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24-EPIBRASSINOLIDE: A MOLECULE WITH MULTIPLE BENEFITS AGAINST HEAVY METAL TOXICITY IN PLANTS

BELÉM-PA
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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.
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RESUMO

Vários poluentes têm sido lançados na atmosfera por meio de inúmeras atividades humanas, entre esses estão os metais pesados (HMs), que são as principais ameaças ao meio ambiente por suas características potencialmente citotóxicas, genotóxicas e mutagênicas. Dentre os HM, Ni e Pb são elementos que podem causar fitotoxicidade, afetando o desempenho fisiológico e o acúmulo de biomassa. Para superar essas interferências negativas, os brassinosteróides (BRs) surgem como reguladores de crescimento de plantas que desempenham uma variedade de funções fisiológicas que também conferem resistência às plantas contra vários estresses bióticos e abióticos, sendo 24-epibrassnolide (EBR) um dos mais biologicamente ativos. Nesse contexto, o objetivo deste estudo foi verificar se a aplicação exógena de EBR pode amenizar os danos provocados pelo estresse induzido por metais pesados, especificamente por Ni e Pb, em tomateiro, avaliando as respostas bioquímicas, fisiológicas, anatômicas e nutricionais. Assim, dois experimentos foram planejados e executados, o primeiro analisou a ação da EBR sobre os efeitos deletérios do excesso de Ni (experimento I), e o segundo avaliou o papel desse esteroide na mitigação da toxicidade do Pb (experimento II). Os dois experimentos foram randomizados com quatro tratamentos cada, o experimento I com duas concentrações de níquel (0 e 400 μM Ni, descritos como - Ni e + Ni, respectivamente), e o experimento II também com duas concentrações de chumbo (0 e 200 μM Pb, descritos como - Pb e + Pb, respectivamente), foram utilizados em ambos os experimentos dois níveis de EBR (0 e 100 nM EBR, descritos como - EBR e + EBR, respectivamente). Em relação ao experimento I, os resultados mostraram que o EBR aliviou o estresse do Ni por meio da regulação positiva do sistema antioxidante com incrementos de 44%, 27%, 46% e 35% em SOD, CAT, APX e POX, respectivamente, auxiliando na proteção do maquinário fotossintético e estimulando o acúmulo de biomassa. Já para o experimento II, os resultados demonstram as interferências causadas pelo estresse de Pb em tomateiro, porém a aplicação exógena de EBR também mitigou os efeitos negativos, confirmados pela melhora na anatomia radicular com aumentos de 23%, 24% e 20% em RET, RDT e RMD, respectivamente, consequentemente promovendo ganhos de 95%, 115% e 92%, na biomassa foliar, radicular e total, respectivamente. Portanto, esta pesquisa demonstrou que a EBR amenizou os danos provocados pelo estresse de Ni e Pb em tomateiros.

Palavras-chave: Poluente, Brassinosteróide, Dano oxidativo, Aparelho fotossintético.

ABSTRACT

Several pollutants have been released into the atmosphere through numerous human activities, among these are heavy metals (HMs), which are the main threats to the environment due to their potentially cytotoxic, genotoxic and mutagenic characteristics. Among of HM, Ni and Pb are elements that can cause phytotoxicity, affecting the physiological performance and biomass accumulation. To overcome these negative interferences, brassinosteroids (BRs) emerge as plant growth regulators that perform a variety of physiological functions such as growth, and that also confer resistance to plants against various biotic and abiotic stresses, being 24-epibrassnolide (EBR) one of the most biologically active. In these contexts, the aim of this study was to verify whether the exogenous application of EBR can alleviate the damage provoked by heavy metals induced stress, precisely by Ni and Pb, in tomato plants, evaluating the biochemical, physiological, anatomical, and nutritional responses. Thus, two experiments were planned and executed, the first analyzed the EBR action on deleterious effects of excess Ni (experiment I), and the second evaluated the role of this steroid in mitigating the Pb toxicity (experiment II). The two experiments were randomized with four treatments each, the first with two concentrations of nickel (0 and 400 μM Ni, described as - Ni and + Ni, respectively), and the second also with two concentrations of lead (0 and 200 μM Pb, described as - Pb and + Pb, respectively), two levels of EBR were used in both experiments (0 and 100 nM EBR, described as - EBR and + EBR, respectively). Regarding experiment I, the results showed that EBR alleviated Ni stress through upregulating the antioxidant system, with increments of 44%, 27%, 46% and 35% in SOD, CAT, APX and POX, respectively, assisting to protect photosynthetic machinery and stimulating the accumulation of biomass. While for experiment II, the results demonstrate the interferences caused by Pb stress in tomato plants, however the exogenous application of EBR also mitigated the negatives effects, confirmed by the improvement in root anatomy with increases of 23%, 24% and 20% in RET, RDT and RMD, respectively, consequently promoting gains of 95%, 115% and 92% in leaf, root and total biomass, respectively. Therefore, this research demonstrated that EBR alleviated the damage provoked by Ni and Pb stress in tomato plants.

Keywords: Pollutant, Brassinosteroids, Oxidative damage, Photosynthetic apparatus.

SUMMARY

CONTEXTUALIZATION

In the last decade, soil heavy metal (HM) contamination has become a primary and severe problem in many regions of the world. Agricultural and urban soil contamination by HMs of natural and anthropogenic sources are being studied and received a special attention (ADIMALLA; QIAN; WANG, 2019; SONG *et al.*, 2018). Activities such as mining, use of fertilizer and pesticides, paper and electronic industries have accounted for the release of large amounts of HMs into the natural ecosystem, possibly causing an interruption in physiological functions in biological systems (JACOB *et al.*, 2018; TAIWO *et al.*, 2016).

HM is a general term assigned to the group of metals and metalloids that have an atomic density greater than 4 g cm^{-3} (EDELSTEIN; BEN-HUR, 2018; HAWKES, 1997), some of the examples of these include copper (Cu), cadmium (Cd), zinc (Zn), chromium (Cr), arsenic (As), boron (B), cobalt (Co), titanium (Ti), tin (Sn) nickel (Ni), molybdenum (Mo), mercury (Hg), lead (Pb), etc. (VARDHAN; KUMAR; PANDA, 2019). These elements can be classified in many ways, the Lewis acid classification indicates the formation of bonding in metal complexes, another way of grouping is based on their toxicity to humans and aquatic biota (AFROZE; SEN, 2018). In addition, they can be divided into two main groups: elements essential for plant growth, but toxic in excessive concentrations; and non-essential for plants and animals, where recommended and toxic concentrations are quite narrow (EDELSTEIN; BEN-HUR, 2018).

Although they occur naturally and some are biologically essential, dangerous concentrations of HM can be caused by pollution emissions and the distribution these elements depends on the proximity of the emitting sources, as well as the media evaluated (VAREDA; VALENTE; DURÃES, 2019). There are different sources of HM in the environment, their natural occurrence in soil is simply a product of weathering process, however major anthropogenic sources are agriculture, industry, mining, transportation, fuel consumption, residual organic matter, and sewage water (EDELSTEIN; BEN-HUR, 2018; GILL, 2014).

HMs are inorganic non-biodegradable substances that tend to accumulate in living organisms and some of these are considered carcinogens (AFROZE; SEN, 2018). Like all living organisms, plants are often sensitive to inappropriate amounts of heavy metals, being strongly harmful to the metabolic activities at higher concentrations, become a critical environmental concern due to their widespread occurrence and their acute and chronic toxic effect on plants grown (NAGAJYOTI; LEE; SREEKANTH, 2010).

Unlike several HM, such as Cd, Cr and Hg, that have no biological function in plant (SEREGIN; KOZHEVNIKOVA, 2006), Ni is considered an essential element for plant growth and development, being a component of several enzymes, like urease and glyoxalase-I, significantly contributing to nitrogen assimilation (DRĄŻKIEWICZ; BASZYŃSKI, 2010; SHAHZAD *et al.*, 2018). This element is incredibly important for plant nutrition, however, due to be required in small amounts, at high concentration, it becomes toxic for plants (HASSAN *et al.*, 2019; SREEKANTH *et al.*, 2013).

Ni is a trace metal which is extensively disseminated in the environment through both natural and anthropogenic sources (AMEEN *et al.*, 2019; SREEKANTH *et al.*, 2013), being released into the environment through various human activities like industries, burning of fossil fuels, application of phosphatic fertilizers, smelting, sludge water, electroplating industries, and vehicle emissions (HASSAN *et al.*, 2019; SHAHZAD *et al.*, 2018). One of the aspects of this metal is to be uniformly distributed through the soil profile, but usually, its concentration is high at soil surface due to the deposition of industrial waste and agricultural activities (HASSAN *et al.*, 2019; YUSUF *et al.*, 2011).

An accumulation beyond the beneficial Ni window can cause toxicity, affecting various physico-biochemical processes, including mineral absorption, photosynthesis, membrane permeability, nitrogen metabolism, and senescence in plants (KÜPPER; ANDRESEN, 2016; MIR *et al.*, 2018; SIRHINDI *et al.*, 2016). Kumar and Prasad (2015) presents reports that higher Ni concentrations disturb physiological and biochemical processes in the plant, which include reduced growth (MOLAS, 2002), photosynthesis and water relationships (CHEN, C.; HUANG; LIU, 2009), alteration of enzymatic activities (GAJEWSKA *et al.*, 2006), interference with the uptake and translocation of others nutrients (CHEN, Cuiyun; HUANG; LIU, 2009) and causes initiation of oxidative damage, through increase in lipid peroxidation products, membrane permeability and chlorophyll degradation (CHEN, Cuiyun; HUANG; LIU, 2009; GAJEWSKA *et al.*, 2012), as a result negatively affect the crop yield and quality (GAJEWSKA *et al.*, 2006).

Among HM, Pb is a naturally occurring non-biodegradable, non-essential metal and considered one of major pollutant substances for all living organisms (KHAN *et al.*, 2018). This element is one of the most widely distributed trace metals, has become ubiquitous in the soil and in the environment due to natural deposits and increasing human activities, such as mining, smelting, fuel combustion, synthetic fertilizers, and various industrial processes: building construction, Pb-acid batteries, bullets and shot, solder,

pewter, and fusible alloys (FAHR *et al.*, 2013; MUKAI *et al.*, 2001). The extensive use Pb in many industries is mainly due to its low melting point, high density, ease of casting, acid resistance, ease of fabrication and chemical stability in the environment (KUMAR, Abhay; PRASAD, 2018).

