – Review and Editing:** Rodrigues F. A.; **Funding Acquisition:** Rodrigues F. A.; **Resources:** Rodrigues F. A.; **Supervision:** Rodrigues F. A.

DATA AVAILABILITY STATEMENT

Data will be available upon request.

FUNDING

Conselho Nacional de Desenvolvimento Científico e Tecnológico
[<https://doi.org/10.13039/501100003593>]

Fundação de Amparo à Pesquisa do Estado de Minas Gerais
[<https://doi.org/10.13039/501100004901>]

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
[<https://doi.org/10.13039/501100002322>]
Finance Code 001

ACKNOWLEDGMENTS

Prof. Rodrigues thanks to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for his fellowship.

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