MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL RURAL DA AMAZÔNIA
PROGRAMA DE PÓS-GRADUAÇÃO EM AGRONOMIA

MARCELO PIRES SARAIVA

24-EPIBRASSINOLIDE INDUCES PROTECTION AGAINST NICKEL EXCESS IN SOYBEAN PLANTS

BELÉM-PA
2021

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Thesis submitted to Universidade Federal Rural da Amazônia, as part of the requirements for obtaining the *Doctor Scientiae* degree in Agronomy. Concentration area: Agronomy.
Advisor: Prof. Dr. Allan Klynger da Silva Lobato

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Firstly, to God for being Always in my heart; to my wife Bárbara and my children Artur and Ana Alice for being the reason of my existence. To my parents for their love and dedication given to me.

I DEDICATED

ACKNOWLEDGEMENTS

To Universidade Federal Rural da Amazônia (UFRA) and to Museu Paraense Emílio Goeldi (MPEG) for the formation and infrastructure;

To Dr. Allan Klynger da Silva Lobato for the orientation and all the support in my formation;

To the examining board for accepting the invitation and to have contributed with my thesis;

To the professors of the graduation courses and the associate professors for the disciplines, discussions, and teaching;

To the users of the Plant Physiology Laboratory (LAVEG) and Applied and Basic Plant Research Center (NPVBA) for exchanging information and leisure times which made the work environment a pleasant place and with mutual support;

To my Family, especially my parents Francisco Max and Ana Maria for my upbringing and my brother Michel Saraiva and my sister Márcia Saraiva,

To all my friends, especially Orivaldo Gomes da Silva who has always believed in my potential and opened my eyes to the importance of education. And to all those who directly and indirectly took part in this process.

Thank you!

RESUMO

A soja [*Glycine max (L.) Merrill*] é uma leguminosa de grande relevância socioeconômica tanto nacional quanto internacional. O excesso de Ni pode afetar seriamente a germinação, o crescimento da planta e prejudicar a atividade enzimática. Os EBRs são apresentados como um novo grupo de fito hormônios esteroides que atuam no estímulo de uma infinidade de reações fisiológicas na planta incluindo funções celulares e metabólicas. Nosso objetivo foi revelar o comportamento estrutural, fisiológico, bioquímico, nutricional e de crescimento de soja submetidas ao estresse por alta dose de Ni e identificar os possíveis benefícios provocados pelos EBR. O experimento foi randomizado com quatro tratamentos, incluindo duas concentrações de Ni (0 e 200 µM Ni, descrito como - $Ni^{2\ +}$ e + $Ni^{2\ +,}$ respectivamente) e duas concentrações de 24-epibrassinolídeo (0 e 100 nM EBR, descrito como - EBR e + EBR , respectivamente. O excesso de $Ni^{2\ +}$ provocou danos nas estruturas radiculares e foliares, causando alterações anatômicas nestes tecidos. Na raiz, a EBR aumentou a espessura da epiderme (27%), protegendo a raiz do íon $Ni^{2\ +.}$ Para o tecido foliar, aumentos significativos na espessura do parênquima paliçadico (11%) e parênquima esponjoso (29%). As plantas submetidas ao tratamento com EBR sob estresse de Ni apresentaram incrementos de 50%, 27%, 40% e 19% nas enzimas antioxidantes como SOD, CAT, APX e POX, respectivamente, promovendo reduções significativas em O^{2-}, H2O2, MDA e EL de 20%, 5%, 9% e 10%, respectivamente, quando comparado com o tratamento $Ni^{2\ +}$ a 0 nM de EBR. Nossos resultados confirmam que o pré-tratamento com 100 nM de EBR mitigou claramente os distúrbios anatômicos ocasionados pelo excesso de Ni nas estruturas das folhas e raízes com desempenho positivo também na atenuação de alta dosagem de níquel nas características nutricionais, fisiológicos, bioquímicos e de crescimento na cultura da soja.

Palavras chave: Anatômica da Raiz, Alta dose de Ni, Enzimas antioxidantes e 24- Epibrassinolideo.

ABSTRACT

Soybean [*Glycine max* (L.) Merrill] is a legume of great socioeconomic relevance, both nationally and internationally. The excess of Ni can seriously affect germination, plant growth, and impair enzyme activity. EBRs are introduced as a new group of plant steroid hormones that act in stimulating a wide range of physiological reactions in the plant, including cellular and metabolic functions. Our objective was to reveal the structural, physiological, biochemical, nutritional, and growth behavior of soybean plants subjected to stress by a high dose of Ni and to identify the possible benefits caused by EBR. The experiment was randomized with four treatments, including two concentrations of Ni (0 and 200 µM Ni, described as - Ni^{2+} and + Ni^{2+}, respectively) and two concentrations of 24-epibrassinolide (0 and 100 nM EBR, described as - EBR and + EBR, respectively. The excess of Ni^{2+} caused damage to the root and leaf structures, resulting in anatomical changes in these tissues. In the roots, the EBR increased the thickness of the epidermis (27%), protecting the root from the Ni^{2+} ion. In relation to the leaf tissue, significant increases in palisade parenchyma thickness (11%) and spongy parenchyma (29%) were observed. Plants submitted to EBR-treatment under Ni stress showed increases by 50%, 27%, 40%, and 19% in antioxidant enzymes such as SOD, CAT, APX, and POX, respectively, promoting significant reductions in O^{2-}, H_2O_2, MDA, and EL by 20%, 5%, 9%, and 10%, respectively, when compared with the Ni^{2+}- treatment at 0 nM of EBR Our results confirm that pretreatment with 100 nM of EBR clearly reduced the anatomical disturbances caused by the excess of Ni in the structures of the leaves and roots, with positive performance, also attenuating the high content of nickel in the nutritional, physiological, biochemical and growth characteristics in the soybean crop.

Keywords: Antioxidant enzymes, Anatomical of the root, high dose of Ni, 24-Epibrassinolide.

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SUMMARY

1 CONTEXTUALIZATION

Soybean [*Glycine max* (L.) Merrill] is a legume of great national and global socioeconomic relevance (FAO, 2017) due to the versatility of its products which range from the manufacture of medicines, industrial products to biofuels (STACHIW et al., 2016), with an important demand in the supply of animal feed and human food (RENNÓ et al., 2015; ZAKIR; FREITAS, 2015).

This legume is originally from China and belongs to the Fabaceae family (BRAGA; PISSOLATO; SOUZA, 2017). It is attractive for consumption because it contains important nutritional components such as carbohydrates, proteins, and oils (MAJUMDAR et al., 2019). Also, it has great morphological variability, sensitive to different environmental conditions. It is an annual, autogamous, herbaceous, and erect plant (MORAES, 2015) with a life-cycle that lasts 75 to 200 days from emergence to maturity (SEDIYAMA, 2009).

The advance of soybean production is a reality that is added to the Brazilian trade balance and extends to all regions of the country, with a strong growth trend in the Northern and Northeastern regions (CONAB, 2016). Records from 2017 point out that in the northern region, the municipalities of Paragominas, Dom Elizeu, Rondon do Pará, Ulianópolis, Goianésia do Pará and Abel Figueiredo together represent the value of 302.74 thousand hectares of the area cropped with soybeans and total productivity corresponding to 1.064,349 tons of grain (IBGE, 2017). However, the constant and excessive application of pesticides, irrigation, and fertilizers in the production environment (PEDROTTI et al., 2015) besides the rise and accumulation of heavy metals in the soil over the years, as well as the deposition of domestic and industrial waste (APRILE; DE BELLIS, 2020), has represented a major phytotoxicity risk for agriculture.

Heavy metal is the element of the periodic table that has the property of binding strongly to its atoms (metal connection) and also shows the following characteristic: malleability, flexibility, shine, and electricity conduction (BACCAN, 2004). In plants, some metals are essential in small quantities and contribute to their development (SANTOS; BATISTA; LOBATO, 2018).

Nickel is a heavy metal recently considered an essential micronutrient for plants in adequate quantity (0,05 and 10 mg/kg^{-1} dry mass), as it constitutes the active site of two metalloenzymes: urea (DIXON et al., 1975) and hydrogenase (EVANS et al., 1987), which are enzymes with an important role in the nitrogen metabolism, especially in legumes such as soybean (SIQUEIRA FREITAS et al., 2018). The excess of Ni can seriously affect germination, plant growth, and impair enzyme activity (KURAMSHINA; SMIRNOVA; KHAIRULLIN, 2018).

Previous studies have specified that the toxicity of Ni affects the growth of the root and stem and may lead to a reduction in the thickness of the mesophyll, the intercellular palisadic and spongy spaces causing a decrease in the diameter of the vessels, in addition to affecting the width of the leaf epidermis (MOLAS, 1998; REIS et al., 2017; SEREGIN; KOZHEVNIKOVA, 2006). Besides, this metal impairs photosynthetic efficiency by reducing the chlorophyll content, therefore reducing CO_2 absorption (KHALIQ et al., 2016; SREEKANTH et al., 2013; YUSUF; FARIDUDDIN; AHMAD, 2011). Changing gas exchange parameters with evident limitations in stomatal performance and decrease in transpiration (RIBEIRO et al., 2020), thus seriously constraining the absorption of water and mineral nutrients (TORRES et al., 2016), impairing plant growth and reducing the biomass (SIQUEIRA FREITAS et al., 2018).

Also, there are reports that Ni, although indirectly, causes the production and concentration of reactive oxygen species (ROS) (GAJEWSKA; SKŁODOWSKA, 2008; SHAHZAD et al., 2018) which negatively impact the regular flow of electrons in photosynthesis and the transport of electrons harming the reaction center (P680 for PSII and P700 for PSI) (SIRHINDI et al., 2016). In response to the damaging effects of ROS, plants use as a defense mechanism the production of antioxidant enzymes (ANJUM et al., 2017; ASHRAF et al., 2015) including Superoxide dismutase (SOD); Catalase (CAT); peroxidase (POX); ascorbate peroxidase (APX). These enzymes are important examples in eliminating and decreasing the formation of free radicals (AMARI; GHNAYA; ABDELLY, 2017).