Pb is a type of phytotoxic element and different plants response processes depends on the plant's genotype and physiological characteristics. Several studies indicate that this metal in general adversely affects plants morpho-physiological and biochemical processes such as seed germination and seedling growth, plant phenology, and root/shoot ratio; disrupts cell membrane permeability, photosynthesis, plant respiratory processes, chlorophyll contents, chloroplastic lamellar organization and cell division; and cause growth and developmental abnormalities as well as ultrastructure changes (ASHRAF *et al.*, 2015; GUPTA; HUANG; CORPAS, 2013).

By acting as an active non-redox molecule, Pb alters this redox balance through the different indirect mechanisms like replacement of essential cations from cellular biomolecules and alteration of metal containing enzymes activities, which enhances the generation of ROS (CHEN, Q. *et al.*, 2017; KUMAR, Abhay; PRASAD, 2018; SHU *et al.*, 2012). Thus, Pb exposure can induce the accumulation of singlet oxygen (O_2^-) and hydrogen peroxide (H_2O_2), causing changes membrane structure and function, starting lipid peroxidation (KUMAR, Abhay; PRASAD; SYTAR, 2012), hence resulting in oxidative damages to membrane lipids, proteins, chloroplast pigments, enzymes and nucleic acids (SRIVASTAVA *et al.*, 2014).

Moreover, imbalance in the uptake, assimilation, and translocation of nutrients result of Pb excess interfering in the permeability of the plasma membrane and influencing the processes involved in the transfer of these elements across the root membrane (KHAN *et al.*, 2018). Photosynthesis is impaired through a series of processes affected by Pb toxicity, such as disruption photosynthetic pigments synthesis, injury of chloroplast ultrastructure, changes in lipid and protein composition of thylakoid membrane, restricted electron transport, inhibited activities of Calvin cycle enzymes, besides deficiency of CO_2 in result of stomatal closure (AHMAD *et al.*, 2011; ALI; NAS, 2018).

In this context, the exogenous application of BRs emerges as a strategy to stimulate plant resilience since these substances are involved in the regulation of several physiological processes, such as the activation of antioxidant enzymes and protection of the photosynthetic apparatus, providing tolerance in environmental stress (DIVI; KRISHNA,

2009; FARIDUDDIN *et al.*, 2014). A study using *Brassica napus* pollen extract, identified BRs as the most active growth promoter discovered, showing increases in stem elongation and cell division in bean internodes (MITCHELL *et al.*, 1970), and so it was prematurely concluded that BRs are specific translocable organic compounds isolated from a plant that allowed a measurable growth control when applied in minimal amounts in another plant (CLOUSE, 2011; OKLESTKOVA *et al.*, 2015). In responses to HM stress, some studies report BRs can regulate the uptake of ions into the plant cells and can be used to reduce the accumulation of these metals, through regulating the electrical characteristics of membranes, cell membrane permeability and ion transport (AHAMMED *et al.*, 2020; DALYAN; YÜZBAŞIOĞLU; AKPINAR, 2018). Moreover, this steroid also increase the activities of some antioxidant enzymes detoxifying the increased production of reactive oxygen species (ROS) generated by heavy metal stress (VÁZQUEZ *et al.*, 2013; XIA *et al.*, 2009).

BR correspond to a group of natural steroidal lactones/ketones that are profusely distributed in the plant kingdom that initially, were considered as plant growth regulators compounds, but now are established as the sixth plant hormone class. (BAJGUZ, 2011; KANWAR *et al.*, 2017). Among all isolated and characterized, Brassinolide (BL), 24-epibrassinolide (24-EBL) and 28-homobrassinolide (28-HBL) are the main bioactive BRs (JOO *et al.*, 2015; VARDHINI; ANJUM, 2015), being reported to be associated with a wide range of biological responses, regulating physiological and developmental processes, such as cell elongation (stem, root), leaf expansion, photomorphogenesis, flower developmental processes, male sterility, stomatal developmental processes, and resistance to stress (ANWAR *et al.*, 2018; NAWAZ *et al.*, 2017).

To better assimilate the action of this steroid on heavy metals, specifically Ni and Pb, tomato (*Solanum lycopersicum* L.) plants were used in this research because this species has been considered the best model organism for fleshy-fruited plants to be used in basic or applied research (MAIA; SILVA; LOBATO, 2018; SURESH *et al.*, 2014). Tomato stands out for having sequenced and small genome (THE TOMATO GENOME CONSORTIUM.; INSTITUTE.; SATO, 2012), a short life cycle and specific morphological traits that are not shared with other model plants (GERSZBERG *et al.*, 2015), besides being able to be cultivated in different conditions, be propagated asexually by grafting, or regenerated from distinct parts of the plant, and to grow in space limited with controlled conditions, like a greenhouse (FLORES *et al.*, 2016).

Our hypothesis considers the negative interferences that Ni and Pb toxicity can causes in antioxidant metabolism, gas exchange, photosynthetic pigments, including the ROS overproduction, resulting in severe oxidative damages (JAHAN *et al.*, 2020; KUMAR, Abhay; PRASAD, 2018) and inhibition of photosynthesis (ALI; NAS, 2018; SREEKANTH *et al.*, 2013). In other hand, diverse studies are demonstrating the potential of BR in ameliorates of heavy metal stress in plants (RIBEIRO *et al.*, 2020; ZHONG *et al.*, 2020), however due to some processes performed by this steroid on heavy metal stress are still unclear, further research is needed. Thus, the aim of this study was to verify whether the exogenous application of EBR can alleviate the damage provoked by heavy metals induced stress oxidative, precisely by Ni and Pb, in tomato plants, evaluating the biochemical, physiological, anatomical, and nutritional responses.

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Exogenously Applied 24-Epibrassinolide Favours Stomatal Performance, ROS Detoxification and Nutritional Balance, Alleviating Oxidative Damage Against the Photosynthetic Apparatus in Tomato Leaves Under Nickel Stress

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Abstract
Soil contamination by toxic heavy metals (HMs) represents a serious global ecological problem. Among the HMs often verified in agricultural soils, nickel (Ni) excess can cause phytotoxicity, affecting essential anatomical structures and photochemical reactions connected to the photosynthetic apparatus. 24-Epibrassinolide (EBR) is an environmentally friendly plant growth regulator in which this natural steroid can stimulate plant metabolism. The experiment was randomized with four treatments: two nickel concentrations (0 and 400 µM Ni, described as − Ni and + Ni, respectively) and two EBR concentrations (0 and 100 nM EBR, described as − EBR and + EBR, respectively). The objective of this research was to evaluate whether exogenously applied 24-epibrassinolide can mitigate oxidative damage against the photosynthetic apparatus in tomato leaves under excess Ni and to evaluate the leaf structures, stomatal variables, reactive oxygen species (ROS), antioxidant enzymes and nutritional status. The results proved that EBR alleviated Ni stress by protecting the photosynthetic machinery, upregulating the antioxidant system, improving leaf anatomy and favouring stomatal performance. This steroid relieves Ni-induced oxidative stress, stimulating superoxide dismutase (44%), ascorbate peroxidase (46%) and peroxidase (35%), which are enzymes involved in ROS detoxification. In addition, exogenous EBR alleviates oxidative damage against the photosynthetic apparatus, promoting increases in the effective quantum yield of PSII photochemistry (33%), photochemical quenching (20%) and electron transport rate (33%). In parallel, this steroid triggered improvements in leaf anatomy and stomatal performance that resulted in increases in net photosynthetic rate (52%) and water-use efficiency (29%). Simultaneously, the multiple functions of this steroid in the antioxidant system, photosynthetic machinery, gas exchange and anatomical characteristics worked towards the amelioration of nutritional status and to increase the biomass verified in our results. Therefore, this research demonstrated that EBR alleviated the negative interferences caused by Ni stress in tomato plants.

Keywords Brassinosteroids · Environmental contamination · Photosynthesis · *Solanum lycopersicum* · Stomatal density · Superoxide dismutase

Abbreviations

APX	Ascorbate peroxidase
Ca	Calcium
CAR	Carotenoids
CAT	Catalase
Cd	Cadmium
Chl *a*	Chlorophyll a
Chl *b*	Chlorophyll b
C_i	Intercellular CO_2 concentration
CO_2	Carbon dioxide
Cu	Copper
E	Transpiration rate

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EBR	24-Epibrassinolide
EDS	Equatorial diameter of the stomata
EL	Electrolyte leakage
ETAb	Epidermis thickness from abaxial leaf side
ETAd	Epidermis thickness from adaxial leaf side
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
HMs	Heavy metals
H_2O_2	Hydrogen peroxide
LDM	Leaf dry matter
MDA	Malondialdehyde
Mn	Manganese
Ni	Nickel
$NiCl_2$	Nickel chloride
NPQ	Nonphotochemical quenching
O_2^-	Superoxide anion
P	Phosphorus
PDS	Polar diameter of the stomata
P_N	Net photosynthetic rate
P_N/C_i	Instantaneous carboxylation efficiency
POX	Peroxidase
PPT	Palisade parenchyma thickness
PSII	Photosystem II
q_P	Photochemical quenching
RCT	Root cortex thickness
RET	Root epidermis thickness
RDM	Root dry matter
RDT	Root endodermis thickness
RMD	Root metaxylem diameter
ROS	Reactive oxygen species
S	Sulphur
SD	Stomatal density
SDM	Stem dry matter
SF	Stomatal functionality
SI	Stomatal Index
SOD	Superoxide dismutase
SPT	Spongy parenchyma thickness
TDM	Total dry matter
Total Chl	Total Chlorophyll
VCD	Vascular cylinder diameter
WUE	Water-use efficiency
Zn	Zinc
Φ_{PSII}	Effective quantum yield of PSII photochemistry

Introduction

Tomato (*Solanum lycopersicum* L.) is currently one of the most cultivated and consumed vegetables worldwide, as well as the best model organism for fleshy-fruited plants to be used in basic or applied research due to its sequenced and small genome (The Tomato Genome Consortium et al. 2012), short life cycle, photoperiod insensitivity, and specific morphological traits that are not shared with other model plants (Gerszberg et al. 2015). Furthermore, tomato can be cultivated under different conditions, propagated asexually by grafting, regenerated from distinct parts of the plant, and is able to grow in space limited to controlled conditions, such as a greenhouse (Flores et al. 2016).

