

Ionic Homeostasis and Redox Metabolism Upregulated by 24-Epibrassinolide are Crucial for Mitigating Nickel Excess in Soybean Plants, Enhancing Photosystem II Efficiency and Biomass

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Research Article

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Abstract

Nickel (Ni) excess often generates oxidative stress in chloroplasts, causing redox imbalance, membrane damage and negative impacts on biomass. 24-Epibrassinolide (EBR) is a plant growth regulator of great interest in the scientific community because it is a natural molecule extracted from plants that is biodegradable and environmentally friendly. This study aimed to determine whether EBR can induce benefits on ionic homeostasis and antioxidant enzymes and convey possible repercussions on photosystem II efficiency and biomass, more specifically evaluating nutritional, physiological, biochemical and morphological responses in soybean plants subjected to Ni excess. The experiment was randomized with four treatments, including two Ni concentrations (0 and 200 μM Ni, described as $- \text{Ni}^{2+}$ and $+ \text{Ni}^{2+}$, respectively) and two concentrations of 24-epibrassinolide (0 and 100 nM EBR, described as $- \text{EBR}$ and $+ \text{EBR}$, respectively). In general, Ni caused deleterious modulatory effects on chlorophyll fluorescence and gas exchange. In contrast, EBR enhanced the effective quantum yield of PSII photochemistry (15%) and electron transport rate (19%) due to upregulation of superoxide dismutase, catalase, ascorbate peroxidase and peroxidase. Exogenous EBR application promoted significant increases in biomass, and these results were explained by the benefits on nutrient contents and ionic homeostasis, demonstrated by increased $\text{Ca}^{2+}/\text{Ni}^{2+}$, $\text{Mg}^{2+}/\text{Ni}^{2+}$ and $\text{Mn}^{2+}/\text{Ni}^{2+}$ ratios.

Introduction

Soybean [*Glycine max* (L.) Merr.] is a leguminous species that is highly relevant to the Brazilian economy and other producer countries because grains are rich in proteins and oils (Bamji and Corbitt, 2017) and are considered a commodity with several possibilities, primarily human nutrition and animal feed (Majumdar et al., 2019). However, environmental problems connected to heavy metals have been verified in cultivated areas with soybean crops, reducing plant performance and subsequent yield (Küpper and Andresen, 2016; Reis et al., 2017).

Soil contamination by heavy metals often occurs due to inadequate crop management and intensively applied pesticides, fertilizers, and petroleum products (Ayangbenro and Babalola, 2017; Mir et al., 2018), in which these compounds are applied indiscriminately and can cause deleterious effects on plants (Aprile and De Bellis, 2020). Nickel (Ni) excess in agronomic crops is a theme of great importance for food security, attracting the attention of researchers worldwide due to representing a recurrent problem in modern agriculture (M. Yusuf et al., 2011), in which this element is found in contaminated environments as Ni^{2+} (Ameen et al., 2019).

Ni excess often impacts biomass, which is correlated with inadequate uptake, transport and distribution of macro- and micronutrients (Matraszek et al., 2016), including strong limitations on the absorption of Mg, Mn, Zn and Fe (Palacios et al. 1998; Torres et al. 2016). Phytotoxicity linked to Ni negatively modulates photochemical efficiency (Ribeiro et al., 2020), gas exchange (Nazir et al., 2019), water relations and protein biosynthesis (Azeem, 2018). These deleterious effects are occasioned by the overproduction of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide (O_2^-)

and hydroxyl radicals (-OH) (Amari et al., 2017; Yan et al., 2010). Oxidative stress generated in chloroplasts causes redox imbalance and membrane damage (Gajewska et al., 2006; Israr et al., 2011; Pietrini et al., 2015; Rizwan et al., 2018; Sreekanth et al., 2013).

Brassinosteroids are plant growth regulators that act by stimulating biochemical reactions and physiological responses and modulating cellular functions (Jan et al., 2018; Rahman et al., 2017). Among steroids, there are more than 50 natural and synthetic forms, of which 24-epibrassinolide (EBR) is of great interest to the scientific community because it is a natural molecule extracted from plants that is both biodegradable and environmentally friendly. This molecule plays multiple roles, stimulating chloroplast pigments (Parmoon et al., 2018), light capture (Lima and Lobato, 2017; Yusuf et al., 2017) and CO₂ fixation (Kohli et al., 2017; Sharma et al., 2016), biosynthesis of nucleic acids (Bajguz, 2000; Tanveer et al., 2018) and tissue structures (Fonseca et al., 2020; Rajewska et al., 2016; Ribeiro et al., 2019). Focusing on plant nutrition, Lima et al. (2018) verified that EBR increased the content of essential elements, improving the nutritional balance (Oliveira et al., 2018) and the activities of H⁺-ATPase enzymes in the root system (Song et al., 2016). In parallel, the literature has verified that EBR positively modulates the antioxidant system (Ahanger et al., 2020; Rodrigues et al., 2020), more specifically controlling ROS overaccumulation (Anjum et al., 2017; Ashraf et al., 2015).

The hypothesis of this research was developed on the deleterious effects provoked by Ni on photochemical efficiency (Drażkiewicz and Baszyński, 2010; Pietrini et al., 2015) and growth (Parida et al., 2003; Rahman et al., 2005) verified in plants grown in environments contaminated by this heavy metal. However, relevant roles linked to EBR in nutritional status (Lima et al., 2018) and the antioxidant system (Santos et al., 2020) suggest that this growth regulator may represent an interesting option to mitigate the negative impacts induced by Ni. This study aimed to determine whether EBR induces benefits for ionic homeostasis and antioxidant enzymes and to reveal possible repercussions on photosystem II efficiency and biomass, more specifically evaluating nutritional, physiological, biochemical and morphological responses in soybean plants subjected to Ni excess.

Materials And Methods

Location and growth conditions

This experiment was performed at the Campus of Paragominas of the Universidade Federal Rural da Amazônia, Paragominas, Brazil (2°55' S, 47°34' W). This study was conducted in a greenhouse with controlled temperature and humidity. The minimum, maximum, and median temperatures were 23.4, 29.8 and 26.3°C, respectively. The relative humidity during the experimental period varied between 60% and 80%.

Plants, containers and acclimation

Seeds of *Glycine max* (L.) Merr. var. M8644RR Monsoy™ were germinated and grown in 1.2-L pots filled with a mixed substrate of sand and vermiculite at a ratio of 3:1. The plants were cultivated under semi-hydroponic conditions containing 500 mL of distilled water for four days. A nutritive solution described by Pereira et al. (2019) was used for plant nutrition, with ionic strength beginning at 50% (4th day) and later modified to 100% after two days (6th day). After this period, the nutritive solution remained at total ionic strength.

Experimental design

The experiment was randomized with four treatments, including two Ni concentrations (0 and 200 μM Ni, described as $-\text{Ni}^{2+}$ and $+\text{Ni}^{2+}$, respectively) and two concentrations of 24-epibrassinolide (0 and 100 nM EBR, described as $-\text{EBR}$ and $+\text{EBR}$, respectively). Five replicates for each of the four treatments were conducted, yielding a total of 20 experimental units, with one plant per unit.