In the last years, many researchers have published their studies addressing the application of plant hormones as an efficient strategy to mitigate the effects of abiotic stress. In this line of research, EBRs have successfully acted in resistance to high and low temperatures, soil salinity, drought, and toxicity caused by heavy metals (LARRÉ et al., 2014). However, Brassinosteroids are gaining more and more space as an alternative to control the adverse effects of abiotic stresses, especially the 24-Epibrassinolid (EBR) form.

EBRs are introduced as a new group of phytohormonal steroids that act to stimulate a multitude of physiological reactions in the plant, including cellular and metabolic functions (JAN et al., 2018; RAHMAN et al., 2017) occurring in various parts of the plants as a leaf, root, flower, and seeds (BAJGUZ; HAYAT, 2009; KAGALE et al., 2007; LIMA; LOBATO, 2017; SASSE, 2003). There are also reports of important contributions by the EBRs at the cellular, biochemical, physiological, and anatomical level, calling attention to the stimulation of cell division, antioxidant activation, gas exchange, and plant growth (DE OLIVEIRA et al., 2019; VRIET; RUSSINOVA; REUZEAUA, 2012; YUAN et al., 2012).

Its mitigating action was approached in the work of Da Silva Cunha et al.,(2020) in the application of EBR in *Eucalyptus urophylla* under Cd stress conditions, significantly alleviating the damage to leaf anatomy, photosynthetic pigments, and gas exchange.

There is strong evidence that EBRs can positively contribute to the tolerance of plants subjected to stress by heavy metals; however, there is little or no information about the action of this hormone and its benefits in soybean [*Glycine max* (L.) *Merrill*] plants exposed to stress by Ni.

The overall hypothesis of this work considers the deleterious effects caused by the stress of Nickel in high concentration in the plant; on the other hand, treatment with EBR has been showing its efficiency in mitigating toxicity with important results in structural characteristics, chlorophyll fluorescence, gas exchange, pigments, the activity of antioxidant enzymes, the concentration of nutrients and growth of agricultural plants.

Nevertheless, our general objective is to clarify the structural, physiological, biochemical, nutritional, and growth behavior of soybean plants subjected to stress by high doses of Ni and to identify the possible benefits caused by EBR.

The present work is divided into two articles, one with the title: 24-Epibrassinolide induce protection against nickel excess in soybean plants: Anatomical evidences and the second Ionic homeostasis and redox metabolism upregulated by 24-Epibrassinolide are crucial to mitigate nickel excess in soybeam plants, enhancing photosystem II efficiency and biomass.

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3 PAPER- 1

24-EPIBRASSINOLIDE INDUCE PROTECTION AGAINST NICKEL EXCESS IN SOYBEAN PLANTS: ANATOMICAL EVIDENCES

Brazilian Journal of Botany
https://doi.org/10.1007/s40415-021-00701-3

STRUCTURAL BOTANY - ORIGINAL ARTICLE

24-Epibrassinolide induces protection against nickel excess in soybean plants: anatomical evidences

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Received: 3 November 2020 / Revised: 7 December 2020 / Accepted: 14 January 2021

Abstract

Nickel (Ni) excess delays plant growth due to damage to root and leaf structures, reducing nutrient uptake, and carbon fixation, respectively. 24-Epibrassinolide (EBR) is a biodegradable plant growth regulator extracted from plant tissues and is highly efficient against oxidative stress. The objective of this research is to determine whether EBR can improve tolerance to toxic metals and the possible mechanism involved by evaluating the root and leaf structures of soybean plants under high Ni concentrations. The experiment was randomized with four treatments, including two Ni concentrations (0 and 200 µM Ni, described as – Ni^{2+} and + Ni^{2+}, respectively) and two concentrations of 24-epibrassinolide (0 and 100 nM EBR, described as – EBR and+ EBR, respectively). Ni^{2+} excess provoked damage to root and leaf structures, causing anatomical disorders in these tissues. In roots, EBR increased the epidermis (27%), protecting the root against Ni^{2+} ions. For leaf tissue, significant increases in palisade (11%) and spongy parenchyma (29%) were detected in plants sprayed with EBR and exposed to Ni^{2+}, which were intrinsically related to stomatal density and stomatal functionality. Our results confirm that pretreatment with 100 nM EBR clearly mitigated the anatomical disorders occasioned by excess Ni on the leaf and root structures of soybean plants.

Keywords Brassinosteroids · *Glycine max* (L.) Merr. · Leaf anatomy · Root structures · Toxic metal

1 Introduction

Soybean [*Glycine max* (L.) Merr.] is one of the main crops of agribusiness worldwide, with high importance to Brazil. Grains have high protein and oil contents (Nishinari et al. 2014) and are intended for human and animal consumption (Sanjukta and Rai 2016). This leguminous product has been produced in several countries, mainly the USA, Brazil, Argentina, and China, which together accounted for 90% of the global production in the 2017/2018 harvest (Zhan et al. 2019). However, soybean also suffers from toxicity due to the excess of toxic metals, a problem that has been recurrent in recent years and is connected to soil pollution (Huang et al. 2015).

Nickel (Ni) has been frequently found in agricultural areas (Ramzani et al. 2017; Turan et al. 2018) due to anthropogenic action, more specifically the intensive and indiscriminate use of agrochemicals (Correia et al. 2018), especially pesticides and fertilizers (Chen et al. 2009; Rizwan et al. 2018; Turan et al. 2018), along with the burning of fuels with Ni in their structure (Wuana and Okieimen 2011). Ni is considered one of the most common toxic metals in the environment (Hussain et al. 2013).

Ni excess causes interference in plant metabolism (Yusuf et al. 2011; Bazihizina et al. 2015) and negatively impacts plant anatomy, generating inhibition in root growth due to significant damage to root tissues (Sirhindi et al. 2016) and reductions in uptake, transport, and accumulation of water and nutrients (Aroca et al. 2012; Garcia et al. 2018). In leaves, high Ni concentrations can decrease the thickness of the epidermis and disorganize the leaf structures (Reis et al. 2017), resulting in changes in the density and size of the stomata (Sagardoy et al. 2010). Ribeiro et al. (2020) described

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that excess Ni^{2+} provokes decreases in root structures, more specifically the epidermis, metaxylem and vascular cylinder, with negative impacts on the uptake of water and nutrients. Akhtar et al (2018) studied the interferences occasioned by Ni toxicity in leaves of *Typha domingensis* (Pers.) and found significant reductions in leaf thickness and photosynthetic rate. Sharma et al. (2011) verified that the oxidative stress generated by excess Ni delays growth in *Raphanus sativus* L. seedlings, resulting in lower root length, shoot length and fresh biomass.

Brassinosteroids (BRs) are plant growth regulators that can be found in several plant organs, such as leaves, roots, stems, seeds, pollen, and flowers (Sasse 2003; Kagale et al. 2007; Bajguz and Hayat 2009). In this context, 24-epibrassinolide (EBR) is one of the most active forms of BR and has the advantages of being biodegradable and extracted from plant tissues (Azhar et al. 2017; Santos et al. 2020). EBR is a molecule that has multiple functions in metabolism (Khripach et al. 2000; Vriet et al. 2012), including benefits in antioxidant metabolism (Ahanger et al. 2018; Kaya et al. 2019) and the photosynthetic apparatus (Yuan et al. 2012; Lima and Lobato 2017), in which it is a response molecule that can improve tolerance to abiotic stresses, including stress by Ni. Exogenous EBR treatment has been highly efficient in mitigating the oxidative stress caused by toxic metals (Kaya et al. 2020), such as *Eucalyptus urophylla* S. T. Blake under Cd toxicity (Cunha et al. 2020), *Indian mustard* plants exposed to Pb (Kohli et al. 2018) and Ni-stressed *Solanum nigrum* L. (Soares et al. 2016).

The hypothesis of this research considered the deleterious effects provoked by excess Ni on anatomical structures. Recent results indicate that EBR stimulates essential structures in the root tissue connected to nutrient uptake, more specifically the vascular cylinder and metaxylem (Pereira et al. 2020). Synergistically, this steroid acts on leaves, improving stomatal responses and carbon dioxide (CO_2) fixation (Maia et al. 2018). However, there is no knowledge in the available literature on the anatomical responses triggered by EBR in soybean plants under Ni excess. Thus, our objective is to determine whether EBR can improve tolerance to toxic metals and the possible mechanism involved by evaluating the root and leaf structures of soybean plants under high Ni concentrations.

2 Materials and methods

Location and growth conditions – The experiment was performed at the Campus of Paragominas of the Universidade Federal Rural da Amazônia, Paragominas, Brazil (2°55′ S, 47°34′ W). The 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 nutrient solution described below was used, with the ionic strength beginning at 50% (4th day) and later modified to 100% after two days (6th day). After this period, the nutrient solution remained at the total ionic strength.

Experimental design – The experiment was randomized with four treatments, including two Ni concentrations (0 and 200 µM Ni, described as—Ni^{2+} and + Ni^{2+}, respectively) and two concentrations of 24-epibrassinolide (0 and 100 nM EBR, described as—EBR and + EBR, respectively). Five replicates for each of the four treatments were conducted, yielding a total of 20 experimental units used in the experiment, with one plant in each 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. The 0 and 100 nM EBR (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: 8.75 mM·KNO_3, 7.5 mM·$Ca(NO_3)_2 \cdot 4H_2O$, 3.25 mM·$NH_4H_2PO_4$, 1.5 mM·$MgSO_4 \cdot 7\ H_2O$, 62.50 µM KCl, 31.25 µM·H_3BO_3, 2.50 µM·$MnSO_4 \cdot H_2O$, 2.50 µM Zn $SO_4 \cdot 7H_2O$, 0.63 µM $CuSO_4 \cdot 5H_2O$, 0.63 µM $NaMoO_4 \cdot 5H_2O$, and 250.0 µM NaEDTAFe·$3H_2O$. To simulate Ni excess, $NiCl_2$ was used at concentrations of 0 and 200 µM Ni and 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, root and leaf tissues were harvested for anatomical analyses.