Environmental stresses, especially soil contamination by toxic heavy metals (HMs), represent a serious global ecological problem. Soil contamination by Nickel (Ni) can be natural, such as weathering of ultrabasic igneous rocks (Yusuf et al. 2011) and anthropogenic activities, including fossil fuel burning, metallurgical, electrical and electronics industries, chemical and food industry, inadequate utilizations of agricultural sewage sludge, organic and mineral fertilizers (Nie et al. 2015; Khaliq et al. 2016), negatively impacting the crop yield. Ni excessive levels can cause phytotoxicity, affecting growth and development, restricting nutrient absorption, destroying the photosystem and membrane integrity (Yusuf et al. 2011), interfering with chloroplast structures (Hermle et al. 2007) and decreasing chlorophyll biosynthesis (Seregin and Kozhevnikova 2006). The toxic effect of Ni can result in plant nutrient deficiency by considerably inhibiting the absorption and translocation of some nutrients, such as Cu, Zn and Mn (Valivand et al. 2019; Jahan et al. 2020). In the same way, high Ni concentrations increase ROS overproduction, leading to oxidative stress (Turan et al. 2018), consequently decreasing the supply of electrons in the electron transport chain during photosynthesis, combined with damage to lipids, proteins and DNA (Küpper and Andresen 2016; Sirhindi et al. 2016).

In leaves, excess Ni causes chlorosis and necrosis, inhibiting several physiological processes, such as photosynthesis and transpiration (Kanwar et al. 2012; Küpper and Andresen 2016; Abd Allah et al. 2019). Ni toxicity can trigger damage to thylakoid structures, decreasing ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) activity, limiting chlorophyll biosynthesis and inducing stomatal closure, which are deleterious effects intrinsically related to the photosynthesis process (Molas 2002; Sreekanth et al. 2013; Hassan et al. 2019).

In parallel, excess Ni often promotes disorders of mesophilic cells and epidermal tissues, consequently reducing epidermal cell size and leaf area (Yusuf et al. 2011). These negative interferences lead to disturbance of the photosynthetic apparatus (Hassan et al. 2019). However, the damage intensities occasioned by Ni on photosynthetic capacity depend on the plant species and phenological stage, Ni concentration and exposure time of the plant to heavy metals (Drążkiewicz and Baszyński 2010; Chen et al. 2009).

Several studies have been carried out aiming to find strategies to stimulate plant resilience in situations of environmental stress, including the use of plant growth regulators that can be applied exogenously, such as brassinosteroids (BRs), considered a promising alternative. BRs are a group of polyhydroxy steroids found in plant tissues and were first isolated from *Brassica napus* pollen (Mitchell et al. 1970; Fridman and Savaldi-Goldstein 2013). These steroids are environmentally friendly, connected to stimulation of plant metabolism and involved in the mitigation of several environmental stresses (Hussain et al. 2019). Among the various BRs identified, 24-epibrassinolide (EBR) is one of the most biologically active (Azhar et al. 2017), maximizing biomass accumulation, gas exchange and defence mechanisms (Hussain et al. 2020). More specifically, BRs have achieved prominence for their potential to regulate, under stress conditions, several physiological processes, including chloroplast synthesis, stomatal movement, root morphology, photosynthesis and antioxidant enzymes (Wei and Li 2016; Sanjari et al. 2019; Ahammed et al. 2020), promoting tolerance to various abiotic factors, including heavy metals (Nawaz et al. 2017; Kour et al. 2021).

The hypothesis of this research takes into account the negative interference that excess Ni provokes in several metabolic processes, including ROS overproduction and inhibition of light capture and carbon dioxide fixation (Sreekanth et al. 2013; Jahan et al. 2020). Tomato plants were used because this species is considered an ideal dicotyledonous model to investigate physiological phenomena (Carvalho et al. 2011; Gerszberg et al. 2015). On the other hand, pretreatment with EBR reduced the photoinhibition in young *Eucalyptus urophylla* plants exposed to excess Ni (Ribeiro et al. 2020) and attenuated the damage generated by Mn stress in *Glycine max* plants, contributing to the maintenance of chloroplastic pigments (Rodrigues et al. 2020). Therefore, the aim of this research was to evaluate whether exogenously applied 24-epibrassinolide can mitigate oxidative damage against the photosynthetic apparatus in tomato leaves under excess Ni and to evaluate the leaf structures, stomatal variables, reactive oxygen species (ROS), antioxidant enzymes and nutritional status.

Materials and Methods

Location and Growth Conditions

The experiment was performed at the Universidade Federal Rural da Amazônia, Paragominas, Brazil (2°55′ S, 47°34′ W). The study was conducted in a greenhouse with temperature and humidity controlled. The minimum, maximum and median temperatures were 24.1, 30.5 and 25.8 °C, respectively. The relative humidity during the experimental period varied between 60 and 80%.

Plants, Containers and Acclimation

Seeds of *Solanum lycopersicum* L. cv Santa Clara were germinated using vegetable substrate and transplanted on the 13th day into 1.2-L pots filled with a mixed substrate of sand and vermiculite at a ratio of 3:1. Plants were cultivated under semi-hydroponic conditions containing 500 mL of nutritive solution. A modified Hoagland and Arnon (1950) solution was used as a source of nutrients; the ionic strength started at 50% and was modified to 100% after 2 days.

Experimental Design

The experiment was randomized with four treatments: two with nickel concentrations (0 and 400 µM Ni, described as − Ni and + Ni, 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 20 experimental units used in the experiment, with three plants in each experimental unit. EBR level was defined in agreement with Maia et al. (2018) and Ni concentrations were chosen based on research of Ribeiro et al. (2020) and Saraiva et al. (2021).

24-Epibrassinolide (EBR) Preparation and Application

Fifteen-day-old seedlings were sprayed with 24-epibrassinolide (EBR) or Milli-Q water (containing a proportion of ethanol that was equally used in preparation of the EBR solution) for 20 days (Days 15–35 after the start of the experiment), and this steroid was applied at intervals of five days. 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 Nutrition and Ni Excess

Plants received the following macro- and micronutrients contained in the nutrient solution in agreement with Pereira et al. (2019). To simulate excess Ni, $NiCl_2$ was used at concentrations of 0 and 400 µM Ni and was applied over 10 days (Days 25–35 after the start of the experiment). During the study, the pH of the nutrient solution was adjusted daily to 5.5. Thirty-five-day-old plants were used to measure the physiological and morphological parameters, and leaf, stem and root tissues were harvested for anatomical, biochemical and nutritional analyses.

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), with equipment calibration described by Lobato et al. (2021). 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 (twelve-g CO_2 cylinder), photosynthetically active radiation, air-flow rate and temperature conditions at 450 µmol mol^{-1} CO_2, 800 µmol photons m^{-2} s^{-1}, 300 µmol s^{-1} and 28 °C, respectively, between 10:00 and 12:00 h. The water-use efficiency (WUE) was estimated according to Ma et al. (2004) and the instantaneous carboxylation efficiency (P_N/C_i) was calculated using the formula described by Aragão et al. (2012).

Anatomical Measurements

Samples were collected from the middle region of the 2nd leaflet inserted in third node and roots 5 cm from the root apex. Subsequently, all collected botanical material was immersed in 70% (v/v) fixation solution (formaldehyde at 37%, acetic acid and ethanol at 70% in proportions of 0.5, 0.5 and 9.0, respectively) for 24 h, dehydrated in ethanol and embedded in historesin Leica™ (Leica, Nussloch, Germany). Transverse sections with a thickness of 5 µm were obtained with a rotating microtome (model Leica RM 2245, Leica Biosystems) and 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 (Model Motic 2500; Motic Group Co., LTD.). The images were analysed with a Moticplus 2.0 previously calibrated with a micrometer slide from the manufacturer. The anatomical parameters evaluated were as follows: the polar diameter of the stomata (PDS), the equatorial diameter of the stomata (EDS), the epidermis thickness from the adaxial leaf side (ETAd), the epidermis thickness from the abaxial leaf side (ETAb), the palisade parenchyma thickness (PPT), the spongy parenchyma thickness (SPT) and the PPT/SPT ratio. For both leaf faces, the stomatal density (SD) was calculated as the number of stomata per unit area, and the stomatal functionality (SF) was calculated as the PDS/EDS ratio, as described by Castro et al. (2009). The stomatal index (SI) was calculated as the percentage of stomata in relation to total epidermal cells by area. In the root samples, the root epidermis thickness (RET), root endodermis thickness (RDT), root cortex thickness (RCT), vascular cylinder diameter (VCD) and root metaxylem diameter (RMD) were measured.

Enzymatic Assays and Superoxide Anion

Extraction was performed using 500 mg plant material homogenized with 5 ml of extraction buffer [50 mM phosphate buffer (pH 7.6), 1.0 mM ascorbate and 1.0 mM EDTA] and subsequently centrifuged at 14,000×*g* for 4 min at 3 °C. Finally, the supernatant was collected (Badawi et al. 2004). In determinations, superoxide dismutase (SOD) activity was measured with 0.2 ml supernatant and 2.8 ml reaction mixture [50 mM phosphate buffer (pH 7.6), 0.1 mM EDTA, 13 mM methionine (pH 7.6), 75 µM NBT and 4 µM riboflavin], expressed in units of mg^{-1} protein (Giannopolitis and Ries 1977). Catalase (CAT) activity was evaluated using 0.2 ml of supernatant and 1.8 ml of reaction mixture [50 mM phosphate buffer (pH 7.0) and 12.5 mM hydrogen peroxide], presented in µmol H_2O_2 mg^{-1} protein min^{-1} (Havir and McHale 1987). Ascorbate peroxidase (APX) activity was determined with 0.2 ml of supernatant and 1.8 ml of reaction mixture [50 mM phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM EDTA and 1.0 mM hydrogen peroxide], expressed in µmol AsA mg^{-1} protein min^{-1} (Nakano and Asada 1981). Peroxidase (POX) activity was measured using 0.2 ml of supernatant and 1.78 ml of a reaction mixture [50 mM phosphate buffer (pH 7.0) and 0.05% guaiacol and 20 µl of 10 mM hydrogen peroxide] presented in µmol tetraguaiacol mg^{-1} protein min^{-1} (Cakmak and Marschner 1992). Superoxide anion (O_2^-) was determined using 1 ml of supernatant extracted above and incubated with a reaction mixture [30 mM phosphate buffer (pH 7.60), 0.51 mM hydroxylamine hydrochloride, 17 mM sulfanilamide, 7 mM α-naphthylamine and ethyl ether] (Elstner and Heupel 1976). Total soluble proteins were analysed using the methodology described by Bradford (1976).