24-Epibrassinolide (EBR) preparation and application

Ten-day-old plants were sprayed with 24-epibrassinolide (EBR) or Milli-Q water (containing a proportion of ethanol that was equal to that used to prepare the EBR solution) at 5-d intervals until day 30. EBR (0 and 100 nM, Sigma-Aldrich, USA) solutions were prepared by dissolving the solute in ethanol followed by dilution with Milli-Q water [ethanol:water (v/v) = 1:10,000] (Ahammed et al., 2013).

Plant conduction and Ni treatment

Plants received the following macro- and micronutrients contained in the nutrient solution in agreement with Pereira et al. (2019). To simulate high Ni concentration, NiCl_2 was used at concentrations of 0 and 200 μM Ni, which was applied over 8 days (days 22–30 after the start of the experiment). During the study, the nutrient solutions were changed at 07:00 h at 3-day intervals, with the pH adjusted to 5.5 using HCl or NaOH. On day 30 of the experiment, physiological and morphological parameters were measured for all plants, and leaf tissues were harvested for biochemical and nutritional analyses.

Determination of Ni and nutrients

Milled samples (100 mg) of root, stem and leaf tissues were pre-digested in conical tubes (50 mL) with 2 ml of sub boiled HNO_3 . Subsequently, 8 ml of a solution containing 4 ml of H_2O_2 (30% v/v) and 4 ml of ultra-pure water were added and transferred to a Teflon digestion vessel in agreement with Paniz et al. (2018). Determination of Ni, P, Ca, Mg, Mn, Zn and Fe was performed using an inductively coupled plasma mass spectrometer (model ICP-MS 7900; Agilent).

Measurement of chlorophyll fluorescence and gas exchange

Chlorophyll fluorescence was measured in fully expanded leaves under light using a modulated chlorophyll fluorometer (model OS5p; Opti-Sciences). Preliminary tests determined the location of the leaf, the part of the leaf and the time required to obtain the greatest F_v/F_m ratio; therefore, the acropetal third of the leaves, which was the middle third of the plant and was adapted to the dark for 30 min, was used in the evaluation. The intensity and duration of the saturation light pulse were $7,500 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 0.7 s, respectively. Gas exchange was evaluated in all plants and measured in the expanded leaves in the middle region of the plant using an infrared gas analyser (model LCPro⁺; ADC BioScientific) in a chamber under constant CO_2 , photosynthetically active radiation, air-flow rate and temperature conditions at $360 \mu\text{mol mol}^{-1} \text{CO}_2$, $800 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, $300 \mu\text{mol s}^{-1}$ and 28°C , respectively, between 10:00 and 12:00 h.

Determination of antioxidant enzymes, superoxide and soluble proteins

Antioxidant enzymes (SOD, CAT, APX, and POX), superoxide, and soluble proteins were extracted from leaf tissues according to the method of Badawi et al. (2004). Total soluble proteins were quantified using the methodology described by Bradford (1976). SOD activity was measured at 560 nm (Giannopolitis and Ries, 1977), and SOD activity is expressed in mg^{-1} protein. The CAT assay was detected at 240 nm (Havir and McHale, 1987), and the CAT activity is expressed in $\mu\text{mol H}_2\text{O}_2 \text{mg}^{-1} \text{protein min}^{-1}$. The APX assay was measured at 290 nm (Nakano and Asada, 1981), and APX activity is expressed in $\mu\text{mol AsA mg}^{-1} \text{protein min}^{-1}$. The POX assay was detected at 470 nm (Cakmak and Marschner, 1992), and activity is expressed in $\mu\text{mol tetraguaiacol mg}^{-1} \text{protein min}^{-1}$. O_2^- was measured at 530 nm (Elstner and Heupel, 1976).

Quantification of hydrogen peroxide, malondialdehyde and electrolyte leakage

Stress indicators (H_2O_2 and MDA) were extracted using the methodology described by Wu et al. (2006). H_2O_2 was measured using the procedures described by Velikova et al. (2000). MDA was determined by the method of Cakmak and Horst (1991) using an extinction coefficient of $155 \text{mM}^{-1} \text{cm}^{-1}$. EL was measured according to Gong et al. (1998) and was calculated by the formula $\text{EL (\%)} = (\text{EC}_1/\text{EC}_2) \times 100$.

Determination of photosynthetic pigments and biomass

Chlorophyll and carotenoid determinations were performed using a spectrophotometer (model UV-M51; Bel Photonics) according to the methodology of Lichtenthaler and Buschmann (2001). The biomass of roots and shoots was measured based on constant dry weights (g) after drying in a forced-air ventilation oven at 65°C.

Data analysis

Data were subjected to an analysis of variance, and significant differences between the means were determined using the Scott-Knott test at a probability level of 5% (Steel et al., 2006). Standard deviations were calculated for each treatment.

Results

Plants pretreated with EBR exhibited reduced Ni²⁺ content in tissues

Ni content was significantly increased in the root, stem and leaf tissues (Table 1). However, EBR spray on plants exposed to Ni reduced ($P < 0.05$) its content in the root, stem and leaf by 38%, 16% and 25%, respectively, compared to plants that received Ni without EBR pretreatment.

Steroids mitigate the stress caused by Ni on nutritional status

Ni excess caused reductions in nutrient content (Table 2). In contrast, treatment with EBR and Ni²⁺ resulted in increased P, Ca, Mg, Mn, Zn and Fe by 14%, 9%, 13%, 21%, 24% and 19% in root tissue, respectively; 18%, 11%, 81, 16%, 18% and 8% in stem, respectively; and 11%, 12%, 39%, 13%, 31% and 17% in leaf, respectively, compared to plants that received Ni²⁺ without EBR. For ionic ratios (Table 3), plants treated with Ni²⁺ excess and sprayed with EBR resulted in increments in Ca²⁺/Ni²⁺, Mg²⁺/Ni²⁺ and Mn²⁺/Ni²⁺ ratios of 77%, 84% and 100% in root, 33%, 115% and 42% in stem and 49%, 86% and 55% in leaf, respectively, compared to equal treatment without EBR.

Pretreatment with EBR minimizes the impacts of Ni on photosynthetic machinery

Ni caused negative changes in chlorophyll fluorescence (Fig. 1). However, EBR spray in plants exposed to Ni²⁺ produced significant increases of 4%, 5% and 1% in the values of F_m , F_v and F_v/F_m , respectively, compared to the same treatment without EBR. For chlorophyll fluorescence (Table 4), treatment with Ni²⁺ + EBR, the variables Φ_{PSII} , q_p and ETR exhibited increments ($P < 0.05$) of 15%, 32% and 19%, respectively.

Plants pretreated with EBR and submitted to Ni^{2+} exhibited reductions of 21% and 5% in NPQ and EXC, respectively, compared to equal treatment in the absence of EBR. Ni generated deleterious effects on gas exchange (Table 4). However, pre-treatment with EBR in plants stressed with Ni resulted in significant increases in P_N , g_s , WUE, and P_N/C_i , corresponding to 20%, 19%, 16%, and 23%, respectively. Additionally, increased E (3%) and reduced C_i (4%) were verified compared to plants that received the same treatment without EBR. With respect to photosynthetic pigments, a high Ni supply occasioned damage to chlorophyll and carotenoids (Table 4). On the other hand, the EBR and Ni^{2+} treatment significantly increased Chl *a*, Chl *b*, total Chl and Car by 37%, 40%, 37% and 31%, respectively, as well as an increase in total Chl/Car (4%) and a decrease in Chl *a*/Chl *b* (2%) compared to the same treatment without EBR application.