Ni determination – Samples with 100 mg of milled samples were weight in 50 ml conical tubes (Falcon^R, Corning, Mexico) and pre-digested (48 h) with 2 ml of sub-boiled HNO_3 (DST 1000, Savillex, USA). After, 8 ml of a solution containing 4 ml of H_2O_2 (30% v/v, Synth, Brasil) and 4 ml of ultra-pure water (Milli-Q System, Millipore, USA) were added and the mixture was transferred to a teflon digestion vessel, closed and heated in a block digester (EasyDigest®, Analab, France) according to the following program: (1) 100 °C during 30 min; (2) 150 °C during 30 min; (3) 130 °C during 10 min; (4) 100 °C during 30 min and, (5) left to cool. The volume was made up to 50 mL with ultra-pure water, and iridium was used as internal standard at 10 µg l^{-1}, in agreement with Batista et al. (2014). The determinations of Ni was carried out by using an inductively coupled plasma mass spectrometer (ICP-MS 7900, Agilent, USA).

Measurements of anatomical parameters – Samples were collected from the middle region of the leaf limbs of fully expanded leaves of the third node and roots 5 cm from the root apex. Subsequently, all collected botanical material was fixed in FAA 70 for 24 h (Johansen 1940) and dehydrated in ethanol and butanol for embedding in historesin (Leica, Nussloch, Germany). Transverse sections with a thickness of 5 µm were obtained with a rotating microtome (model Leica RM 2245, Leica Biosystems), stained with toluidine blue (O'Brien et al. 1964). For stomatal characterization, the epidermal impression method was used according to Segatto et al. (2004). The slides were observed and photomicrographed under an optical microscope (Motic BA 310, Motic Group Co. LTD.) coupled to a digital camera (Motic 2500, Motic Group Co., LTD.). The images were analyzed with Moticplus 2.0 previously calibrated with a micrometer slide from the manufacturer.

Scanning electron microscopy – The middle region of the leaf limb previously fixed in FAA was dehydrated in an ethyl series, processed in a critical point CO_2 dryer and metallized with gold (one layer approximately 20 nm thick) under a current of 25 mA. The micrographs were obtained by scanning electron microscopy (model LEO 1450 VP, Zeiss).

Data analysis – The 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.

3 Results

EBR improved the protection of root structures against excess Ni – Ni contents provoked increases ($P < 0.05$) in both tissues evaluated (Fig. 1), but the pretreatment with EBR minimized the Ni contents in roots (38%) and leaves (25%), respectively, comparing plants that received Ni and without EBR. Ni stress-induced damage to root anatomy (Table 1 and Fig. 2). However, plants that received the exogenous EBR and Ni^{2+} had increases in root epidermis thickness (RET), root endodermis thickness (RDT), root cortex thickness (RCT), vascular cylinder diameter (VCD), and root metaxylem diameter (RMD) of 7%, 3%, 16%, 17%, and 37%, respectively, when compared to treatment without EBR. In the root cross section, Ni^{2+} promoted changes in the cortical parenchyma, revealing cells that were slightly plasmolyzed. For the vascular cylinder, early maturation and deformations of the xylem cells that caused a reduction in the thickness of the vessel elements were observed. However, EBR attenuated these negative effects through apparent improvements in the turgidity of cortex cells and the maturation of the vascular cylinder, with vessel elements presenting greater thickness (Fig. 2).

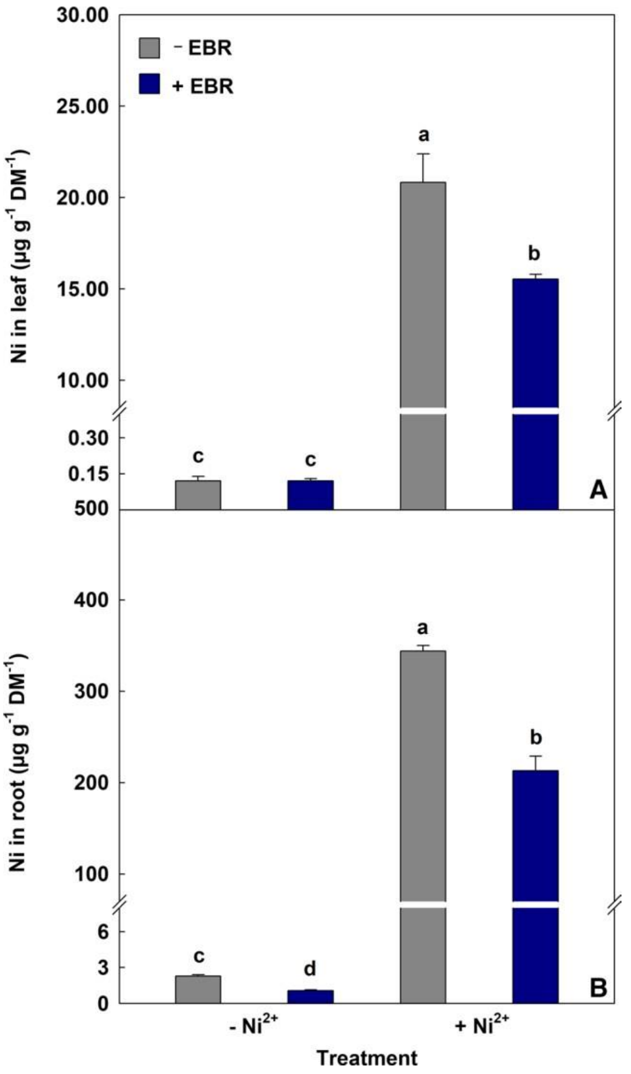


Fig. 1 Ni contents in soybean plants sprayed with EBR and exposed to high Ni concentration. Columns with different letters indicate significant differences from the Scott–Knott test ($P < 0.05$). Columns corresponding to means from five repetitions and standard deviations

Table 1 Root anatomy in soybean plants sprayed with EBR and exposed to Ni excess

Ni^{2+}	EBR	RET (µm)	RDT (µm)	RCT (µm)	VCD (µm)	RMD (µm)
–	–	14.3 ± 0.3b	18.9 ± 0.5b	347 ± 6b	260 ± 8b	44.4 ± 1.4b
–	+	15.7 ± 0.5a	19.9 ± 0.3a	370 ± 9a	303 ± 11a	48.9 ± 2.0a
+	–	10.3 ± 0.2d	17.8 ± 0.6b	287 ± 5d	209 ± 7d	29.1 ± 2.0d
+	+	13.1 ± 0.3c	18.4 ± 0.5b	333 ± 5c	245 ± 4b	40.0 ± 1.0c

RET = Root epidermis thickness; RDT = Root endodermis thickness; RCT = Root cortex thickness; VCD = Vascular cylinder diameter; RMD = Root metaxylem diameter. Columns with different letters indicate significant differences from the Scott–Knott test ($P < 0.05$). Values correspond to means from five repetitions and standard deviations

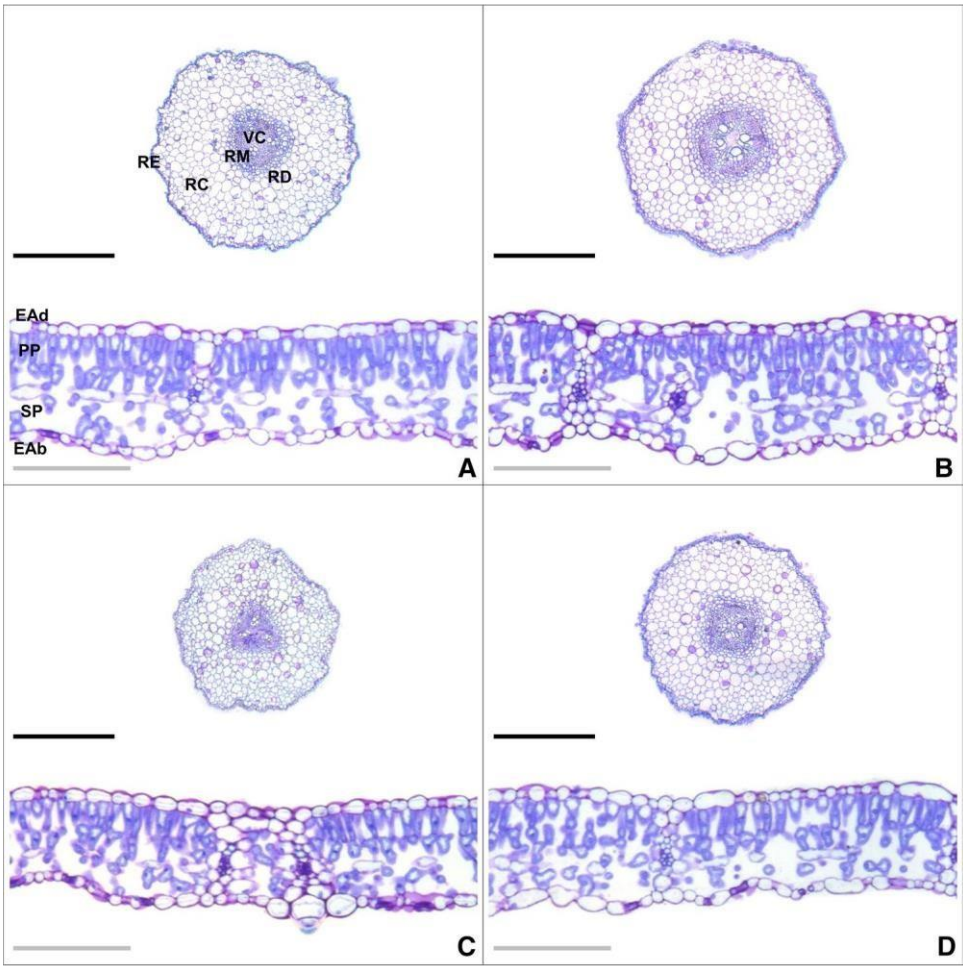


Fig. 2 Root and leaf cross sections in soybean plants sprayed with EBR and Ni excess.—$Ni^{2\pm}$ EBR (**A**),—Ni^{2+} /+ EBR (**B**), + $Ni^{2\pm}$ EBR (**C**) and + Ni^{2+} / + EBR (**D**). Legends: RE = Root epidermis; RC = Root cortex; RD = Root endodermis; VC = Vascular cylinder; RM = Root metaxylem; EAd =adaxial epidermis; EAb = Adaxial epidermis; PP =Palisade parenchyma; SP = Spongy parenchyma. Black bars = 500 µm and gray bars: 150 µm

Ni phytotoxicity on leaf structures was minimized by EBR – The treatment with only Ni caused changes in the leaf anatomy variables (Table 2 and Fig. 2). However, the EBR spray in plants under Ni toxicity reversed the damage, conferring increments of 30%, 23%, 11%, and 29% in epidermis thickness from adaxial leaf side (ETAd), epidermis thickness from abaxial leaf side (ETAb), palisade parenchyma thickness (PPT), and spongy parenchyma thickness (SPT), respectively, and a reduction in PPT/SPT (14%) compared to treatment with Ni and without EBR. For the leaf cross section, plants exposed to excess Ni^{2+} presented mesophyll with compact arrangement. In contrast, leaves treated with EBR presented an arrangement of mesophyll that was more elongated. In the frontal view, EBR promoted an apparent expansion of epidermal cells, mainly on the abaxial surface (Fig. 2).