Determining of Ni and Nutrients

Milled samples (100 mg) of root, stem and leaf tissues were predigested 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, S, Ca, Mn, Cu and Zn was carried out using an inductively coupled plasma mass spectrometer (model ICP-MS 7900; Agilent). All found values were in agreement with certified values (NIST 1570a and NIST 1577c).

Stress Indicators, Chloroplastic Pigments and Biomass

Stress indicators were extracted using 500 mg of fresh material and 5 ml of 5% (w/v) trichloroacetic acid and subsequently centrifuged at 15,000×*g* for 15 min at 3 °C to collect the supernatant (Wu et al. 2006). Hydrogen peroxide (H_2O_2) was measured with 0.2 ml of supernatant and 1.8 ml of reaction mixture [2.5 mM potassium phosphate buffer (pH 7.0) and 500 mM potassium iodide] (Velikova et al. 2000). Malondialdehyde (MDA) was determined using 0.5 ml of supernatant and 1 ml of the reaction mixture (0.5% (w/v) thiobarbituric acid in 20% trichloroacetic acid) based on the methodology of Cakmak and Horst (1991). Electrolyte leakage (EL) was performed according to the protocol described by Gong et al. 1998). Photosynthetic pigments chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), carotenoid (Car) and total chlorophyll (total Chl) were extracted with 40 mg of leaf tissue homogenized in 8 ml of 90% methanol (Lichtenthaler and Buschmann 2001). The biomass of roots, stems and leaves was measured based on constant dry weights (g) after drying in a forced-air ventilation oven at 65 °C.

Data Analysis

The normality of residues was verified with the Shapiro–Wilk test. Data were subjected to one-way ANOVA, 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. Statistical analysis of the data was performed using R™ software (Venables et al. 2021).

Results

EBR Minimized the Ni Contents in Plants Exposed to Excess Ni

Plants subjected to 400 µM Ni treatment presented increases in Ni content in their tissues (Table 1). However, the treatment with EBR (100 nM) promoted root, stem and leaf decreases of 8%, 14% and 23%, respectively, in comparison with the treatment with Ni without EBR.

Pretreatment with EBR Promoted Protection Against Excess Ni in Roots and Leaves

RET, RDT, RCT, VCD and RMD values decreased in plants exposed to excess Ni (Table 2; Fig. 1). However, plants treated with EBR and exposed to Ni stress had increases of 26%, 10% and 4% in RET, RDT and RMD, respectively, when compared to the same treatment without EBR. On leaf structures, excess Ni clearly caused deleterious effects (Table 2; Fig. 1). The application of EBR induced decreases in ETAd, ETAb and SPT of 6%, 18% and 8%, respectively, relative to the treatment with Ni and in the absence of EBR.

Steroids Favoured Nutritional Balance and Metal Homeostasis

The stress caused by Ni decreased the macronutrients and micronutrients, while the application of EBR increased the nutrient contents in the evaluated tissues (Table 3). For root tissue, the increases obtained by the treatment exposed to steroids + Ni were 34%, 41%, 16%, 15%, 24% and 12% for P, S, Ca, Mn, Cu and Zn, respectively. In leaves, the increases were 12%, 10%, 9%, 71%, 15% and 25%, respectively, and in stems, the increases were 10%, 91%, 16% and 10% for Ca, Mn, Cu and Zn, respectively, in comparison with plants treated only with Ni.

Stomatal Performance was Upregulated by EBR

The stress caused by Ni promoted reductions in stomatal characteristics (Table 4). On the adaxial face, the EBR

Table 1 Ni contents in tomato plants sprayed with EBR and exposed to Ni stress

Ni^{2+}	EBR	Ni in root (µg g DM^{-1})	Ni in stem (µg g DM^{-1})	Ni in leaf (µg g DM^{-1})
−	−	7.69 ± 0.33c	0.23 ± 0.02c	0.27 ± 0.02c
−	+	7.42 ± 0.29c	0.21 ± 0.01c	0.22 ± 0.02c
+	−	229.30 ± 8.18a	74.89 ± 1.72a	104.07 ± 4.91a
+	+	210.93 ± 6.77b	64.50 ± 1.81b	80.59 ± 2.76b

Ni^{2+} = 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 Structures of root and leaf in tomato plants sprayed with EBR and exposed to Ni stress

Ni^{2+}	EBR	RET (µm)	RDT (µm)	RCT (µm)	VCD (µm)	RMD (µm)
−	−	19.76 ± 1.06a	17.54 ± 0.68a	190 ± 3b	175 ± 10a	30.39 ± 1.22b
−	+	20.18 ± 1.31a	17.73 ± 0.72a	198 ± 3a	179 ± 10a	33.18 ± 1.36a
+	−	14.04 ± 1.11c	14.78 ± 0.60c	176 ± 3c	145 ± 11b	24.84 ± 1.12d
+	+	17.74 ± 0.93b	16.19 ± 0.65b	183 ± 3d	151 ± 11b	27.58 ± 1.28c
Ni^{2+}	EBR	ETAd (µm)	ETAb (µm)	PPT (µm)	SPT (µm)	Ratio PPT/SPT
−	−	21.42 ± 0.50a	15.29 ± 0.59a	65 ± 3a	68 ± 2a	0.95 ± 0.07a
−	+	21.71 ± 0.44a	15.41 ± 0.75a	66 ± 3a	69 ± 3a	0.96 ± 0.06a
+	−	19.32 ± 0.52c	11.65 ± 0.62c	60 ± 4a	59 ± 2c	1.02 ± 0.07a
+	+	20.42 ± 0.42b	13.73 ± 0.60b	63 ± 4a	64 ± 1b	0.99 ± 0.06a

RET root epidermis thickness, *RDT* root endodermis thickness, *RCT* root cortex thickness, *VCD* vascular cylinder diameter, *RMD* root metaxylem diameter, *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 described corresponding to means from five repetitions and standard deviations

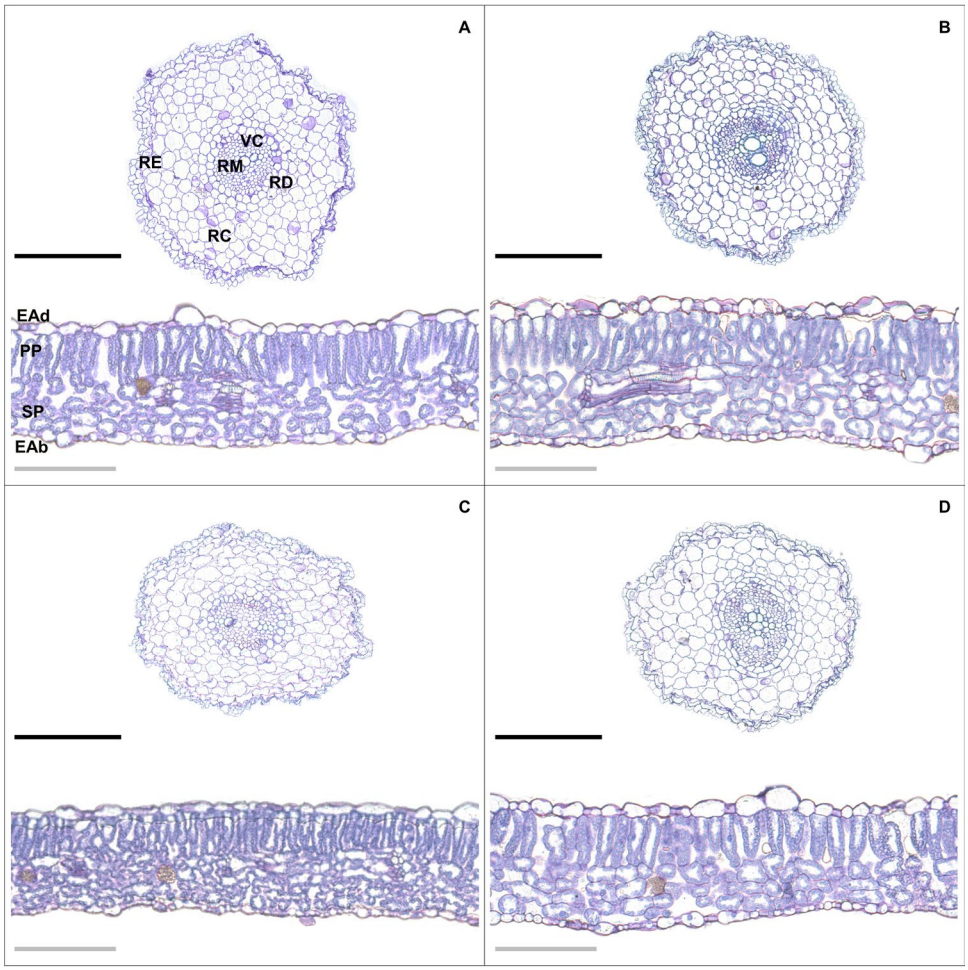

Fig. 1 Root and leaf cross sections in tomato plants sprayed with EBR and exposed to Ni stress. − Ni / − EBR (**A**), − Ni / + EBR (**B**), + Ni / − EBR (**C**) and + Ni / + EBR (**D**). *RE* root epidermis, *RC* root cortex, *RD* root endodermis, *VC* vascular cylinder, *RM* root metaxylem, *EAd* adaxial epidermis, *EAb* abaxial epidermis, *PP* palisade parenchyma, *SP* spongy parenchyma. Black bars = 300 µm and grey bars: 150 µm

treatment in plants under Ni stress increased the SD and SI by 18% and 10%, respectively. For abaxial faces, the increments were 19% and 99%, respectively. For EDS, reductions of 7% were detected on both sides of the leaf (adaxial and abaxial) compared to the equal treatment without EBR.