Antioxidant responses are upregulated in plants treated with EBR and exposed to Ni

Adverse effects promoted by Ni increased the activities of antioxidant enzymes (Fig. 2). However, plants treated with both EBR and Ni presented significant increases in SOD, CAT, APX and POX of 50%, 27%, 40% and 19%, respectively, compared to treatment without EBR. Ni stress induced increases in stress indicators (Fig. 3), but EBR treatment in plants under Ni excess promoted significant reductions in O_2^- , H_2O_2 , MDA and EL of 20%, 5%, 9% and 10%, respectively, compared to the $\text{Ni}^{2+} + 0$ nM EBR treatment.

EBR suppresses the negative impacts on biomass caused by Ni excess

Toxic action promoted by Ni provoked a decrease in biomass (Fig. 4), but EBR treatment in plants under Ni stress mitigated these effects, with increases of 7%, 12%, 25% and 7% in LDM, RDM, SDM and TDM, respectively, compared to the same treatment without EBR.

Discussion

Soybean plants pretreated with EBR exhibited reductions in Ni content in the roots, stems and leaves. These results indicate that this steroid likely maximizes the endogenous levels of phytochelatin, acting on immobilization and detoxification of excess Ni^{2+} ions into the plant cells (Rajewska et al., 2016). Concomitantly, there was a decrease in the negative effects of this heavy metal on Fe, Zn, Mn and Mg contents, improving the absorption and accumulation of these metals, corroborated by the results obtained in this research. Ahmad et al. (2018b) studied the effects of EBR application (10^{-6} M) in *Cicer arietinum* seedlings subjected to Hg toxicity (15 μM and 30 μM), observing significant reductions in Hg contents in roots and leaves, in addition to increases in Mg, Mn and Ca contents. Surgun et al. (2016) evaluated B toxicity in *Arabidopsis thaliana*-treated EBR and observed a reduction in B contents in the

tissues of the leaf, root and inflorescence, and these results were explained by the authors as due to the better selectivity of the membrane enzymes. Sharma et al. (2011) studied the mechanisms of action connected to EBR in *Raphanus sativus* seedlings under Ni stress and found increases in root length and activities of antioxidant enzymes in shoot.

Steroid mitigated the stress caused by Ni on the contents of macro- (P, Ca, Mg) and micronutrients (Mn, Fe, Zn), improving nutritional status. These results can be explained by the systemic role promoted by the EBR, stimulating root structures intrinsically related to selectivity and protection of the root tissue against Ni (Ranathunge et al., 2003; Saraiva et al., 2021), as well as protection against biotic and abiotic stress (Barberon et al., 2016; Cui et al., 2016). Matraszek et al. (2017) studied EBR's effects on the nutritional status of *Sinapis alba* exposed to four Ni concentrations (0, 0.0004, 0.04 and 0.08 mM Ni) and observed reductions in P, Ca and Mg contents. Yuan et al. (2015) evaluated the roles of EBR in nutrient accumulation in *Cucumis sativus* plants under $\text{Ca}(\text{NO}_3)_2$ stress and described increases in K, P, Mg, Fe and Mn contents in shoot and root tissues. Jan et al. (2018) reported increases in macronutrient contents, more specifically Mg, Ca and P, in *Pisum sativum* pretreated with EBR (individual or combined with silicon) under cadmium (Cd) stress.

$\text{Ca}^{2+}/\text{Ni}^{2+}$, $\text{Mg}^{2+}/\text{Ni}^{2+}$ and $\text{Mn}^{2+}/\text{Ni}^{2+}$ ratios were increased after EBR spray in leaves, stems and roots. These results reveal multiple benefits of this steroid on homeostasis, increasing ionic ratios and decreasing the stress generated by Ni (Reis et al., 2017; Ribeiro et al., 2020), confirmed by increases in Ca, Mg and Mn contents and other elements evaluated in this research. Hu et al. (2016) studied the EBR effects in *Solanum tuberosum* plants under salt stress conditions and described positive effects of this steroid on homeostasis connected to the K^+/Na^+ ratio, combined with higher root efficiency and improvement in antioxidant capacity in shoots.

Pretreatment with EBR minimized the impacts of Ni on F_v , F_m and F_v/F_m . Our results demonstrated that this steroid provided protection for photosynthetic machinery, including benefits on the absorption of light energy by chloroplasts. EBR clearly alleviated oxidative damage due to increases in the activities of antioxidant enzymes (SOD, CAT, APX and POX) measured in this research, resulting in reduced concentrations of oxidative compounds, such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), revealing a protective role for EBR on chloroplast ultrastructure (Sadeghi and Shekafandeh, 2014). According to Wani et al. (2017), BRs protect PSII against excessive excitation in abiotic stress conditions, preventing possible damage to thylakoid membranes. The positive effects of EBR also induced increases in Φ_{PSII} , ETR and q_p , which were related to the benefits of F_0 and F_m , as verified in this study. This result demonstrates better absorption and photon capture and maintenance of Q_A oxidation, improving the flow of electrons through PSII. Additionally, EBR reduced EXC, ETR/P_N and NPQ, demonstrating higher efficiency in the use of light and decreased use in secondary processes. Palliotti et al. (2015) studied *Vitis vinifera* genotypes under conditions of water restriction and reported an increase in ETR/P_N ; this result was associated with a possible imbalance in the production of electrons during water photolysis and use in photosynthetic machinery, suggesting an increase in alternative drains, including photorespiration. The

stress generated by Ni reduced F_v/F_m and NPQ, indicating inhibition of light absorption and energy accumulation in the antenna complex and developing favourable conditions for the overproduction of reactive oxygen species (ROS), which in contact with the membrane cause severe damage to thylakoid structures and pigments (Anjum et al., 2016). Bukhari et al. (2016) described that EBR application attenuated the stress generated by Cr and increased the F_v/F_m values in *Nicotiana tabacum* seedlings. Pietrini et al. (2015) studied the deleterious effects caused by Ni on chlorophyll fluorescence in plants of *Amaranthus paniculatus* and detected reductions in the values of Φ_{PSII} , q_p and NPQ, compromising the functioning of PSII. Research conducted by Santos et al. (2018) using *Vigna unguiculata* plants sprayed with EBR and under Cd toxicity obtained significant improvements in the values of EXC, ETR, ETR/P_N and NPQ.

EBR mitigated the negative effects caused by Ni on gas exchange. Increases in P_N and WUE promoted by EBR can be explained by the positive effects on PPT and SPT observed in this study. These tissues have a large amount of chloroplasts and contribute to the formation of intercellular spaces that accumulate CO_2 essential for the photosynthetic process (Sorin et al., 2015). Increments observed in E and g_s after EBR application are related to increases in SD and SI detected in this research, suggesting higher efficiency in gas exchange, including the transpiration process and CO_2 assimilation. Our results also indicate that the increase in P_N/C_i values and reduction in C_i occurred due to EBR actions on possible increases in ribulose-1,5-bisphosphate carboxylase/oxygenase activity (reduction in C_i) and by CO_2 fixation (increase in P_N) during the photosynthetic process (Farooq et al., 2009; Shu et al., 2016; X.-J. Xia et al., 2009). Ni interference impaired gas exchange, reducing P_N , g_s , and E , which was related to stomatal limitations, confirmed by reductions in SF and SI, and the non-stomatal implications, corroborated by the overproduction of O_2^- and H_2O_2 , were verified in this research. Shah et al. (2019) investigated photosynthetic responses and the antioxidant system in *Cucumis sativus* plants treated with EBR (5 μM) that were subjected to Cd stress (2.5 mM) and confirmed that steroids attenuated ($P < 0.05$) the effects of heavy metals on P_N , C_i , g_s , and E . Santos et al. (2020) evaluated gas exchange and anatomical structures in *Glycine max* plants exposed to Zn stress and treated with EBR, obtaining increased P_N , E , g_s , WUE and P_N/C_i and reduced C_i . Khan et al. (2017) measured the deleterious effects provoked by soil contamination with Ni (50, 100 and 200 mg Ni kg^{-1} soil) on gas exchange in *Vinca rosea* plants and described reductions in g_s , E and P_N . Khaliq et al. (2016) studied alterations caused by Ni toxicity (50 and 100 μM) on carbon fixation in *Gossypium hirsutum* plants and verified significant reductions in E and P_N .