Steroid stimulated the stomatal performance in Ni^{2+}-stressed plants – Stomatal characteristics were affected by Ni excess (Table 3 and Fig. 3). On the adaxial face, plants under exogenous application of steroids+ Ni^{2+} had significant increases of 20%, 7%, and 27% in stomatal density (SD), stomatal

Table 2 Leaf anatomy in soybean plants sprayed with EBR and exposed to Ni excess

Ni^{2+}	EBR	ETAd (μm)	ETAb (μm)	PPT (μm)	SPT (μm)	Ratio PPT/SPT
–	–	18.7 ± 1.4a	17.1 ± 2.0b	67 ± 1b	56 ± 2b	1.22 ± 0.14b
–	+	19.2 ± 1.2a	19.1 ± 1.3a	72 ± 2a	62 ± 3a	1.18 ± 0.11b
+	–	14.1 ± 1.3b	11.5 ± 1.3d	55 ± 1d	38 ± 1d	1.45 ± 0.12a
+	+	18.3 ± 1.3a	14.2 ± 0.9c	61 ± 1c	49 ± 1c	1.25 ± 0.04b

ETAd = Epidermis thickness from adaxial leaf side; ETAb = Epidermis thickness from abaxial leaf side; PPT=Palisade parenchyma thickness; SPT = Spongy parenchyma thickness. Columns with different letters indicate significant differences from the Scott–Knott test ($P < 0.05$). Values correspond to means from five repetitions and standard deviations

Table 3 Stomatal characteristics in soybean plants sprayed with EBR and exposed to Ni excess

Ni^{2+}	EBR	SD (stomata per mm^2)	PDS (μm)	EDS (μm)	SF	SI (%)
Adaxial face						
–	–	186 ± 6b	4.32 ± 0.10c	8.7 ± 0.4c	0.50 ± 0.02b	9.8 ± 0.2b
–	+	202 ± 7a	3.71 ± 0.11d	6.5 ± 0.3d	0.56 ± 0.03a	10.3 ± 0.2a
+	–	106 ± 3d	4.87 ± 0.14a	11.5 ± 0.4a	0.42 ± 0.01d	5.9 ± 0.1d
+	+	127 ± 5c	4.55 ± 0.11b	9.9 ± 0.3b	0.45 ± 0.01c	7.5 ± 0.1c
Abaxial face						
–	–	364 ± 10b	4.82 ± 0.14c	9.5 ± 0.3c	0.50 ± 0.01b	16.5 ± 0.4a
–	+	386 ± 11a	3.90 ± 0.11d	7.4 ± 0.3d	0.54 ± 0.02a	17.1 ± 0.4a
+	–	293 ± 8d	5.78 ± 0.22a	12.7 ± 0.2a	0.44 ± 0.01d	13.6 ± 0.3c
+	+	338 ± 9c	5.39 ± 0.15b	11.5 ± 0.4b	0.47 ± 0.01c	15.7 ± 0.3b

SD = Stomatal density; PDS = Polar diameter of the stomata; EDS = Equatorial diameter of the stomata; SF = Stomatal functionality; SI = Stomatal index. Columns with different letters indicate significant differences from the Scott–Knott test ($P < 0.05$). Values correspond to means from five repetitions and standard deviations

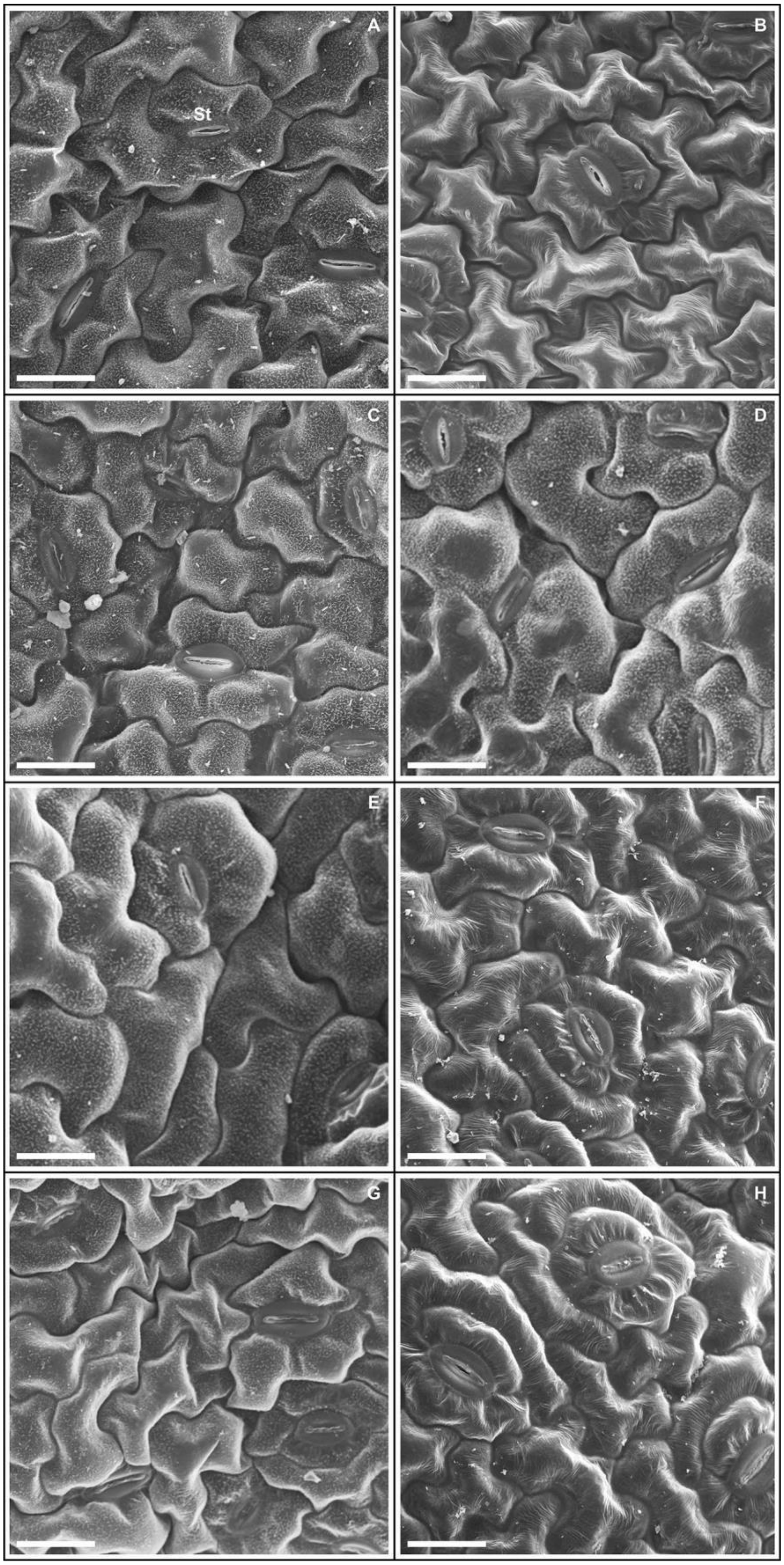
functionality (SF), and stomatal index (SI), in this order, but decreases in polar diameter of the stomata (PDS) and equatorial diameter of the stomata (EDS) of 7% and 14%, respectively. On the abaxial face, the treatment exposed to Ni and EBR had increases in the values of SD (15%), SF (4%) and SI (15%) and reductions in PDS (7%) and EDS (9%) compared to plants under Ni and without EBR.

4 Discussion

Plants exposed to Ni^{2+} excess presented increases in Ni contents in both tissues (leaf and root), revealing that Ni concentration available to plants was efficient to simulate the stress connected to toxic metal. Interestingly, EBR reduced the Ni contents in roots and leaves, modulating protection on root structures and improving the stomatal density, confirmed by the anatomical evidences described in this research. Abd Allah et al. (2019) studying *Brassica juncea* (L.) Czern. plants submitted to different Ni stress (0, 50, 100, and 150 μM) found high Ni contents in roots (118%) and leaves (49%), evidencing that this toxic metal can be transported to shoot, occasioning deleterious effects in leaf structures. Mir et al. (2018) described significant Ni accumulations in root and leaf of soybean plants Ni stressed, being these results explained by the lower biomass and higher Ni contents.