EBR Alleviated the Oxidative Impacts on the Photosynthetic Apparatus

Excess Ni caused reductions in photosynthetic pigments (Table 5). However, 100 nM EBR in plants stressed by Ni

Table 3 Nutrient contents in tomato plants sprayed with EBR and exposed to Ni stress

Ni^{2+}	EBR	P (mg g DM^{-1})	S (mg g DM^{-1})	Ca (mg g DM^{-1})	Mn (µg g DM^{-1})	Cu (µg g DM^{-1})	Zn (µg g DM^{-1})
Contents in root							
−	−	4.48 ± 0.17b	3.59 ± 0.11a	4.65 ± 0.16b	409.26 ± 29.13a	13.90 ± 0.55a	35.43 ± 1.02a
−	+	4.87 ± 0.21a	3.62 ± 0.13a	5.01 ± 0.18a	414.56 ± 24.68a	14.38 ± 0.69a	35.69 ± 1.15a
+	−	2.86 ± 0.16d	1.76 ± 0.10c	3.43 ± 0.20d	199.55 ± 10.53c	9.34 ± 0.70c	26.70 ± 0.95c
+	+	3.83 ± 0.24c	2.49 ± 0.11b	3.98 ± 0.17c	228.83 ± 10.52b	11.58 ± 0.91b	29.80 ± 1.10b
Contents in stem							
−	−	7.62 ± 0.21a	2.61 ± 0.15a	8.55 ± 0.24a	76.02 ± 3.67a	4.53 ± 0.15a	19.36 ± 0.96a
−	+	7.58 ± 0.31a	2.66 ± 0.21a	8.81 ± 0.29a	77.57 ± 2.53a	4.42 ± 0.22a	20.20 ± 0.99a
+	−	7.40 ± 0.15a	2.43 ± 0.14a	7.29 ± 0.27c	17.71 ± 1.55c	3.06 ± 0.14c	16.50 ± 0.62c
+	+	7.51 ± 0.30a	2.56 ± 0.15a	7.99 ± 0.26b	33.80 ± 2.54b	3.54 ± 0.15b	18.20 ± 0.85b
Contents in leaf							
−	−	7.72 ± 0.35a	5.15 ± 0.20a	19.20 ± 0.71a	152.28 ± 8.78a	6.90 ± 0.40a	20.33 ± 0.46a
−	+	7.89 ± 0.32a	5.39 ± 0.23a	20.04 ± 0.61a	153.14 ± 7.65a	7.06 ± 0.42a	20.46 ± 0.87a
+	−	6.20 ± 0.34c	4.15 ± 0.18c	16.31 ± 0.62c	38.05 ± 2.84c	5.40 ± 0.23c	13.35 ± 0.70c
+	+	6.94 ± 0.26b	4.58 ± 0.18b	17.78 ± 0.68b	65.08 ± 3.31b	6.21 ± 0.19b	16.71 ± 0.74b

P phosphorus, *S* Sulphur, *Ca* calcium, *Mn* manganese, *Cu* copper, *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 4 Stomatal variables in tomato plants sprayed with EBR and exposed to Ni stress

Ni^{2+}	EBR	SD (stomata per mm^2)	PDS (µm)	EDS (µm)	SF	SI
Adaxial face						
−	−	16.32 ± 0.94b	12.34 ± 0.33b	20.95 ± 0.85c	0.59 ± 0.04a	11.71 ± 0.80a
−	+	17.84 ± 1.15a	11.91 ± 0.60b	20.00 ± 1.04c	0.60 ± 0.03a	12.07 ± 0.79a
+	−	11.21 ± 0.93d	13.78 ± 0.56a	24.46 ± 0.79a	0.56 ± 0.04a	7.78 ± 0.61d
+	+	13.24 ± 1.03c	13.01 ± 0.30a	22.79 ± 0.83b	0.57 ± 0.05a	8.55 ± 0.60c
Abaxial face						
−	−	33.13 ± 1.76a	13.91 ± 0.91a	22.05 ± 0.78c	0.63 ± 0.01a	14.95 ± 0.62a
−	+	33.64 ± 1.33a	13.85 ± 0.62a	21.80 ± 0.85c	0.64 ± 0.02a	14.53 ± 0.55a
+	−	24.46 ± 1.06c	14.65 ± 1.06a	25.16 ± 0.81a	0.58 ± 0.03b	6.79 ± 0.50c
+	+	29.05 ± 1.71b	14.13 ± 1.02a	23.51 ± 0.61b	0.60 ± 0.01b	13.48 ± 0.49b

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 described corresponding to means from five repetitions and standard deviations

promoted increases of 19%, 23%, 19% and 55% in Chl *a*, Chl *b*, Chl Total and Car, respectively, and a 24% reduction in Chl Total/Car, when compared to the same treatment without EBR. In relation to chlorophyll fluorescence, plants under Ni stress presented an increase in F_0 and a reduction in F_v and F_v/F_m (Fig. 2). However, plants treated with Ni + 100 nM EBR suffered a decrease of 4% in F_0 and increases of 6% and 2% in F_v and F_v/F_m, respectively, in relation to the same treatment without EBR. Excess Ni caused reductions in Φ_{PSII}, q_P and ETR and increases in NPQ, EXC and ETR/P_N (Table 5). However, plants treated with Ni^{2+} + 100 nM EBR achieved increases of 33%, 20% and 33% in Φ_{PSII}, q_P and ETR, respectively, and decreases in NPQ, EXC and ETR/P_N of 33%, 6% and 12%, respectively, when comparing the same treatment in the absence of the steroid. For gas exchange, Ni^{2+} caused negative interferences (Table 5). However, plants subjected to Ni stress when sprayed with EBR obtained increases in P_N, E, g_s, WUE and P_N/Ci of 52%, 17%, 34%, 29% and 92%, respectively, and a loss of 22% in C_i compared to plants treated with Ni^{2+} + 0 nM EBR.

Benefits on Antioxidant Defence Induced by EBR

Plants exposed to Ni stress had increases in SOD, CAT, APX and POX activities (Fig. 3). However, treatment with 100 nM EBR combined with Ni^{2+} promoted increases of 44%, 27%, 46% and 35% in SOD, CAT, APX and POX,

Table 5 Photosynthetic pigments, chlorophyll fluorescence and gas exchange in tomato plants sprayed with EBR and exposed to Ni stress

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
−	−	7.18 ± 0.31a	1.35 ± 0.10a	8.53 ± 0.39a	0.75 ± 0.03b	5.32 ± 0.37a	11.39 ± 0.84b
−	+	7.40 ± 0.38a	1.40 ± 0.10a	8.80 ± 0.47a	0.83 ± 0.06a	5.30 ± 0.17a	10.66 ± 0.90b
+	−	5.54 ± 0.35c	0.88 ± 0.05c	6.42 ± 0.35c	0.40 ± 0.04d	6.30 ± 0.45b	16.18 ± 1.55a
+	+	6.58 ± 0.27b	1.08 ± 0.08b	7.65 ± 0.43b	0.62 ± 0.02c	6.12 ± 0.32b	12.32 ± 1.40b

Ni^{2+}	EBR	Φ_{PSII}	q_P	NPQ	ETR (μmol m^{-2} s^{-1})	EXC (μmol m^{-2} s^{-1})	ETR/P_N
−	−	0.216 ± 0.007b	0.299 ± 0.010b	0.64 ± 0.03c	31.77 ± 1.05b	0.733 ± 0.009c	1.77 ± 0.03b
−	+	0.232 ± 0.006a	0.316 ± 0.009a	0.62 ± 0.05c	34.08 ± 1.23a	0.716 ± 0.008d	1.76 ± 0.05b
+	−	0.129 ± 0.012d	0.212 ± 0.016d	1.20 ± 0.09a	18.98 ± 1.82d	0.833 ± 0.015a	2.10 ± 0.17a
+	+	0.172 ± 0.007c	0.254 ± 0.010c	0.80 ± 0.05b	25.25 ± 1.05c	0.783 ± 0.008b	1.84 ± 0.07a

Ni^{2+}	EBR	P_N (μmol m^{-2} s^{-1})	E (mmol m^{-2} s^{-1})	g_s (mol m^{-2} s^{-1})	C_i (μmol mol^{-1})	WUE (μmol $mmol^{-1}$)	P_N/C_i (μmol m^{-2} s^{-1} Pa^{-1})
−	−	17.96 ± 0.63b	2.54 ± 0.10a	0.230 ± 0.012a	178 ± 8c	7.09 ± 0.42a	0.101 ± 0.007b
−	+	19.36 ± 0.48a	2.60 ± 0.08a	0.242 ± 0.019a	161 ± 7d	7.45 ± 0.27a	0.120 ± 0.007a
+	−	9.05 ± 0.45d	1.95 ± 0.12c	0.152 ± 0.013c	254 ± 9a	4.67 ± 0.36c	0.036 ± 0.001d
+	+	13.75 ± 0.54c	2.29 ± 0.13b	0.204 ± 0.010b	199 ± 8b	6.01 ± 0.23b	0.069 ± 0.003c

Chl a chlorophyll *a*, *Chl b* chlorophyll *b*, *Total chl* total chlorophyll, *Car* carotenoids, 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. 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.