Ni excess was partially suppressed by exogenous application of EBR, with increases in SOD, CAT, APX and POX activities. EBR clearly improved the performance of the antioxidant system, resulting in more efficient elimination of reactive oxygen species (ROS) due to the positive regulation exerted by this steroid on gene expression connected to these enzymes and subsequent quantitative activation of the antioxidant system (Mohammad Yusuf et al., 2011), reducing damage to the structure of chloroplast cells (Sharma et al., 2017). Increases linked to these enzymes are intrinsically related to the maintenance of photosynthetic pigments (Chl *a*, Chl *b* and Car), and significant reductions in stress indicators (H_2O_2 and

O_2^-) were verified in our research. Cao et al. (2005) investigated the biochemical and molecular responses in *Arabidopsis thaliana* with loss of function for the *DET2* gene and interestingly described that the mutation of this gene increased the transcripts linked to the antioxidant system, reducing simulated oxidative stress. Xia et al. (2009b) observed higher tolerance to stress in *Cucumis sativus* leaves treated with EBR, positively regulating the gene expression and activities of enzymes related to antioxidant metabolism. Oliveira et al. (2019) described that pretreatment with 100 nM EBR in Na^+ stressed *Eucalyptus urophylla* plants resulted in increased SOD, CAT, APX and POX, in which the authors found that this steroid minimized deleterious effects on photosynthetic machinery.

In the literature, there are several studies describing the benefits of this steroid, more specifically potentiating antioxidant enzymes, such as Hussain et al. (2019), who evaluated the antioxidant system in *Triticum aestivum* under EBR application and Mn stress, reporting increases in SOD, CAT and POX enzymes. These authors suggested that EBR likely stimulated the expression of regulatory genes involved in antioxidant defence. Fariduddin et al. (2015) studied the responses of *Brassica juncea* seedlings sprayed with EBR under Mn toxicity to photosynthetic attributes and redox metabolism, revealing evidence that this natural steroid acts as an efficient stress alleviator.

Exogenous EBR induced decreases in oxidative compounds and cellular damage generated by Ni. These findings reveal that EBR improved the performance of the antioxidant system, controlled H_2O_2 and O_2^- overproduction, and subsequently mitigated oxidative damage, as confirmed by the lower MDA and EL values described in this study. H_2O_2 and O_2^- are omnipresent in cellular compartments; however, these toxic compounds accumulate during adverse environmental stresses, including Ni excess (Ahmad et al., 2010; Gill and Tuteja, 2010; Gupta et al., 2016). Chandrakar et al. (2017) evaluated *Glycine max* seedlings treated with EBR (0.5 μ M) exposed to As stress and observed decreases in O_2^- , H_2O_2 and MDA, and these authors suggested that EBR promoted tolerance to oxidative stress by accumulating osmolytes and activating the antioxidant defence system in stressed plants. Dalyan et al. (2018) studied the roles of EBR under ROS overproduction in seedlings of *Brassica juncea* seedlings under Pb stress and identified reductions in H_2O_2 and MDA values, suggesting a protective role triggered by steroids. Sreekanth et al. (2013) described that Ni at high concentrations can negatively interfere with the balance between detoxification and the generation of ROS. Sirhindi et al. (2016) and (Mir et al., 2018), working with *Glycine max* plants exposed to Ni, found increases in stress indicators (H_2O_2 , O_2^- , MDA and EL). These authors described that ROS accumulation and the extent of oxidative stress are often correlated with inefficient antioxidant systems in plants under environmental stress conditions.

Steroid positively act on pigments of soybean plants exposed to Ni^{+2} excess. Maintenance of these photopigments after pretreatment with EBR can be explained by the alleviation of oxidative damage and subsequent positive repercussions on chlorophyll fluorescence, as evidenced in this study. In other words, reductions in O_2^- and H_2O_2 , combined with less deleterious effects on membranes (MDA and EL), occurred, resulting in better structural and functional integrity of these pigments associated with increments linked to light absorption, confirmed by the increases in ETR and Φ_{PSII} . Simultaneously,

increases observed in Chl *a*, Chl *b* and Car are likely associated with a positive modulation induced by EBR in the metabolic pathway linked to the biosynthesis of these pigments (Soares et al., 2016) and with increased contents of essential elements, specifically Mg, which compose the structure of the chlorophyll molecule (Jan et al., 2018). There are studies in the literature revealing the direct relationship between Ni excess and ROS overproduction (Dourado et al., 2015; Muhammad et al., 2013) and its potential deleterious effects, such as peroxidation and degradation of membranes in chloroplast pigments (Ahmad et al., 2010; Gajewska and Skłodowska, 2008; Gill and Tuteja, 2010). Results corroborating our research were described by Ahmad et al. (2018a) when studying the effects of EBR application in *Solanum lycopersicon* plants stressed with NaCl, demonstrated increases in photosynthetic pigments (total Chl and Car), which were attributed by the authors to changes in enzymatic and nonenzymatic antioxidants, osmolytes and metabolites. Dong et al. (2019) evaluated the mechanisms of action triggered by EBR (0.1 μM) and nitric oxide on gas exchange in *Arachis hypogaea* plants under Cd toxicity and observed significant increases in Chl *a*, Chl *b*, total Chl and Car.

Pretreatment with EBR spray on soybean plants subjected to Ni stress resulted in significant increases in biomass (LDM, RDM, SDM and TDM), suppressing the negative impacts caused by Ni excess. These results confirm the multiple roles of EBR in plant metabolism, specifically increasing essential nutrient contents, reducing ROS levels, improving gas exchange (P_N , E , g_s and WUE) and attenuating the negative effects connected to Ni on chloroplast pigments (Chl *a*, Chl *b* and Car). Liu et al. (2019) evaluated the effects induced by brassinosteroid mimetics (EBR, bikinin and brazide) in *Zea mays* exposed to nicosulfuron toxicity and found beneficial responses linked to steroids on P_N , increments in chlorophyll and reduction in H_2O_2 , improving biomass. Zhong et al. (2020) investigated *Festuca arundinacea* plants sprayed with three EBR concentrations (0.05, 0.10 and 0.20 mg L^{-1}) and stressed with Pb (100 mg kg^{-1} soil) and detected increments in biomass (shoot and root), similar to the results of this research.