Steroid suppressed the injuries caused by Ni on root anatomical variables, resulting in increases in RET, RDT, and RCT. These results reveal that EBR contributes to improving the selectivity, maximizing the physical barrier of the tissues against toxic metals. EBR increased the diameter of vascular tissues in the root, mainly the vascular cylinder and metaxylem, structures that can maximize the uptake efficiency of nutrients and water in the symplastic pathway (Meyer et al. 2011). Concomitantly, this steroid probably stimulated the expression of the *PIN1* and *PIN2* genes, which are genes that have steroid-mediated regulation, contribute to auxin biosynthesis in the root system (Bao et al. 2004; Li et al. 2005; Smet et al. 2015) and induce the development and elongation of tissues and structures in the root system (Hacham et al. 2011; Xie et al. 2011). EBR also increased VCD and RMD. Maia et al. (2018) evaluated the EBR actions on root structures of two genotypes of *Solanum lycopersicum* L. (wild and dwarf) and described increments in the root anatomical variables (RET, RDT, RCT, VCD, and RMD), mainly in the dwarf genotype. Reis et al. (2017) detected that soybean plants under Ni toxicity suffer negative impacts on root phloem and xylem diameter, reducing the water supply

Fig. 3 Adaxial leaf surface (**A**, **C**, **E** and **G**) and abaxial (**B**, **D**, **F** and **H**) scanning electron microscopy showing stomata in soybean sprayed with EBR and high Ni concentration.—Ni$^{2\pm}$ EBR (A and B),—Ni^{2+} / + EBR (C and D), + Ni$^{2\pm}$ EBR (E and F) and + Ni^{2+} / + EBR (G and H). Legend: St =stomata. Bars: 20 µm



intrinsically necessary to photoassimilate mobility. Interestingly, Kováčik and Babula (2017) using fluorescence microscopy revealed overproduction of hydrogen peroxide and superoxide radical in roots of *Zea mays* L. seedlings exposed to toxic metal, confirming the oxidative stress on this organ.

EBR promoted beneficial effects on leaf structures, minimizing Ni phytotoxicity. In both leaf and root tissue, this steroid stimulated the processes of cell division and elongation (Khripach et al. 2000; Tanveer et al. 2018), inducing increases in ETAd and ETAb and indirectly improving stomatal efficiency (SD and SI), as previously confirmed by our results in this research. EBR clearly contributes to the increases verified in PPT, and SPT, generating better conditions for CO_2 fixation. Santos et al. (2020) investigated the EBR effects on the leaf anatomy of *Glycine max* (L.) Merr. plants under Zn stress (deficiency and toxicity) and reported increases in ETAd, ETAb, PPT and SPT, which were described by the authors as interesting interactions related to PPT and SPT, improving gas exchange, including CO_2 assimilation. Minkina et al. (2018) evaluating the problems occasioned by the toxic metals (Ni and others) on leaves of *Phragmites australis* (Cav.) Trin. ex Steud. plants, these authors detected grana with irregular shape and thylakoid disorders, resulting in chlorophyll degradation with intense negative repercussions on photosynthetic machinery. Ribeiro et al. (2020) detected significant increments in membrane damages (electrolyte leakage and malondialdehyde), being these responses related to oxidative stress generated by the reactive oxygen species (hydrogen peroxide and superoxide) in young *Eucalyptus urophylla* subjected to Ni excess.

Plants sprayed with EBR suffered fewer deleterious effects connected to Ni^{2+}, improving stomatal performance in soybean plants (Fig. 3), more specifically increasing SD, SF, and SI and reducing PDS and EDS on both leaf surfaces. Increments in SD, SF, and SI clearly reveal that this steroid improved stomatal regulation (Franks and Beerling 2009), contributing positively to gas exchange. In other words, EBR provided multiple benefits, improving efficiency in the use of water, increasing transpiration processes and maximizing CO_2 uptake (Luković et al. 2009; Eburneo et al. 2017). For PDS and EDS, the reductions detected are associated with anatomical changes in the stomata, which acquire a more elliptical form that favors stomatal performance (Martins et al. 2015). Balal et al. (2016) evaluated stomatal characteristics and gas exchange in *Lolium perenne* L. plants subjected to Ni^{2+} toxicity and reported significant reductions in the size and number of stomata, resulting in lower values of stomatal conductance, water-use efficiency and net photosynthetic rate.

In conclusion, this study revealed that EBR suppressed the injuries caused by Ni excess in root and leaf tissues. In roots, this steroid induced an increase in the epidermis, maximizing the physical barrier of the tissue against toxic metals. Synergistically, increments in the vascular cylinder and metaxylem can contribute to nutrient uptake in the symplastic pathway. For leaf tissue, significant increases in palisade and spongy parenchyma were detected in plants sprayed with EBR and exposed to Ni2 + ions, which are explained by the improvements observed in stomatal density and stomatal functionality because better stomatal performance facilitates carbon fixation in mesophyll cells (palisade and spongy parenchyma). Our results confirm that pretreatment with 100 nM EBR clearly mitigated the anatomical disorders occasioned by excess Ni on the leaf and root structures of soybean plants.

Acknowledgements This research had financial support from Fundação Amazônia de Amparo a Estudos e Pesquisas (FAPESPA/Brazil), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brazil) and Universidade Federal Rural da Amazônia (UFRA/Brazil) to AKSL.

Author contributions AKSL was the advisor of this project, planned all phases of the research and critically revised the manuscript. MPS, CFM and BRSS conducted the experiment, performed anatomical determinations and wrote and edited the manuscript, while BLB carried out the nutritional determinations. All authors read and approved the final version of the manuscript.

Data availability Data are available upon request to the corresponding author.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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4 PAPER 2

IONIC HOMEOSTASIS AND REDOX METABOLISM UPREGULATED BY 24-EPIBRASSINOLIDE ARE CRUCIAL TO MITIGATE NICKEL EXCESS IN SOYBEAN PLANTS, ENHANCING PHOTOSYSTEM II EFFICIENCY AND BIOMASS

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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.

4.1 Abstract

Nickel (Ni) excess often generates oxidative stress into chloroplast, causing redox imbalance, membrane damages and negative impacts on biomass. 24-Epibrassinolide (EBR) is a plant growth regulator with great interest by the scientific community because is a natural molecule, extracted from plants, biodegradable and environmentally friendly. This study aimed to answer whether EBR can induce benefits on ionic homeostasis and antioxidant enzymes and 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. The experiment was randomized with four treatments, including two Ni concentrations (0 and 200 µM Ni, described as – Ni^{2+} and + Ni^{2+}, respectively) and two concentrations of 24-epibrassinolide (0 and 100 nM EBR, described as – EBR and + EBR, respectively). In general, Ni caused modulated deleterious effects in chlorophyll fluorescence and gas exchange. In other hand, this steroid 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. The exogenous EBR application promoted significant increases in biomass, being these results explained by the benefits on nutrient contents and ionic homeostasis, proved by increases in Ca^{2+}/Ni^{2+}, Mg^{2+}/Ni^{+2} e Mn^{2+}/Ni^{2+} ratios.

Keywords brassinosteroids ● chlorophyll fluorescence ● *Glycine max* ● growth ● heavy metal

Abbreviations

BRs	Brassinosteroids
Ca	Calcium
CAR	Carotenoids
Chl *a*	Chlorophyll a
Chl *b*	Chlorophyll b
C_i	Intercellular CO_2 concentration
CO_2	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
F_0	Minimal fluorescence yield of the dark-adapted state
Fe	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
H_2O_2	Hydrogen peroxide
LDM	Leaf dry matter
MDA	Malondialdehyde
Mg	Magnesium
Mn	Manganese
Ni	Nickel
NPQ	Nonphotochemical quenching
O_2^-	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
Zn	Zinc
Φ_{PSII}	Effective quantum yield of PSII photochemistry

4.2 Introduction

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

Soil contamination by heavy metals often occurs due to inadequate crop management, being intensively applied pesticides, fertilizers, and petroleum products (Ayangbenro and Babalola 2017; Mir et al. 2018), in which they 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 represent a recurrent problem in modern agriculture (Yusuf et al. 2011b), in which this element is found in contaminated environments as Ni^{2+} (Ameen et al. 2019).

Ni excess often impacts the biomass, being this fact connected to inadequate uptake, transport and distribution of macro and micronutrients (Matraszek et al. 2016), including strong limitation on absorption of Mg, Mn, Zn and Fe (Palacios et al. 1998; Torres et al. 2016). Phytotoxicity linked to Ni negatively modulates the 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) (Yan et al. 2010; Amari et al. 2017). Oxidative stress generated into chloroplast cause redox imbalance and membrane damages (Gajewska et al. 2006; Israr et al. 2011; Sreekanth et al. 2013; Pietrini et al. 2015; Rizwan et al. 2018).

Brassinosteroids are plant growth regulators that act stimulating biochemical reactions, physiological responses and modulating cellular functions (Rahman et al. 2017; Jan et al. 2018). Among steroids, there are more than 50 natural and synthetic forms, in which 24-Epibrassinolide (EBR) presents great interest by the scientific community because is a natural molecule, extracted from plants, biodegradable and environmentally friendly. This molecule exercises multiple roles, stimulating the chloroplastic pigments (Parmoon et al. 2018), light capture (Lima and Lobato 2017; Yusuf et al. 2017) and fixation CO_2 (Sharma et al. 2016; Kohli et al. 2017), biosynthesis of nucleic acids (Bajguz 2000; Tanveer et al. 2018) and tissue structures (Rajewska et al. 2016; Ribeiro et al. 2019; Fonseca et al. 2020). Focusing on plant nutrition, Lima et al. (2018) verified that EBR increased the contents of essential elements, improving the nutritional balance (Oliveira et al. 2018) and the activities of the H+-ATPase enzymes in root system (Song et al. 2016). Paralelly, in literature are verified that EBR positively modulates the antioxidant system (Ahanger et al. 2020; Rodrigues et al. 2020), more specifically controlling the ROS overaccumulation (Ashraf et al. 2015; Anjum et al. 2017).

Hypothesis of this research was developed on the deleterious effects provoked by the Ni on photochemical efficiency (Drążkiewicz 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, the relevant roles linked to EBR on nutritional status (Lima et al. 2018) and antioxidant system (Santos et al. 2020) suggest that this growth regulator may represent an interesting option to mitigate the negative impacts induced by the Ni. This study aimed to answer whether EBR can induce benefits on ionic homeostasis and antioxidant enzymes and 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.

4.3 Materials and Methods

4.3.1 Location and growth conditions

The experiment was performed at the Campus of Paragominas of the Universidade Federal Rural da Amazônia, Paragominas, Brazil (2°55’ S, 47°34’ W). The study was conducted in a greenhouse with the temperature and humidity controlled. 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%.