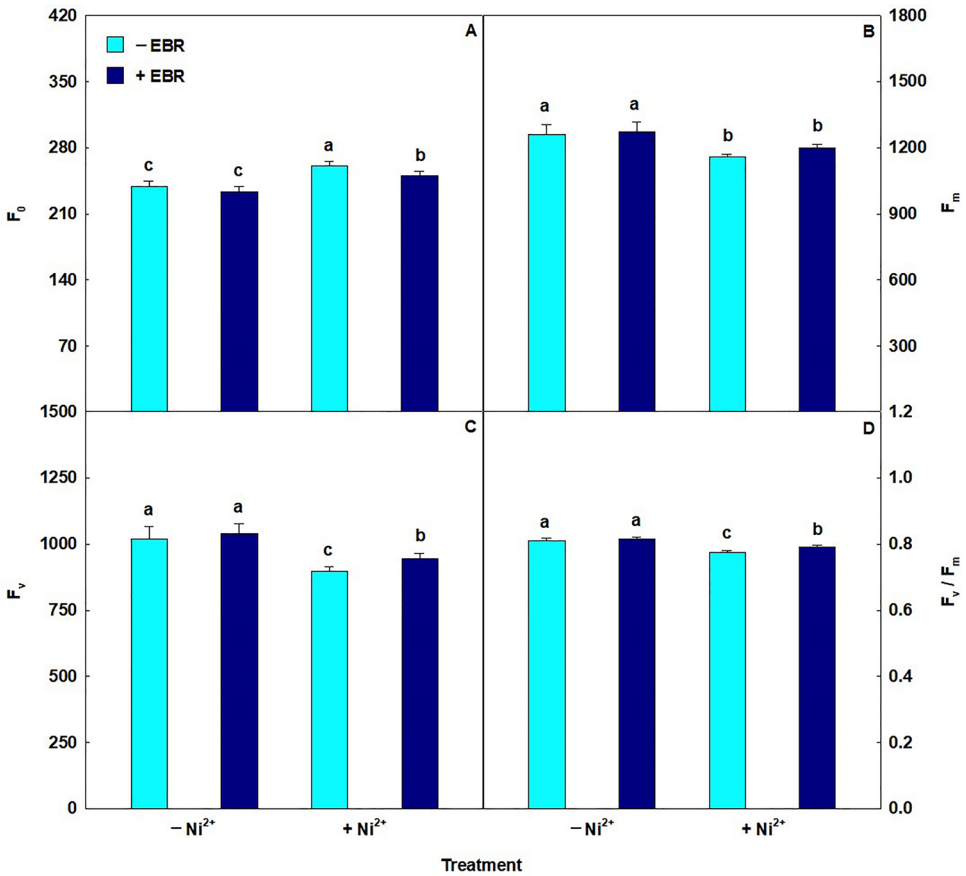

Fig. 2 Minimal fluorescence yield of the dark-adapted state (F_0), maximal fluorescence yield of the dark-adapted state (F_m), variable fluorescence (F_v) and maximal quantum yield of PSII photochemistry (F_v/F_m) in tomato plants sprayed with EBR and exposed to Ni stress. Bars with different letters indicate significant differences from the Scott-Knott test ($P < 0.05$). Bars corresponding to means from five repetitions and standard deviations

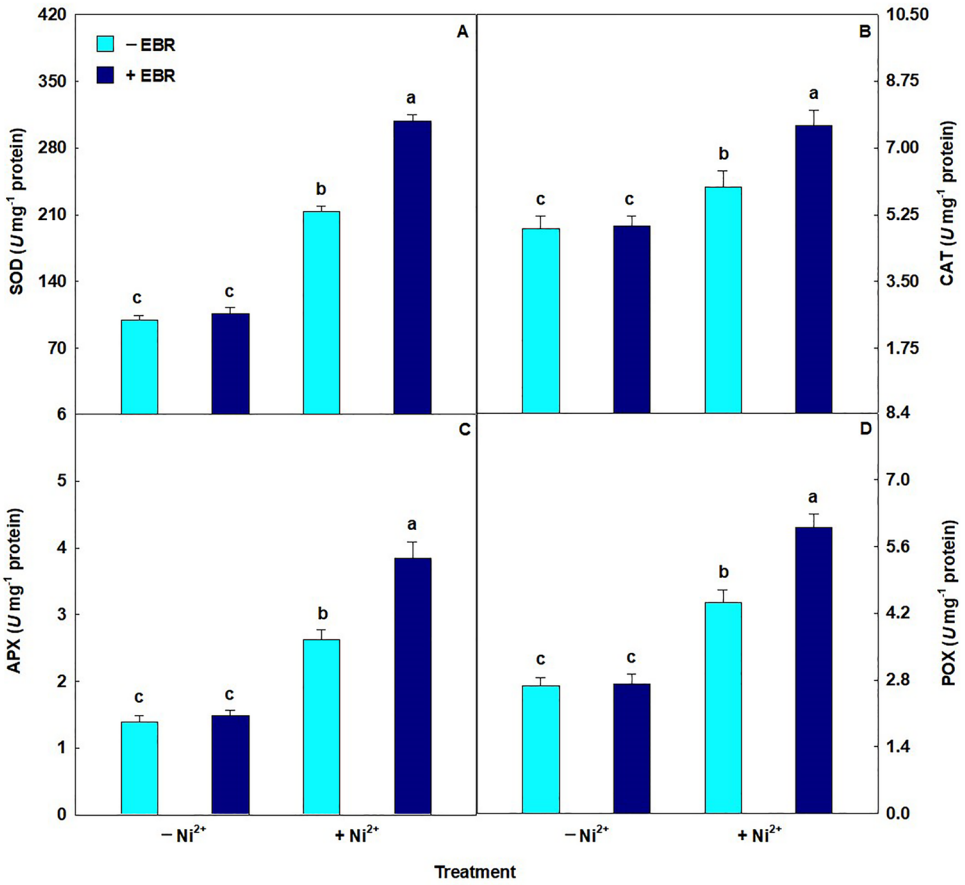

Fig. 3 Activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POX) in tomato plants sprayed with EBR and Ni stress. Columns with different letters indicate significant differences from the Scott-Knott test ($P < 0.05$). Columns corresponding to means from five repetitions and standard deviations

respectively, compared to equal treatment without steroids. Stress indicators caused by Ni stimulated ROS overaccumulation (Fig. 4). However, the application of 100 nM EBR in plants exposed to 400 µM Ni caused decreases in the compounds O_2^-, H_2O_2, MDA and EL of 22%, 19%, 41% and 21%, respectively, in relation to the Ni + 0 nM EBR treatment.

Steroids Reduced the Deleterious Effects Caused by Excess Ni on Biomass

Biomass was significantly reduced in plants exposed to treatment with 400 µM Ni (Fig. 5). However, the application of EBR in plants stressed by Ni promoted increases in LDM, SDM, RDM and TDM of 84%, 93%, 70% and 84%, respectively, when compared to plants exposed only to Ni stress.

Discussion

In this research, the exogenous application of 400 µM Ni caused significant increases in the content of this metal found in leaf, stem and root tissues in tomato plants. On the other hand, plants treated with 100 nM EBR presented reductions in Ni contents, confirming the action of this steroid attenuating the toxic effects caused by this heavy metal. In the literature, EBR exercises multiple roles involved in the regulation of heavy metal effects on plant metabolism, maximizing ROS elimination (Zhong et al. 2020), improving cell membrane permeability (Ramakrishna and Rao 2012) and stimulating the production of phytochelatins (Talarek-Karwel et al. 2019), thus promoting a reduction in the absorption and accumulation of Ni (Rajewska et al. 2016). Kumar et al. (2015) investigated the Ni effects in self-grafted or grafted onto *Solanum lycopersicum* plants and observed significant increases in the Ni contents in plant tissues. Similar to our study, Kanwar et al. (2012) evaluated *Brassica juncea* plants and observed a reduction in Ni uptake in root, stem and leaf tissues after foliar EBR application.

Excess Ni had negative repercussions on root structures, and exogenous EBR treatment alleviated these interferences, modulating increases in RET, RDT, RCT and RMD. These benefits observed in these variables may be related to the actions of this steroid by stimulating the increase in the thickness of root structures, more specifically through cell division and expansion processes in RET, RDT and RCT (Hacham et al. 2011), aiming to minimize metal translocation, acting as a barrier and improving membrane selectivity

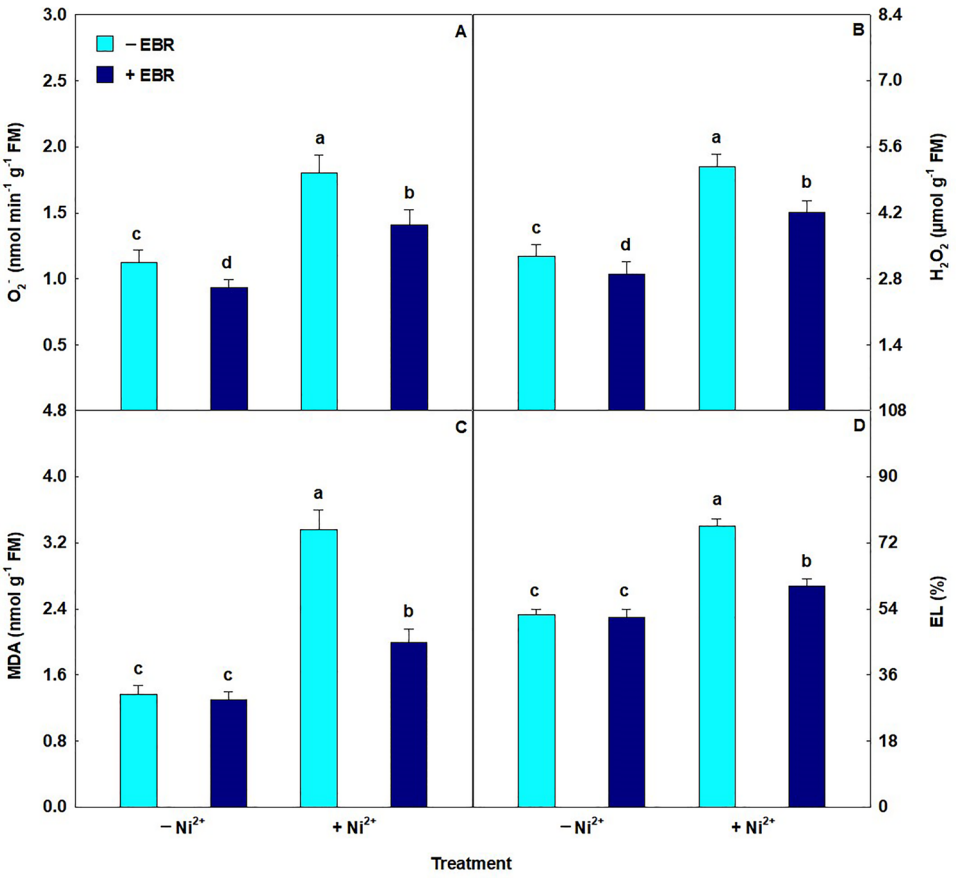

Fig. 4 Superoxide anion (O_2^{-}), hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and electrolyte leakage (EL) in tomato plants sprayed with EBR and Ni stress. Columns with different letters indicate significant differences from the Scott-Knott test ($P < 0.05$). Columns corresponding to means from five repetitions and standard deviations

(Gomes et al. 2011). Another converging point is that the increase observed in RMD after EBR application suggests improvements in nutrient absorption due to the greater permeability of the membranes, which is corroborated by the increase in the content of macronutrients (P, S and Ca) and of micronutrients (Mn, Zn and Cu) found in this study. Saraiva et al. (2021), evaluating *Glycine max* plants under high Ni concentrations, verified increases in root structures after treatment with 100 nM EBR. Jan et al. (2018) tested the individual and combined effects of EBR and Si on *Pisum sativum* seedlings under Cd stress and observed that treatment with EBR improved the nutritional status.