Conclusions

This research demonstrated the positive effects induced by EBR on antioxidant metabolism and ionic homeostasis in soybean plants subjected to Ni excess. This steroid improved the performance of the photosynthetic machinery, more specifically, the effective quantum yield of PSII photochemistry and electron transport rate due to upregulation of antioxidant enzymes (superoxide dismutase, catalase, ascorbate peroxidase and peroxidase), controlling the overproduction of superoxide and hydrogen peroxide, and reducing oxidative damage to chloroplasts. Exogenous EBR application promoted significant increases in biomass (leaves, roots and stems), and these results were explained by the benefits in nutrient content and ionic homeostasis, demonstrated by increases in $\text{Ca}^{2+}/\text{Ni}^{2+}$, $\text{Mg}^{2+}/\text{Ni}^{2+}$ and $\text{Mn}^{2+}/\text{Ni}^{2+}$ ratios. Therefore, our results demonstrated that EBR attenuates oxidative damage caused by Ni in soybean plants.

Abbreviations

BRs Brassinosteroids

Ca Calcium

CAR Carotenoids

Chl *a* Chlorophyll *a*

Chl *b* Chlorophyll *b*

C_i Intercellular CO₂ concentration

CO₂ Carbon dioxide

E Transpiration rate

EBR 24-epibrassinolide

EL Electrolyte leakage

ETR Electron transport rate

ETR/P_N Ratio between the apparent electron transport rate and net photosynthetic rate

EXC Relative energy excess at the PSII level

F₀ Minimal fluorescence yield of the dark-adapted state

F_e Iron

F_m Maximal fluorescence yield of the dark-adapted state

F_v Variable fluorescence

F_v/F_m Maximal quantum yield of PSII photochemistry

g_s Stomatal conductance

H₂O₂ Hydrogen peroxide

LDM Leaf dry matter

MDA Malondialdehyde

Mg Magnesium

Mn Manganese

Ni Nickel

NPQ Nonphotochemical quenching

O₂⁻ Superoxide

P Phosphorus

P_N Net photosynthetic rate

P_N/C_i Instantaneous carboxylation efficiency

PSII Photosystem II

q_p Photochemical quenching

RDM Root dry matter

SDM Stem dry matter

TDM Total dry matter

Total Chl Total Chlorophyll

WUE Water-use efficiency

Z_n Zinc

Φ_{PSII} Effective quantum yield of PSII photochemistry

Declarations

Author contribution statement

AKSL was the advisor of this project, planning all phases of the research and critically revised the manuscript. MPS and CFM conducted the experiment and performed physiological, biochemical and morphological determinations, as well as wrote and edited the manuscript. BLB carried out the nutritional determinations and critically revised the manuscript. All authors read and approved the final version of manuscript.

Data availability statement

Data are available upon request to the corresponding author.

Conflict of interest

The authors declare that they have no competing interests.

Ethical Approval

Not applicable.

Consent to Participate

Not applicable.

Consent to Publish

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Tables

Table 1. Ni contents in soybean plants sprayed with EBR and exposed to high Ni concentration.

Ni ²⁺	EBR	Ni in root ($\mu\text{g g DM}^{-1}$)	Ni in stem ($\mu\text{g g DM}^{-1}$)	Ni in leaf ($\mu\text{g g DM}^{-1}$)
☒	☒	2.29 ± 0.12c	0.11 ± 0.01c	0.12 ± 0.02c
☒	+	1.07 ± 0.08d	0.07 ± 0.01d	0.12 ± 0.01c
+	☒	344.55 ± 6.39a	10.87 ± 0.07a	20.81 ± 1.56a
+	+	213.64 ± 16.14b	9.12 ± 0.47b	15.53 ± 0.27b

Ni = Nickel. Columns with different letters indicate significant differences from the Scott-Knott test ($P < 0.05$). Values described correspond to means from five repetitions with standard deviations.

Table 2. Nutrient contents in soybean plants sprayed with EBR and exposed to high Ni concentration.

Ni ²⁺	EBR	P (mg g DM ⁻¹)	Ca (mg g DM ⁻¹)	Mg (mg g DM ⁻¹)	Mn (µg g DM ⁻¹)	Fe (µg g DM ⁻¹)	Zn (µg g DM ⁻¹)
Contents in root							
⊗	⊗	14.28 ± 0.57b	13.83 ± 0.53b	14.31 ± 0.15b	293.94 ± 16.47b	2539.06 ± 67.09b	30.57 ± 0.83b
⊗	+	16.17 ± 0.39a	15.89 ± 0.47a	14.92 ± 0.06a	354.79 ± 13.45a	2906.85 ± 65.67a	36.47 ± 0.72a
+	⊗	11.16 ± 0.09d	12.24 ± 0.18c	12.29 ± 0.29d	213.41 ± 13.99d	2150.27 ± 72.83c	25.53 ± 0.76c
+	+	12.71 ± 0.91c	13.32 ± 0.87b	13.94 ± 0.18c	258.89 ± 11.91c	2554.58 ± 97.14b	31.55 ± 0.52b
Contents in stem							
⊗	⊗	7.08 ± 0.24b	13.21 ± 0.32b	2.07 ± 0.30c	15.71 ± 0.48b	40.13 ± 0.59b	11.62 ± 0.27b
⊗	+	8.45 ± 0.24a	14.23 ± 0.16a	3.23 ± 0.16a	17.68 ± 0.49a	46.71 ± 0.63a	12.29 ± 0.29a
+	⊗	6.06 ± 0.02c	11.95 ± 0.22c	1.54 ± 0.20d	13.40 ± 0.48c	32.92 ± 0.69d	7.47 ± 0.29d
+	+	7.18 ± 0.60b	13.32 ± 0.43b	2.78 ± 0.16b	15.55 ± 0.68b	35.43 ± 0.90c	8.81 ± 0.21c
Contents in leaf							
⊗	⊗	9.24 ± 0.12b	13.67 ± 0.50b	3.32 ± 0.14b	46.18 ± 1.99b	92.39 ± 1.65b	21.84 ± 0.70b
⊗	+	9.97 ± 0.37a	15.49 ± 0.14a	4.98 ± 0.42a	50.28 ± 0.61a	117.94 ± 0.96a	25.90 ± 0.66a
+	⊗	8.06 ± 0.05c	12.26 ± 0.23c	3.39 ± 0.19b	41.30 ± 0.82c	72.24 ± 0.60d	12.61 ± 0.43d
+	+	8.92 ± 0.05b	13.71 ± 0.22b	4.71 ± 0.33a	46.67 ± 0.28b	84.85 ± 0.85c	16.55 ± 0.19c

P = Phosphorus; Ca = Calcium; Mg = Magnesium; Mn = Manganese; Fe = Iron; Zn = Zinc. Columns with different letters indicate significant differences from the Scott-Knott test ($P < 0.05$). Values described correspond to means from five repetitions with standard deviations.

Table 3. Ionic ratios in soybean plants sprayed with EBR and exposed to high Ni concentration.