4.3.2 *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 to 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.

4.3.3 *Experimental design*

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

4.3.4 *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. The 0 and 100 nM EBR (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).

4.3.5 *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.

4.3.6 Determining of Ni and nutrients

Milled samples (100 mg) of root, stem and leaf tissues were pre-digested using 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). The determination of Ni, P, Ca, Mg, Mn, Zn and Fe were performed using an inductively coupled plasma mass spectrometer (model ICP-MS 7900; Agilent).

4.3.7 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 Fv/Fm 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 µmol m^{-2} 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 CO2, photosynthetically active radiation, air-flow rate and temperature conditions at 360 µmol mol^{-1} CO2, 800 µmol photons m^{-2} s^{-1}, 300 µmol s^{-1} and 28 °C, respectively, between 10:00 and 12:00 h.

4.3.8 Determination of the 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). Thetotal soluble proteins were quantified using the methodology described by Bradford (1976). The SOD assay was measured at 560 nm (Giannopolitis and Ries 1977), and theSOD activity was expressed in mg^{-1} protein. The CAT assay was detected at 240 nm (Havir and McHale 1987), and the CAT activity was expressed in µmol

H_2O_2 mg^{-1} protein min^{-1}. The APX assay was measured at 290 nm (Nakano and Asada 1981), and the APX activity was expressed in µmol AsA mg^{-1} protein min^{-1}. The POX assay was detected at 470 nm (Cakmak and Marschner 1992), and the activity was expressed in µmol tetraguaiacol mg^{-1} protein min^{-1}. O_2^- was measured at 530 nm (Elstner and Heupel1976).

4.3.9 Quantification of hydrogen peroxide, malondialdehyde and electrolyte leakage

Stress indicators (H_2O_2 and MDA) were extracted using the methodology described byWu 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 mM^{-1} cm^{-1}. EL was measured according to Gong et al.(1998) and calculated by the formula EL (%) = $(EC_1/EC_2) \times 100$.

4.3.10 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.

4.3.11 Data analysis

The 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.

4.4 Results

4.4.1 Plants pretreated with EBR had reductions in Ni^{2+} contents in tissues

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

4.4.2 *Steroid mitigated the stress caused by Ni on nutritional status*

The Ni excess caused reductions in nutrient contents (Table 2). In other hand, the treatment with EBR and Ni^{2+} had increases in P, Ca, Mg, Mn, Zn and Fe of 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 the same treatment without EBR. To ionic ratios (Table 3), plants under Ni^{2+} excess and sprayed with EBR resulted in increments in Ca^{+2}/Ni^{+2}, Mg^{+2}/Ni^{+2} and Mn^{+2}/Ni^{+2} ratios of 77%, 84% and 100% in root, 33%, 115% and 42% in stem and 49%, 86% and 55% in leaf , if compared with equal treatment without EBR.

4.4.3 *Pretreatment with EBR minimized the Ni impacts on photosynthetic machinery*

Ni caused negative changes in chlorophyll fluorescence (Fig. 1). However, EBR spray in plants exposed to Ni^{2+} produced significant increases of 4%, 5% and 1% in the values of F_m, F_v and F_v/F_m, when compared to the same treatment without EBR. For chlorophyll fluorescence (Table 4), treatment with Ni^{2+} + EBR, variables Φ_{PSII}, q_P and ETR had increments ($P<0.05$) of 15%, 32% and 19%, respectively. Plants pretreated with EBR and submitted to Ni^{2+} suffered reductions of 21% and 5% in NPQ and EXC, in the same order, if 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 had significant increases in P_N, g_s, WUE, and P_N/C_i, corresponding to 20%, 19%, 16%, and 23%, respectively. Additionally, also were verified increases in E (3%) and reduction in C_i (4%), comparing with plants that received the same treatment without EBR. On photosynthetic pigments, high Ni supply occasioned damages on chlorophylls and carotenoids (Table 4). On the other hand, the treatment EBR and Ni^{2+} had significant increases in Chl *a*, Chl *b*, Total Chl and Car of 37%, 40%, 37% and 31%, respectively, as well as an increase in Total Chl/Car (4%) and decrease in Chl *a*/Chl *b* (2%), when compared to the same treatment without EBR application.

4.4.4 Antioxidant responses were upregulated in plants treated with EBR andexposed to Ni

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

4.4.5 EBR suppressed the negative impacts caused by Ni excess on biomass

Toxic action promoted by Ni provoked decrease in biomass (Fig. 4), but EBR treatment in plants under Ni stress mitigated these effects, being verified increases of 7%, 12%, 25% and 7% in LDM, RDM, SDM and TDM, in this order, when compared to the sametreatment without EBR.

4.5 Discussion

Soybean plants pretreated with EBR had reductions in Ni contents of root, stem and leaf. These results indicate that this steroid probably maximized the endogenous levelsof phytoquelatins, acting on immobilization and detoxification of excess N^{2+} ions into the plant cells (Rajewska et al. 2016). Concomitantly, there was 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 the EBR application (10^{-6} M) in *Cicer arietinum* seedlings submitted to Hg toxicity (15 µM and 30 µM), obtaining significant reductions in the Hg contents in root and leaf, besides increments in Mg, Mn and Ca contents. Surgun et al. (2016) evaluating the B toxicity in *Arabidopsis thaliana* treated EBR observed the reduction of B contents in the tissues of the leaf, root and inflorescence, being these results explained by the authors due to the better selectivity of the membrane enzymes. Sharma et al. (2011) studying the action mechanisms connected to EBR in *Raphanus sativus* seedlings under Ni stress found increases in root length and activities of the antioxidant enzymes in shoot.

Steroid mitigated the stress caused by Ni on 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) studying the EBR effects on the nutritional status of *Sinapis alba* submitted to four Ni concentrations (0, 0.0004, 0.04 and 0.08 mM Ni) observed reductions in P, Ca and Mg contents. Yuan et al. (2015) evaluating the EBR roles on the nutrient accumulations in *Cucumis sativus* plants under $Ca(NO_3)_2$ stress described increases in K, P, Mg, Fe and Mn contents in shoot and in the 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.

Ca^{2+}/Ni^{2+}, Mg^{2+}/Ni^{+2} and Mn^{2+}/Ni^{2+} ratios were increased after EBR spray in leaf, stem and root. These results reveal multiple benefits of this steroid on homeostasis, increasing ionic ratios and decreasing the stress generated by the 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) studying the EBR effects in *Solanum tuberosum* plants under salt stress conditions described positive effects of this steroid on homeostasis connected to K^+/Na^+ ratio, combined with higher root efficiency and improvement on antioxidant capacity in shoot.

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 absorption of light energy by the chloroplasts. EBR clearly alleviated oxidative damages due to increases in the activities of antioxidant enzymes (SOD, CAT, APX and POX) measured in this research, resulting in lower concentrations of oxidative compounds, such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), revealing a protective role of the EBR on chloroplast ultrastructure (Sadeghi and Shekafandeh 2014). According to Wani et al. (2017), BRs protects the PSII against excessive excitation in abiotic stress conditions, preventing possible damages to thylakoid membranes. The positive effects of EBR also induced increases in Φ_{PSII}, ETR and q_P, being related to the benefits in F_0 and F_m, verified in this study. This result reveals better absorption and photon capture and maintenance of Q_A oxidation, improving the flow of electrons through PSII. Additionally, the 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) studying *Vitis vinífera* genotypes under

conditions of water restriction reported an increase in ETR/PN, this result being 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, energy being accumulated in the antenna complex and developing favorable conditions for the overproduction of reactive oxygen species (ROS), which in contact with membrane causes severe damages to thylakoid structures and pigments (Anjum et al. 2016). Bukhari et al. (2016) described that the EBR application attenuates the stress generated by Cr and increased the F_v/ F_m values in *Nicotiana tabacum* seedlings. Pietrini et al. (2015) studying the deleterious effects caused by Ni on the chlorophyll fluorescence in plants of *Amaranthus paniculatus* detected reductions in the values of Φ_{PSII}, q_P and NPQ, compromising the functioning of the 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 the Ni on gas exchange. Increases in P_N and WUE promoted by the EBR can be explained by the positive effects on PPT and SPT detected in this study. These tissues have a large amount of chloroplasts and it 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 the 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 action on possible increases in ribulose-1,5-bisphosphate carboxylase/oxygenase activity (reduction in C_i) and by the CO_2 fixation (increase in P_N) during the photosynthetic process (Farooq et al. 2009; Xia et al. 2009a; Shu et al. 2016). Ni interferences impaired gas exchange, reducing P_N, g_s, and E, being related to the stomatal limitations, confirmed by the reductions in SF and SI, and the non-stomatal implications, corroborated by the overproductions of O_2^- and H_2O_2, verified in this research. Shah et al. (2019) investigating photosynthetic responses and the antioxidant system in *Cucumis sativus* plants treated with EBR (5 µM) and subjected to Cd stress (2.5 mM) confirmed that the steroid attenuated ($P<0.05$) the effects of heavy metal on P_N, C_i, g_s, and *E*. Santos et al. (2020) evaluating gas exchange and anatomical structures in *Glycine max* plants exposed to Zn stress and treated with EBR, obtaining increases in P_N, *E*, g_s, WUE and P_N/C_i and reduction in C_i. Khan et al. (2017) measuring the deleterious effects provoked by the soil contamination by Ni (50, 100 and 200 mg Ni kg^{-1} soil) on gas exchange in *Vinca rosea* plants described reductions in g_s, *E* and P_N. Khaliq et al. (2016) studying the alterations caused by Ni toxicity (50 and 100 µM) on carbon fixation in *Gossypium hirsutum* plants verified significant reductions in *E* and P_N.