Exogenously applied EBR minimized the harmful effects of excess Ni on leaf anatomy. The epidermis is a tissue associated with water use, and it plays an essential role, protecting against excessive water loss during the transpiration process (Javelle et al. 2011). Therefore, increases in ETAd and ETAb in plants treated with 100 nM EBR can be correlated with increments obtained by this steroid on *E* and WUE. The intrinsic transport capacity of the mesophyll and CO_2 conductance from ambient air to carboxylation sites in chloroplasts modulate the photosynthetic process, in which SPT is connected to an intense formation of intercellular spaces directly involved with gas exchange. In other words, the increases promoted by EBR on leaf structures improved the photosynthetic performance, including P_N and P_N/C_i, which was also observed in other studies (Ennajeh et al. 2010; Sorin et al. 2015). A study conducted by Santos et al. (2020) measured the leaf modifications induced by EBR (100 nM) in *Glycine max* plants under three Zn levels (0.2, 20 and 2000 µM) and verified increases in ETAd, ETAb and SPT. Oliveira et al. (2019) obtained increments in ETAd, ETAb and SPT after pretreatment with EBR in young *Eucalyptus urophylla* plants stressed by Na^+.

Treatment with EBR alleviated the negative interference caused by Ni on stomatal characteristics. Increments in SD, EDS and SI detected in this study indicate that exogenous EBR pretreatment stimulated stomatal performance, which was confirmed by the increase obtained in g_s. This steroid plays an important role in the regulation of stomatal development, mediated by specific signaling proteins, such as *BRI1*, *BSU1* and *BIN2* (Kim et al. 2012), positively modulating gas exchange, more specifically increasing CO_2 inflow, corroborated by the maximizations of SPT, P_N and P_N/C_i verified in this research. SD, EDS and SI are variables intrinsically linked to the quantity, size and functionality of the stomata (Franks and Beerling 2009; Maia et al. 2018) and are interesting indicators to measure stomatal behaviour during stress conditions. Saraiva et al. (2021) evaluated the anatomical responses modulated by EBR (100 nM)

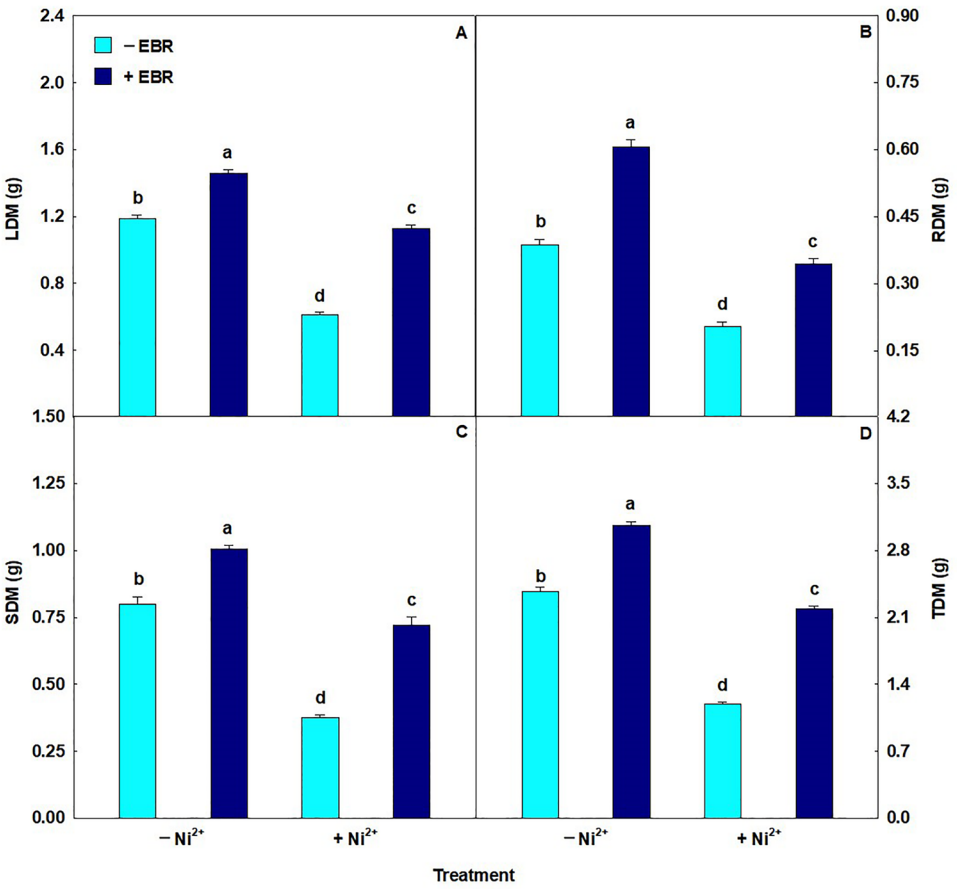

Fig. 5 Leaf dry matter (LDM), root dry matter (RDM), stem dry matter (SDM) and total dry matter (TDM) in tomato plants sprayed with EBR and Ni stress. Columns with different letters indicate significant differences from the Scott-Knott test ($P < 0.05$). Columns corresponding to means from five repetitions and standard deviations

in *Glycine max* plants subjected to Ni stress and detected increases in SD, EDS and SI. A study connected to EBR effects in *Oryza sativa* plants under Fe toxicity. Tadaiesky et al. (2020) reported benefits on stomatal characteristics, including SD.

Steroids in plants under Ni stress induced increases in SOD, CAT, APX and POX activities. These results are clearly related to the participation of this steroid during the regulation, biosynthesis and/or activation of these enzymes, mediated by the processes of expression, transcription and translation of specific genes, such as *RBOH* (respiratory burst oxidase homologue), *MAPK1* (mitogen-activated protein kinase 1) and *MAPK3* (mitogen-activated protein kinase 3), stimulating the antioxidant defence system to eliminate ROS (Xia et al. 2009; Sharma et al. 2016). Yusuf et al. (2014) investigated the interaction of different Ni concentrations and two 28-homobrasinolide levels in *Vigna radiata* plants and found that BR application promoted increases in CAT, POX and SOD. Similar to our results, Dalyan et al. (2018), working with *Brassica juncea* seedlings treated with EBR and under Pb stress, found increases in antioxidant enzymatic activities.

Plants exposed to excess Ni induced an imbalance associated with ROS, strongly confirmed by higher MDA and EL contents, reducing cell membrane integrity and resulting in severe oxidative damage (Jahan et al. 2020). In our study, plants treated with Ni showed increases in the levels of these stress indicators, but EBR application attenuated these deleterious effects, reducing O_2^-, H_2O_2, MDA and EL. EBR restricts the excessive formation of ROS, positively modulates the antioxidant system and stimulates increases in the activities of SOD, CAT, APX and POX, as verified in this research. Mir et al. (2018) observed increases in the contents of O_2^-, H_2O_2, MDA and EL in *Glycine max* plants exposed to Ni stress. Ramakrishna and Rao (2014) studied the toxic effects of Zn on *Raphanus sativus* plants and identified that EBR alleviated oxidative stress by reducing the levels of H_2O_2, MDA and EL.

Ni-induced stress considerably decreased the content of chloroplastic pigments. Ni stress negatively impacts the size and number of chloroplasts, as well as their ultrastructural disorganization, including a decrease in the number of grains and thylakoids and their deformation, together with changes in the lipid composition of the membrane (Sreekanth et al. 2013). On the other hand, pretreatment using EBR promoted increases in Chl *a*, Chl *b*, total Chl and Car, which can be explained by the fact that this steroid acts in the maintenance of chloroplast integrity through the reduction of membrane

damage, corroborated by the decrease in MDA and EL in this study. In a study evaluating the attenuation of chromium stress using polyamine and EBR, Choudhary et al. (2012) identified an increase in pigment contents in *Raphanus sativus* plants treated with 10^{-9} M EBR. Shah et al. (2019) detected increases in the levels of Chl *a*, Chl *b*, Car and total Chl after the application of EBR, mitigating the injuries caused by Cd in *Cucumis sativus* plants.

EBR spray mitigated the interference caused by excess Ni on F_0, F_v and F_v/F_m. The increases obtained in F_v and F_v/F_m and reduction in F_0 signalled the benefits on efficiency of conversion and capture of light energy in the PSII reaction centre. In other words, this steroid can reduce photoinhibition by increasing the proportion of oxidized plastoquinone Q_A, boosting photon capture in the reaction centre and increasing the F_v/F_m ratio, an efficient indicator connected to photosynthetic machinery (Hertle et al. 2013; Li et al. 2015a). Drążkiewicz and Baszyński (2010) evaluated *Zea mays* seedlings under Ni stress and verified reductions in F_v and F_v/F_m. On the other hand, a study with *Oryza sativa* plants subjected to simulated acid rain stress that was pretreated with 100 nM EBR. Fonseca et al. (2020) obtained positive results in F_0, F_v and F_v/F_m. Additionally, corroborating our study, Santos et al. (2018) analysed EBR spray in *Vigna unguiculata* plants under Cd stress and found an increase in F_v/F_m and a reduction in F_0.