Ni ²⁺	EBR	Root	Stem	Leaf
Ca ²⁺ /Ni ²⁺ ratio				
☒	☒	6089.1 ± 470.0b	123359.6 ± 10882.3b	109973.7 ± 12054.5b
☒	+	15009.3 ± 1519.7a	210916.2 ± 24930.8a	126519.1 ± 6810.6a
+	☒	35.5 ± 0.8d	1099.5 ± 22.10d	592.1 ± 48.1d
+	+	62.8 ± 7.4c	1460.0 ± 56.12c	883.0 ± 9.2c
Mg ²⁺ /Ni ²⁺ ratio				
☒	☒	6298.3 ± 396.8b	19267.0 ± 3382.5b	26802.4 ± 3763.7b
☒	+	14067.4 ± 1011.0a	47970.1 ± 6402.1a	40688.5 ± 4174.2a
+	☒	35.7 ± 1.2d	141.7 ± 19.2d	163.5 ± 13.8d
+	+	65.6 ± 5.0c	305.2 ± 28.2c	303.7 ± 25.7c
Mn ²⁺ //Ni ²⁺ ratio				
☒	☒	129.3 ± 13.7b	146.8 ± 15.2b	371.6 ± 14.9b
☒	+	334.9 ± 32.2a	261.8 ± 28.8a	410.1 ± 10.4a
+	☒	0.6 ± 0.1d	1.2 ± 0.1d	2.0 ± 0.2d
+	+	1.2 ± 0.1c	1.7 ± 0.1c	3.1 ± 0.1c

Ca²⁺/Ni²⁺ = Calcium and nickel ratio; Mg²⁺/Ni²⁺ = Magnesium and nickel ratio and Mn²⁺/Ni²⁺ = Manganese and nickel ratio. Columns with different letters indicate significant differences from the Scott-Knott test ($P < 0.05$). Values described correspond to means from five repetitions with standard deviations.

Table 4. Chlorophyll fluorescence, gas exchange and photosynthetic pigments in soybean plants sprayed with EBR and exposed to high Ni concentration.

Ni ²⁺	EBR	Φ_{PSII}	q_P	NPQ	ETR ($\mu\text{mol m}^{-2}$ s^{-1})	EXC (μmol $\text{m}^{-2} \text{s}^{-1}$)	ETR/ P_N
⊠	⊠	0.24 ± 0.01b	0.43 ± 0.05b	0.18 ± 0.01c	36.2 ± 0.7b	0.70 ± 0.01b	2.09 ± 0.04b
⊠	+	0.27 ± 0.01a	0.56 ± 0.03a	0.12 ± 0.01d	39.1 ± 1.0a	0.67 ± 0.01c	2.08 ± 0.04b
+	⊠	0.20 ± 0.01c	0.25 ± 0.02d	0.42 ± 0.02a	29.1 ± 1.4d	0.75 ± 0.01a	2.22 ± 0.07a
+	+	0.23 ± 0.01b	0.33 ± 0.03c	0.33 ± 0.01b	34.5 ± 1.0c	0.71 ± 0.01b	2.19 ± 0.03a
Ni ²⁺	EBR	P_N (μmol $\text{m}^{-2} \text{s}^{-1}$)	E (mmol $\text{m}^{-2} \text{s}^{-1}$)	g_s (mol m^{-2} s^{-1})	C_i (μmol mol^{-1})	WUE (μmol mmol^{-1})	P_N/C_i (μmol $\text{m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$)
⊠	⊠	17.29 ± 0.22b	3.11 ± 0.07a	0.39 ± 0.008a	267 ± 7b	5.56 ± 0.15b	0.065 ± 0.002b
⊠	+	18.80 ± 0.18a	3.14 ± 0.06a	0.40 ± 0.013a	264 ± 8b	6.00 ± 0.14a	0.071 ± 0.002a
+	⊠	13.09 ± 0.64d	2.79 ± 0.26b	0.27 ± 0.009c	281 ± 5a	4.71 ± 0.29c	0.047 ± 0.003d
+	+	15.72 ± 0.31c	2.87 ± 0.10b	0.32 ± 0.011b	271 ± 4b	5.48 ± 0.25b	0.058 ± 0.001c
Ni ²⁺	EBR	Chl <i>a</i> (mg g^{-1} FM)	Chl <i>b</i> (mg g^{-1} FM)	Total Chl (mg g^{-1} FM)	Car (mg g^{-1} FM)	Ratio Chl <i>a</i> /Chl <i>b</i>	Ratio Total Chl/Car
⊠	⊠	11.65 ± 0.19b	2.67 ± 0.08b	14.32 ± 0.21b	0.59 ± 0.02b	4.37 ± 0.16a	24.29 ± 1.36a
⊠	+	12.78 ± 0.21a	3.27 ± 0.21a	16.05 ± 0.39a	0.63 ± 0.01a	3.91 ± 0.20b	25.71 ± 2.09a
+	⊠	6.06 ± 0.37d	1.32 ± 0.07d	7.38 ± 0.42d	0.36 ± 0.08d	4.58 ± 0.25a	20.76 ± 0.92b
+	+	8.27 ± 0.13c	1.85 ± 0.09c	10.11 ± 0.17c	0.47 ± 0.02c	4.49 ± 0.20a	21.51 ± 1.20b

Φ_{PSII} = Effective quantum yield of PSII photochemistry; q_P = Photochemical quenching coefficient; NPQ = Nonphotochemical quenching; ETR = Electron transport rate; EXC = Relative energy excess at the PSII level; ETR/ P_N = Ratio between the electron transport rate and net photosynthetic rate; P_N = Net photosynthetic rate; E = Transpiration rate; g_s = Stomatal conductance; C_i = Intercellular CO₂ concentration; WUE = Water-use efficiency; P_N/C_i = Carboxylation instantaneous efficiency; Chl *a* = Chlorophyll *a*; Chl *b* = Chlorophyll *b*; Total Chl = Total chlorophyll; Car = Carotenoids. Columns with

different letters indicate significant differences from the Scott-Knott test ($P < 0.05$). Values described correspond to means from five repetitions with standard deviations.