Ni excess was partially suppressed by the exogenous application of EBR, with increases on SOD, CAT, APX and POX activities. EBR clearly improved the performance of the antioxidant system, resulting elimination more efficient 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 (Yusuf et al. 2011a), reducing damages on structure of the 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^-) verified in our research. Cao et al. (2005) investigating the biochemical and molecular responses in *Arabidopsis thaliana* with loss of function for *DET2* gene, interestingly described that the mutation of this gene increased the transcripts linked to the antioxidant system, reducing simulated oxidative stress. Xia et al. (2009b) obtained higher tolerance to stress in *Cucumis sativus* leaves treated with EBR, positively regulating gene expression and activities of enzymes related to antioxidant metabolism. Oliveira et al. (2019) described that pretreatment with 100 nM of EBR in *Eucalyptus urophylla* plants Na^+ stressed resulted in increases of SOD, CAT, APX and POX, in which the authors found that this steroid minimized deleterious effects on photosynthetic machinery.

In literature there are several studies describing the benefits of this steroid, more specifically potentiating antioxidant enzymes, such as Hussain et al. (2019) evaluating the antioxidant system in *Triticum aestivum* under EBR application and Mn stress obtaining increases in SOD, CAT and POX enzymes. These authors related that probably the EBR stimulated the expression of regulatory genes involved in antioxidant defense. Fariduddin et al. (2015) studying the responses on photosynthetic attributes and redox metabolism in *Brassica juncea* seedlings sprayed with EBR and under Mn toxicity, revealing evidences that this natural steroid acts as an efficient stress alleviator. EBR exogenous induced decreases in oxidative compounds and cellular damages generated by Ni. These facts reveal that EBR improved the performance of the

antioxidant system, controlling the H_2O_2 and O_2^- overproduction, subsequently mitigating oxidative damages, confirmed by 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) evaluating *Glycine max* seedlings treated with EBR (0.5 µM) and As stressed found decreases in O_2^-, H_2O_2 and MDA, which these authors suggested that the EBR promoted tolerance against oxidative stress by accumulating osmolytes and activating antioxidant defense system in the stressed plants. Dalyan et al. (2018) studying the EBR roles under ROS overproduction in seedlings of *Brassica juncea* seedlings under Pb stress obtained reductions in H_2O_2 and MDA values, suggesting a protective role triggered by the steroid. Sreekanth et al. (2013) described that Ni in high concentrations can interfere negatively on balance between detoxification and 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 often are connected to inefficient antioxidant system in plants under environmental stress conditions.

Steroid positively acts 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 the oxidative damages and subsequent positive repercussions on chlorophyll fluorescence, evidenced in this study. In other words, occurred reductions of O_2^- and H_2O_2, combined with less deleterious effects to membranes (MDA and EL), resulting in better structural and functional integrities of these pigments associated with increments linked to light absorption, confirmed by the increases in ETR and Φ_{PSII}. Simultaneously, increases showed in Chl *a*, Chl *b* and Car are probably 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 a higher contents of essential elements, specifically the Mg that composed the structure of the chlorophyll molecule (Jan et al. 2018). There are studies in the literature that reveal the direct relationship between Ni excess and ROS overproduction (Muhammad et al. 2013; Dourado et al. 2015) and its potential deleterious effects, such as peroxidation and degradation of membranes in chloroplastic pigments (Gajewska and Skłodowska 2008; Ahmad et al. 2010; Gill and Tuteja 2010). Results that corroborate our research were described by Ahmad et al. (2018a) studying the effects of the EBR application in *Solanum lycopersicon* plants stressed by NaCl, obtaining increases in photosynthetic pigments (Total Chl and Car), being attributed by the authors to changes in enzymatic and non-enzymatic antioxidants, osmolytes and metabolites. Dong et al. (2019) evaluating the action mechanisms triggered by the EBR (0.1 µM) and nitric oxide on gas exchange of *Arachis hipogaea* plants under Cd toxicity found significant increases in Chl *a*, Chl *b* Total Chl and Car.

Pretreatment with EBR spray in soybean plants subjected to Ni stress had 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 on plant metabolism, more specifically increasing essential nutrient contents, reduced ROS levels, improved gas exchange (P_N, *E*, g_s and WUE) and attenuated the negative effects connected to Ni on chloroplastic pigments (Chl *a*, Chl *b* and Car). Liu et al. (2019) evaluating the effects induced by brassinosteroid mimetics (EBR, bikinin and brazide) in Zea mays exposed to nicosulfuron toxicity found benefical responses linked steroid on P_N, increments in chlorophylls and reduction in H_2O_2, improving also the biomass. Zhong et al. (2020) investigating 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) detected increments in biomass (shoot and root), similarly with results found in this research.

4.6 Conclusion

This research evidenced the positive effects induced by EBR on antioxidant metabolismand ionic homeostasis in soybean plants submitted to Ni excess. This steroid improved the performance of photosynthetic machinery, more specifically effective quantum yieldof 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 the oxidative damages on chloroplast. The exogenous EBR application promoted significant increases in biomass (leaves, root and stem), being these results explained by the benefits on nutrient contents and ionic homeostasis, proved by increases in Ca^{2+}/Ni^{2+}, Mg^{2+}/Ni^{+2} e Mn^{2+}/Ni^{2+} ratios. Therefore, our results demonstrated that EBR attenuated the oxidative damages caused by Ni in soybean plants.

Acknowledgements

This research had financial supports from Fundação Amazônia de Amparo a Estudos e Pesquisas (FAPESPA/Brazil), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brazil) and Universidade Federal Rural da Amazônia (UFRA/Brazil) to AKSL.

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Figures

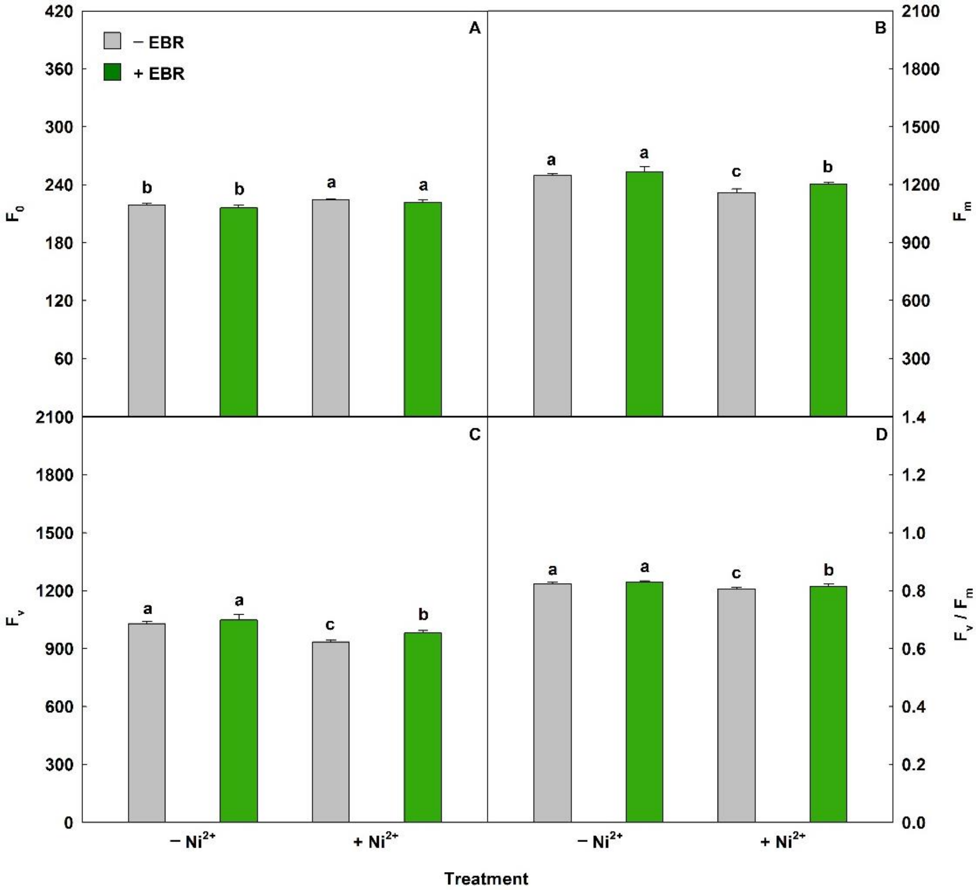

Fig. 1. Minimal fluorescence yield of the dark-adapted state (F_0), maximal fluorescence yield of the dark-adapted state (F_m), variable fluorescence (F_v) and maximal quantum yield of PSII photochemistry (F_v/F_m) in soybean plants sprayed with EBR and high Ni concentration. Columns with different letters indicate significant differences from the Scott-Knott test (*P*<0.05). Columns corresponding to means from five repetitions and standard deviations.

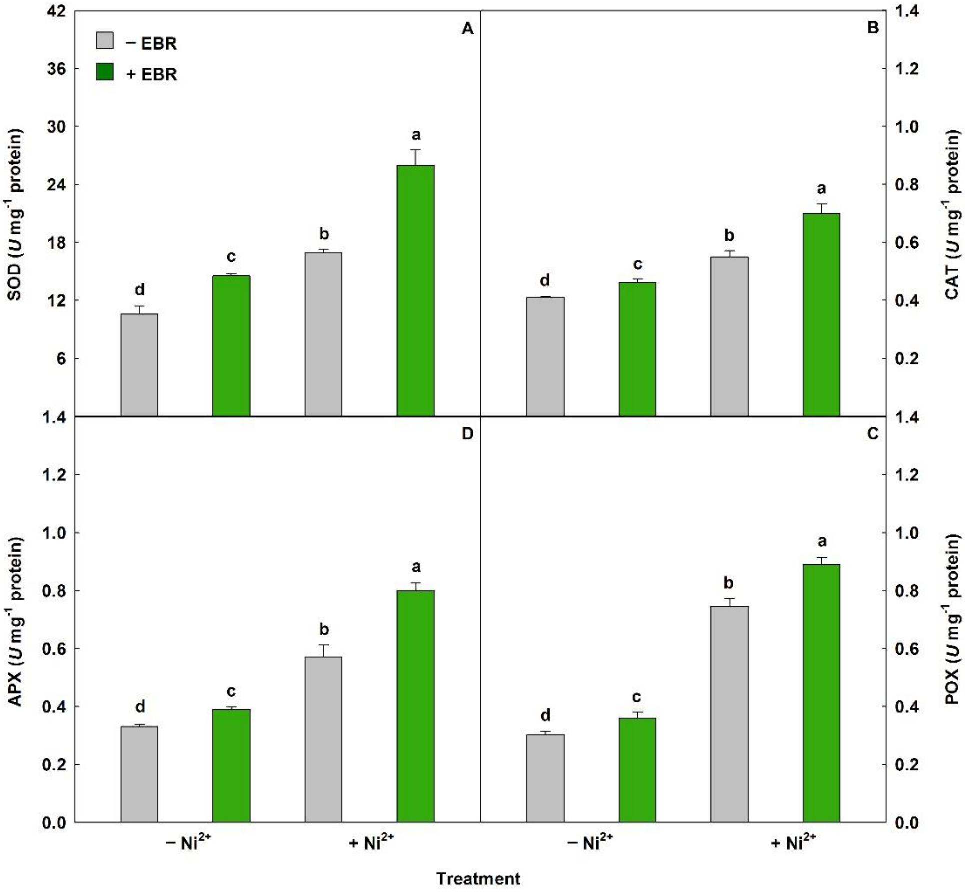

Fig. 2. Activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POX) in soybean plants sprayed with EBR and high Ni concentration. Columns with different letters indicate significant differences from the Scott-Knott test (*P*<0.05). Columns corresponding to means from five repetitions and standard deviations.

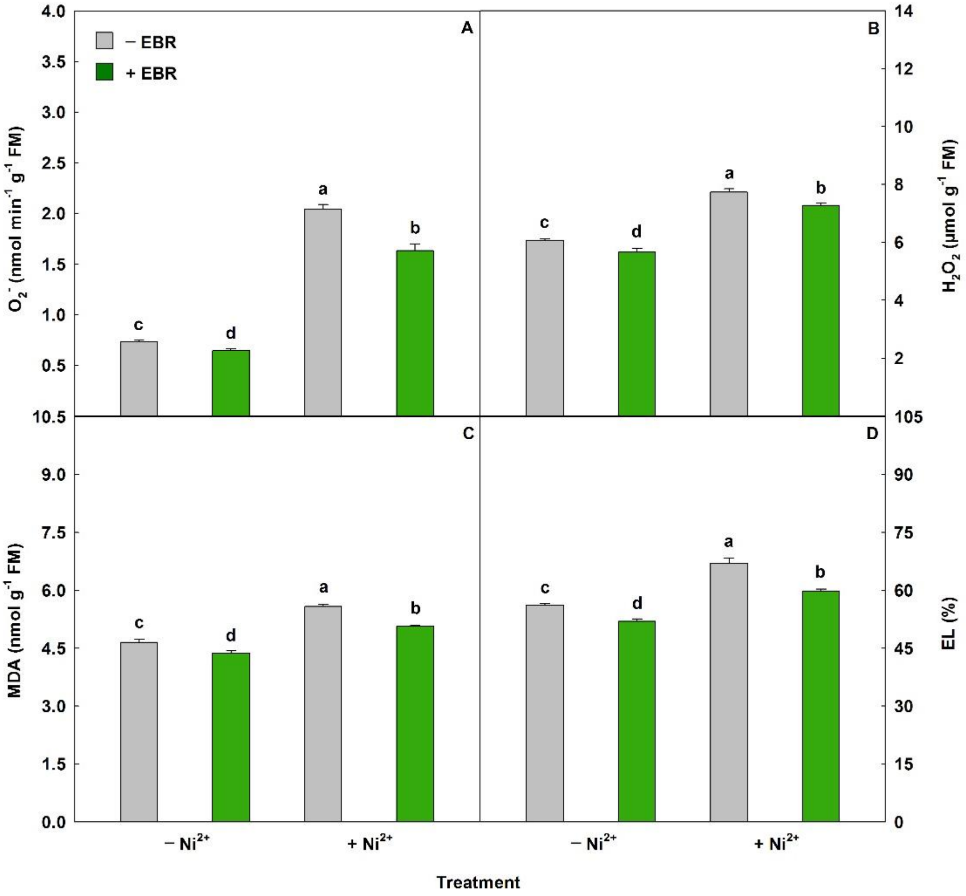

Fig. 3. Superoxide (O_2^-), hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and electrolyte leakage (EL) in soybean plants sprayed with EBR and high Ni concentration. Columns with different letters indicate significant differences from the Scott-Knott test ($P<0.05$). Columns corresponding to means from five repetitions and standard deviations.

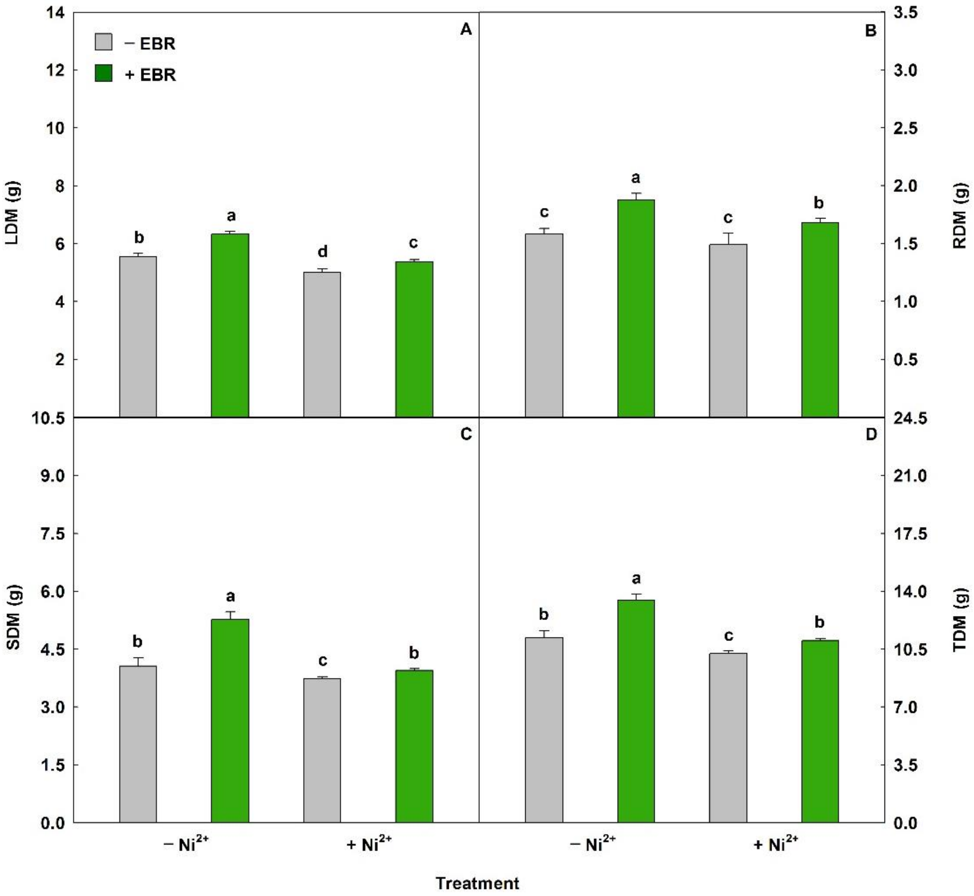

Fig. 4. Leaf dry matter (LDM), root dry matter (RDM), stem dry matter (SDM) and total dry matter (TDM) in soybean plants sprayed with EBR and high Ni concentration. Columns with different letters indicate significant differences from the Scott-Knott test (*P*<0.05). Columns corresponding to means from five repetitions and standard deviations.

Tables

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

Ni^{2+}	EBR	Ni in root (µg g DM^{-1})	Ni in stem (µg g DM^{-1})	Ni in leaf (µg 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 corresponding to means from five repetitions and 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 corresponding to means from five repetitions and 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

Ca^{2+}/Ni^{2+} = Calcium and nickel ratio; Mg^{2+}/Ni^{2+} = Magnesium and nickel ratio and Mn^{2+}/Ni^{2+} = Manganese and nickel ratio. Columns with different letters indicate significant differences from the Scott-Knott test ($P<0.05$).Values described corresponding to means from five repetitions and 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 ($\mu mol\ m^{-2}\ s^{-1}$)	EXC ($\mu mol\ m^{-2}\ s^{-1}$)	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 ($\mu mol\ m^{-2}\ s^{-1}$)	E ($mmol\ m^{-2}\ s^{-1}$)	g_s ($mol\ m^{-2}\ s^{-1}$)	C_i ($\mu mol\ mol^{-1}$)	WUE ($\mu mol\ mmol^{-1}$)	P_N/C_i ($\mu mol\ m^{-2}\ s^{-1}\ Pa^{-1}$)
−	−	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 *a* ($mg\ g^{-1}$ FM)	Chl *b* ($mg\ g^{-1}$ FM)	Total Chl ($mg\ g^{-1}$ FM)	Car ($mg\ g^{-1}$ FM)	Ratio Chl *a*/Chl *b*	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 CO_2 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 corresponding to means from five repetitions and standard deviations.

5 GENERAL CONCLUSION

The treatment with EBR positively influenced the structural, physiological, biochemical, nutritional, and growth characteristics of soybean plants under Ni stress. Its action promoted an increase in the thickness and diameter of the root tissues (epidermis, vascular cylinder, and metaxylem) with indirect effect in improving the physical barriers against the accumulation of Ni ions, and in the absorption of nutrients in the symplastic pathway. In the leaf structure, the EBR increased the thickness of the parenchymal tissues (palisade and spongy) with an influence observed in the density and functioning of the stomata.

Its addition affected the physiology and metabolism of the plants through the protection of photosynthetic machinery; reduced the oxidative compounds with an increase in antioxidant enzymes (SOD, CAT, APX, and POX); preserved the integrity of cell membranes; stimulated the synthesis of photosynthetic pigments and mitigated the negative effect caused by gas exchange.

The EBR regulated the uptake of macro and micronutrients; caused improvements in the use of water; reduced the concentration of Ni in the tissues and finally contributed decisively to the improvements mentioned in the increase in the biomass of soybean plants.