Figures

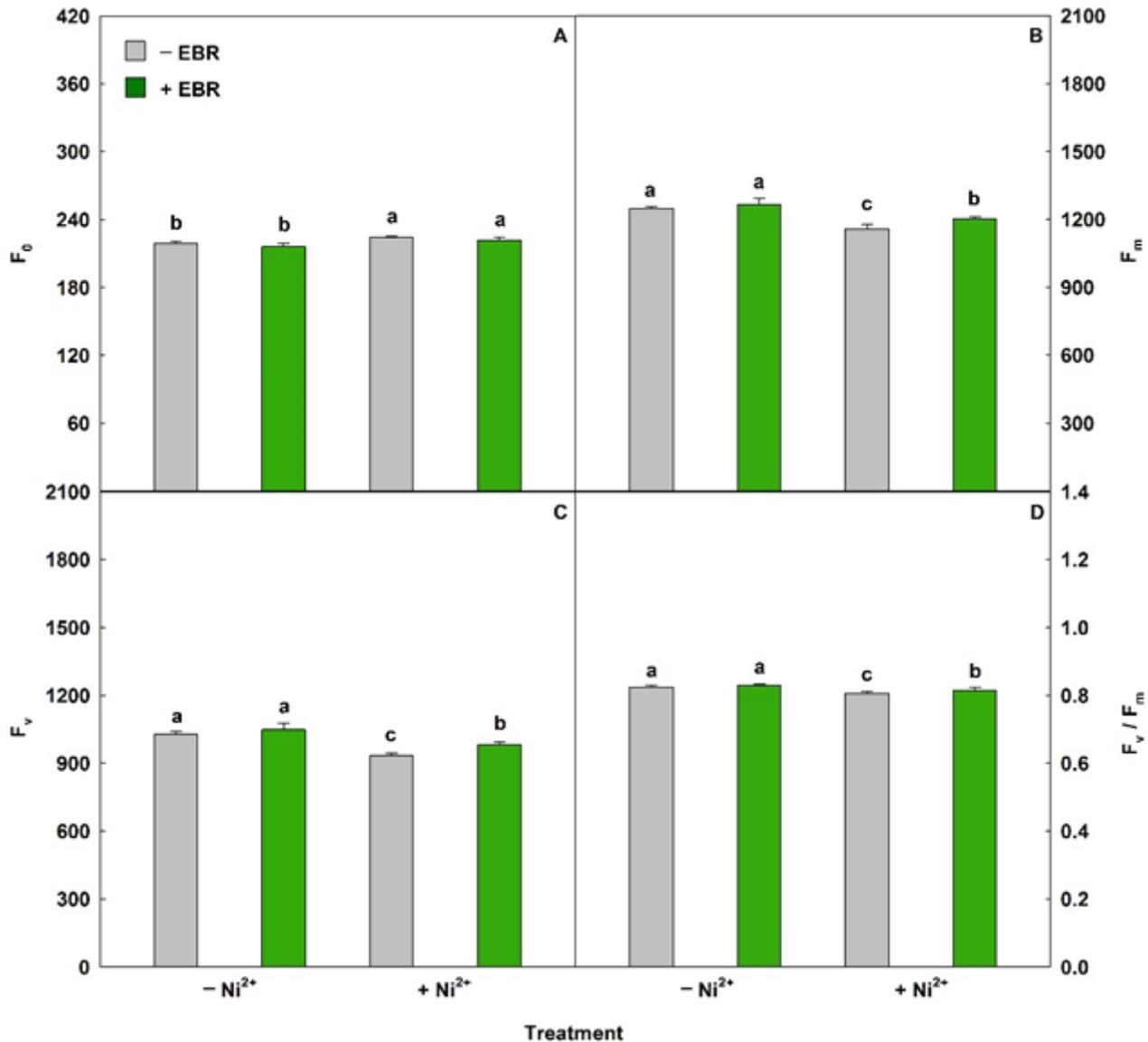


Figure 1

Minimal fluorescence yield of the dark-adapted state (F_0), maximal fluorescence yield of the dark-adapted state (F_m), variable fluorescence (F_v) and maximal quantum yield of PSII photochemistry (F_v/F_m) in soybean plants sprayed with EBR and high Ni concentration. Columns with different letters indicate significant differences from the Scott-Knott test ($P < 0.05$). Columns correspond to means from five repetitions with standard deviations.

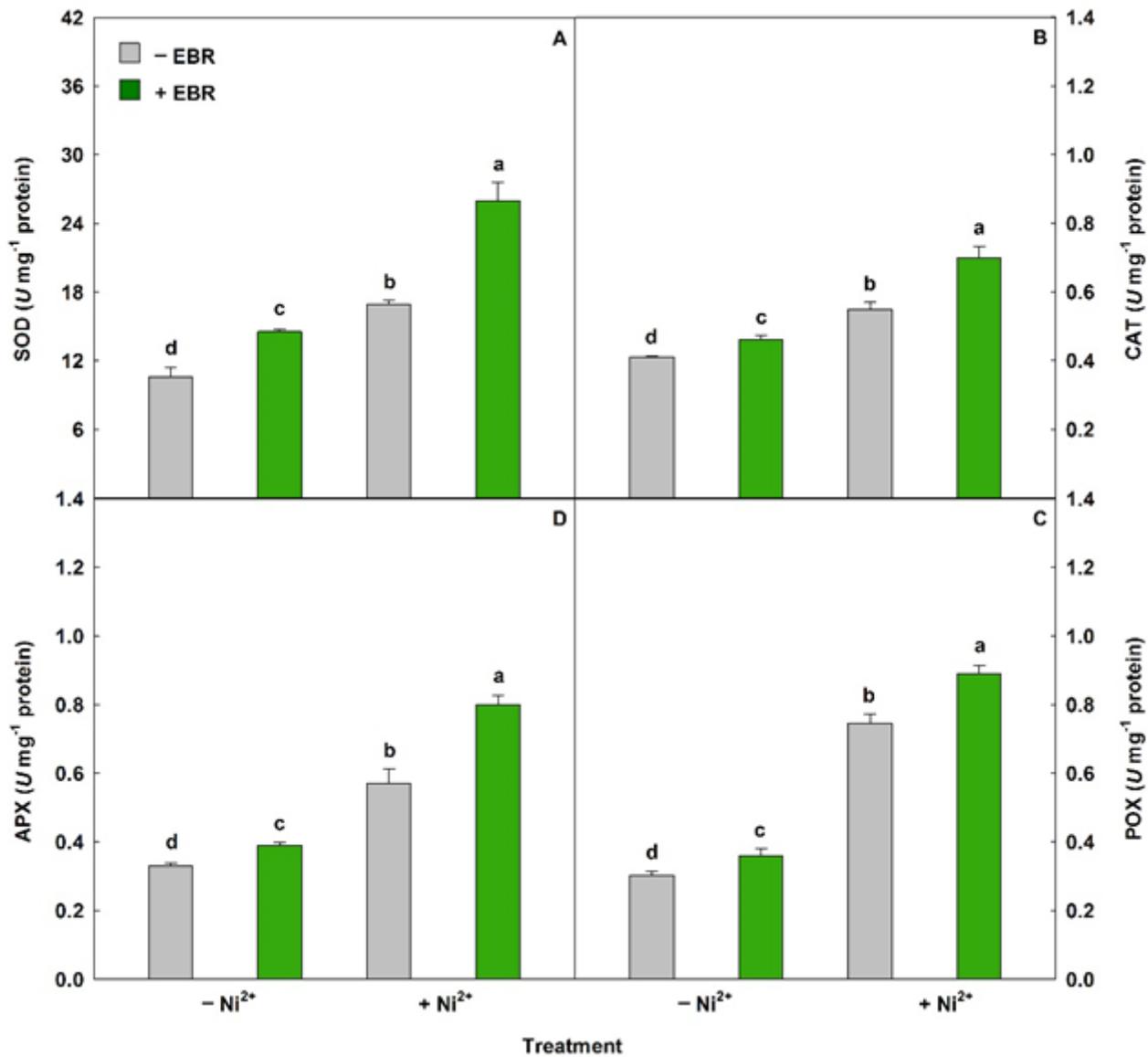


Figure 2

Activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POX) in soybean plants sprayed with EBR and high Ni concentration. Columns with different letters indicate significant differences from the Scott-Knott test ($P < 0.05$). Columns correspond to means from five repetitions with standard deviations.

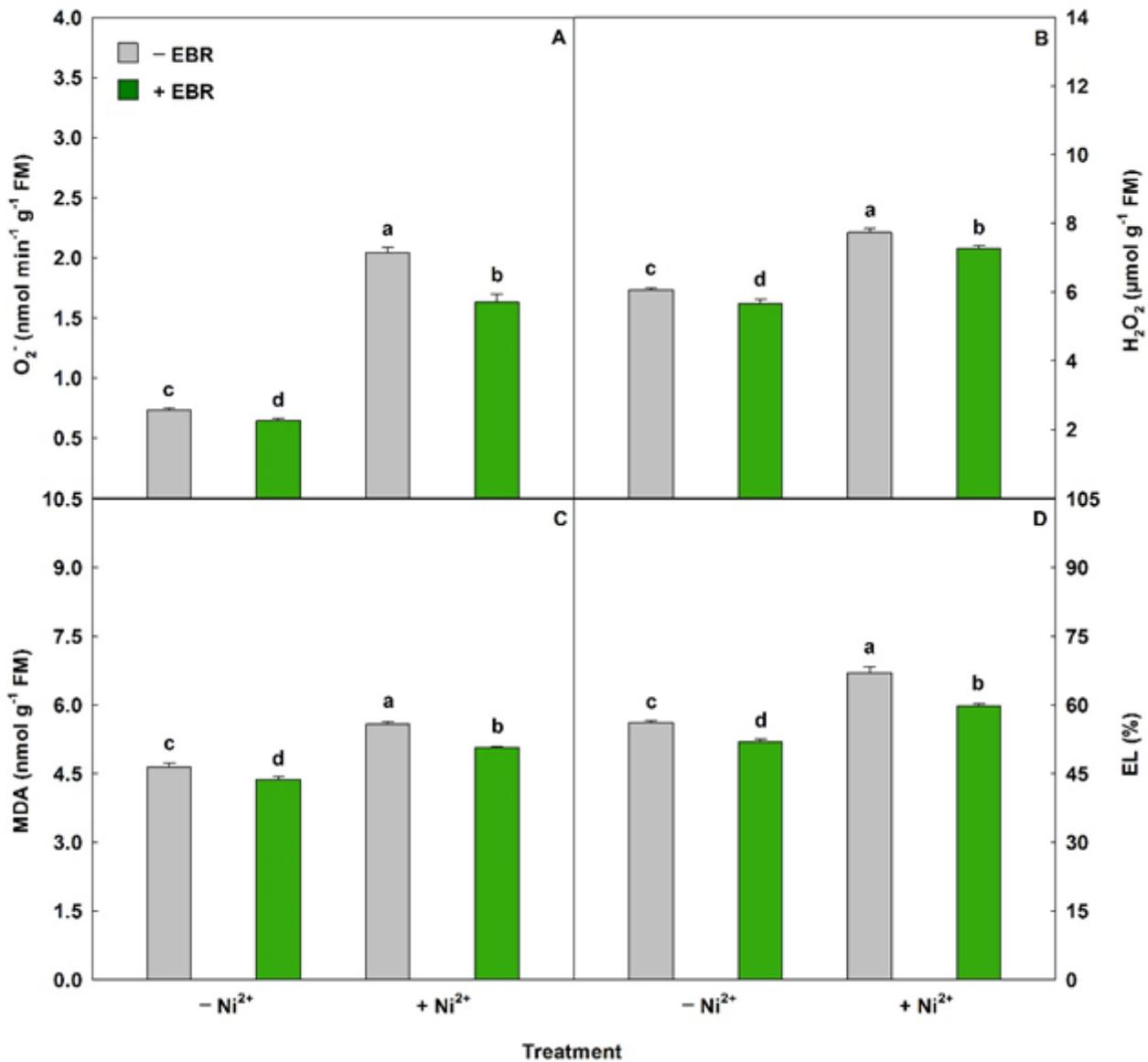


Figure 3

Superoxide (O_2^-), hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and electrolyte leakage (EL) in soybean plants sprayed with EBR and high Ni concentration. Columns with different letters indicate significant differences from the Scott-Knott test ($P < 0.05$). Columns correspond to means from five repetitions with standard deviations.

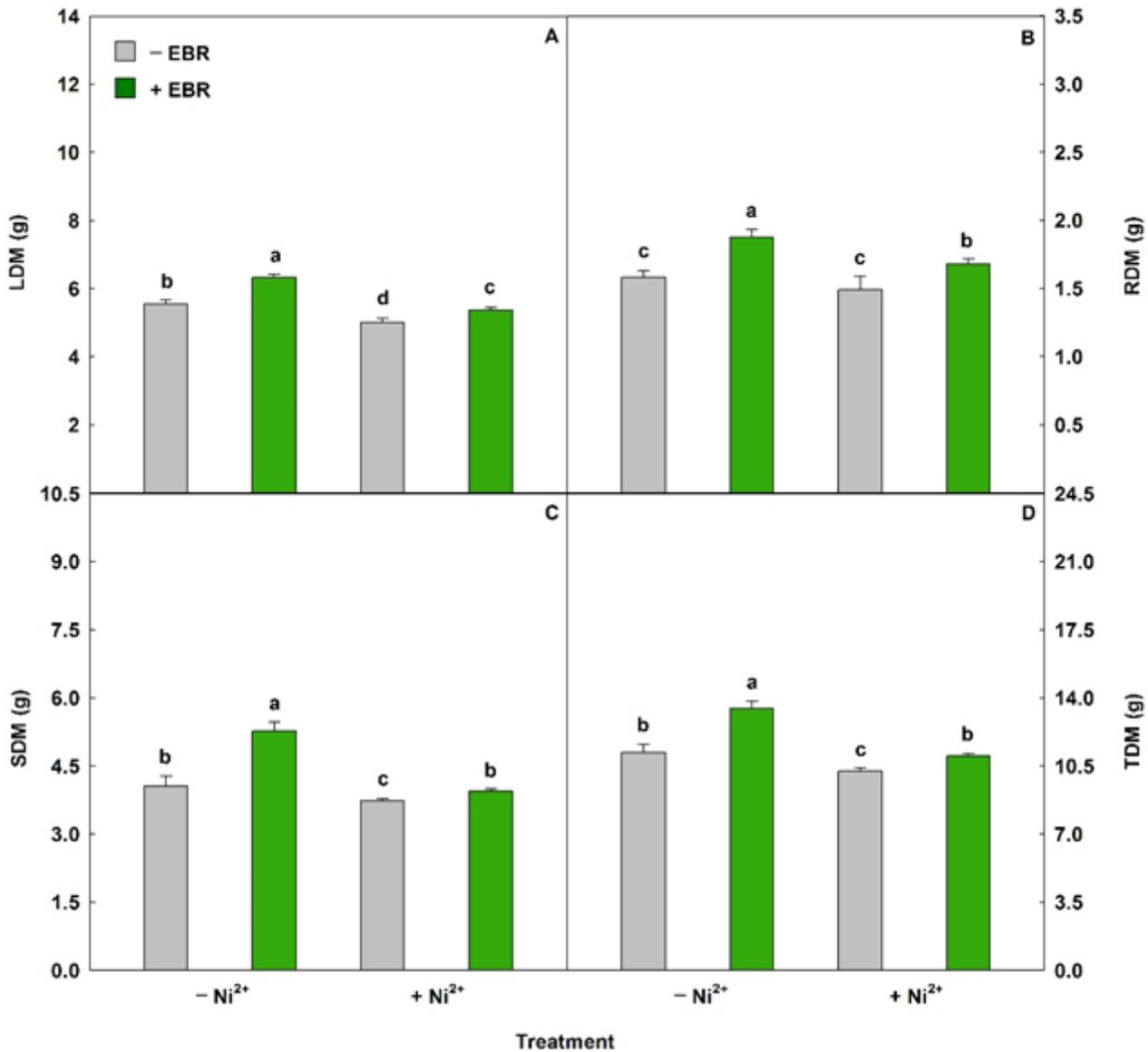


Figure 4

Leaf dry matter (LDM), root dry matter (RDM), stem dry matter (SDM) and total dry matter (TDM) in soybean plants sprayed with EBR and high Ni concentration. Columns with different letters indicate significant differences from the Scott-Knott test ($P < 0.05$). Columns correspond to means from five repetitions with standard deviations.