EBR alleviated the deleterious effects provoked by 400 µM Ni on chlorophyll fluorescence. Excess Ni inhibited/reduced the efficiency of the electron flow from pheophytin via plastoquinone Q_A and Fe to plastoquinone Q_B, changing the structure of carriers, such as Q_B, or reaction centre proteins present in the thylakoids, decreasing the content of cytochromes b_6f and b_{559}, as well as ferredoxin and plastocyanin (Seregin and Kozhevnikova 2006). However, the application of 100 nM EBR promoted increments in Φ_{PSII}, q_p and ETR due to this steroid contributing during the opening process from the PSII reaction centre, improving the efficiency in the capture of light energy and consequently increasing the photosynthetic efficiency of electron transport (Li et al. 2015b). In addition, EBR induced a reduction in NPQ, EXC and ETR/P_N values, confirming the maintenance of excitation energy transfer by the antenna system towards the reaction centres, reducing heat dissipation and protecting PSII from damage caused by excess energy (Ogweno et al. 2008; Thussagunpanit et al. 2015). Reductions in Φ_{PSII} and q_p values in *Amaranthus paniculatus* plants exposed to different Ni concentrations were identified by Pietrini et al. (2015). A study by Siddiqui et al. (2018) comparing the application of two BR isomers (28-homobrassinolide and 24-epibrassinolide) in *Brassica juncea* plants obtained increases in Φ_{PSII}, q_p and ETR and a reduction in NPQ, confirming improvements in chlorophyll fluorescence caused by the action of this steroid.

Plants treated with 100 nM EBR and submitted to 400 µM Ni presented interesting benefits in gas exchange. Ni stress impairs numerous metabolic processes, inhibiting photosynthesis through damage to the electron transport chain, reducing enzyme activities and synthesizing chlorophyll contents, which are associated with decreased g_s, E and WUE (Yusuf et al. 2011). Increases in P_N, E, WUE and P_N/C_i achieved with EBR spray are explained by the beneficial repercussions that this steroid provided on photosynthetic apparatus, through increases in Φ_{PSII}, q_p and ETR. These increases in gas exchange can also reveal an improvement in stomatal performance, enhancing CO_2 fixation, detected by the increase in g_s and reduction in C_i. Research conducted by Reis et al. (2017) investigated the behaviour of *Glycine max* plants exposed to progressive Ni concentrations (0, 0.05, 0.10, 0.50, 10 and 20 µM) and found that P_N, g_s and E decreased but C_i increased in plants under high Ni (20 µM). Similar results were described by Cunha et al. (2020), who evaluated the EBR roles in *Eucalyptus urophylla* plants during Cd stress and described increments in P_N, E, g_s, WUE and P_N/C_i and a reduction in C_i.

Ni stress reduced plant growth, consequently resulting in significant reductions in the biomass of roots, stems and leaves. The decrease in biomass is caused by Ni stress due to its negative interference with nutrient absorption, plant metabolism, water relations, gas exchange and cell permeability (Hassan et al. 2019). On the other hand, plants treated with EBR obtained considerable increases in LDM, SDM, RDM and TDM, and these results were intrinsically connected to the multiple roles of this steroid in the antioxidant system, chloroplastic pigments, chlorophyll fluorescence, gas exchange, nutritional status and anatomical characteristics described in this study. *Solanum lycopersicum* plants treated with 10^{-8} M EBR had increases in RDM and shoot dry matter in a study conducted by Nazir et al. (2019) evaluating the combined effects of EBR and H_2O_2 aiming to mitigate Cu stress. Corroborating our research, Ribeiro et al. (2020) revealed increases in LDM, SDM, RDM and TDM after the application of 100 nM in young *Eucalyptus urophylla* plants subjected to Ni toxicity.

Conclusion

Our results clearly suggest that EBR acted in defence against oxidative damage caused by excess Ni by protecting the photosynthetic machinery, upregulating the antioxidant system and improving leaf anatomy. This steroid relieves Ni-induced oxidative stress, stimulating enzymes connected to redox metabolism, such as SOD, APX and POX, which are involved in the detoxification of reactive oxygen species, including superoxide anion and hydrogen peroxide. Exogenous EBR application alleviated photoinhibition and

contributed to the maintenance of photosynthetic efficiency, as demonstrated in this study through increases in Φ_{PSII}, q_p, ETR and photosynthetic pigments. Additionally, EBR promoted improvements in leaf structures and stomatal performance, as confirmed by the increments in gas exchange detected in this study. Simultaneously, the multiple functions of this steroid in the antioxidant system, photosynthetic machinery, gas exchange and anatomical characteristics worked towards the amelioration of nutritional status and to increase the biomass verified in our results. Therefore, this research demonstrated that EBR alleviated the negative interferences caused by Ni stress in tomato plants, but it is highly recommended in the future studies, molecular approaches aiming to determine the gene expression modulated by EBR in plants under Ni toxicity.

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Author Contributions AKSL was the advisor of this project, planned all phases of the research and critically revised the manuscript. CFM, YCP and BRSS conducted the experiment and performed physiological, biochemical, anatomical and morphological determinations; they wrote and edited the manuscript. BLB involved in nutritional analysis. All authors read and approved the final version of the manuscript.

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

Declarations

Conflict of interest The authors declare that they have no competing interests.

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24-Epibrassinolide Simultaneously Stimulates Photosynthetic Machinery and Biomass Accumulation in Tomato Plants under Lead Stress: Essential Contributions Connected to the Antioxidant System and Anatomical Structures

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Abstract: Several toxic pollutants are released into the atmosphere through human activities. Among these pollutants, lead (Pb) is a non-biodegradable element that can cause reduced cell division, impact negatively on the biosynthesis of photosynthetic pigments, and lower biomass accumulation, which can lead to plant death. 24-epibrassinolide (EBR) is a plant growth regulator with broad benefits on physiological functions and biochemical responses, conferring tolerance to plants against several biotic and abiotic stresses. The experiment was randomized with four treatments, two lead concentrations (0 and 200 μM Pb, described as −Pb and +Pb, respectively) and two EBR (0 and 100 nM EBR, described as −EBR and +EBR, respectively). We detected a negative impact of Pb stress in tomato plants; however, the exogenous application of EBR induced protection on leaf anatomy and photosynthetic apparatus, mitigating the Pb impacts on growth. This steroid enhances the root and leaf structures (in root tissue, the epidermis thickness; and in the leaf, palisade parenchyma, and spongy parenchyma), improving the membrane selectivity, light energy absorption, and CO_2 fixation. Applying 200 μM Pb and 100 nM EBR caused an increase in superoxide dismutase, catalase, ascorbate peroxidase, and peroxidase activity (by 26%, 18%, 25%, and 20%, respectively). Moreover, the improvements obtained on photosynthetic pigments, electron transport rate, the effective quantum yield of photosystem II photochemistry, and net photosynthetic rate prove the benefits and protection of photosynthetic apparatus, resulting in increased biomass accumulation, with increases of 95%, 115%, 74%, and 92% in leaf, root, stem, and the whole plant, respectively. Taken together, our findings confirm that EBR alleviates the damages provoked by Pb stress in tomatoes.

Keywords: brassinosteroids; growth; heavy metal; photosynthesis; *Solanum lycopersicum*

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1. Introduction

Due to fast and uncontrolled industrialization, urbanization, and intensive agriculture, the environment has been under remarkable pressure, with various toxic pollutants released into the atmosphere through human activities [1]. Heavy metals (HMs) are non-biodegradable inorganic chemical constituents and are a major threat to the environment due to their potentially cytotoxic, genotoxic, and mutagenic characteristics. Some of these HMs are not essential since they do not perform any known physiological function in plants, such as arsenic (As), cadmium (Cd), lead (Pb), chromium

(Cr), and aluminum (Al) [2,3]. One of the most toxic HMs, Pb, can cause common toxic effects on plants, such as inhibition of growth, altered nutrient assimilation, low biomass accumulation, and senescence, which ultimately cause plant death [4]. Photosynthetic pathways are also affected by Pb toxicity due to disrupting chloroplast ultrastructure and obstruction of essential pigments synthesis, including chlorophyll and carotenoids, in addition to blocking the Calvin cycle and electron transport chain, which produces a shortage of carbon dioxide [5,6].

Plants under HMs toxicity usually present redox imbalance of the cell, inducing oxidative stress due to overproduction of reactive oxygen species (ROS), more specifically superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-), being highly reactive, toxic, and harmful to plant metabolism [7,8]. Interestingly, Rodrigues et al. [9] described that the activities of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (POX) were stimulated after application of brassinosteroids (BRs), indicating an improvement in O_2^- detoxification. The SOD enzyme is part of the first line of defense of the antioxidant system in plants and is involved in the dismutation of O_2^- into H_2O_2, which will later be decomposed by the CAT, APX, and POX enzymes [10,11].

The leaf anatomy is linked to leaf mass per unit area and correlates well with plant growth rates due to its influence on photosynthesis and hence plant growth; however, HMs, including Pb, can cause damaged and altered ultrastructure of this foliar structure [12,13]. The epidermis is the protective outer layer of cells that acts as a barrier against biotic or abiotic agents and as an active interface that controls the vital exchange of gas, water, and nutrients with the environment [14,15]. Palisade and spongy parenchyma are tissues related to the photosynthetic process and formation of intercellular spaces involved with gas exchange, contributing to the influx and consequent fixation of CO_2 [16].

The chlorophyll content is an important indicator of photosynthetic potential, and carotenoids are the endogenous antioxidant pigments that prevent the peroxidation of lipid membranes by the extinction of ROS [17]. Pb stress causes chloroplast disorganization and damage to its membrane, besides induced increases in chlorophyllase activity, the enzyme responsible for chlorophyll degradation [18,19]. In parallel to this, the stress induced by Pb also causes an imbalance in plastoquinone and electron transport, reducing photosynthesis, which consequently leads to a reduction in biomass [20].

BRs are polyhydroxy steroid phytohormones that perform various physiological functions, such as growth, and confer resistance to plants against various biotic and abiotic stresses [21,22]. Among all isolated and characterized, brassinolide (BL), 24-epibrassinolide (EBR), and 28-homobrassinolide (HBL) are the main bioactive BRs, and several studies are addressing the adaptive responses of plants caused by these steroids to environmental stresses [23], such as heavy metal toxicity [24], salinity [25], temperature extremes [26,27], and drought [28,29].

To overcome the harmful effects of these stresses, especially heavy metals, BRs can perform several roles, such as reducing these toxic elements' absorption by altering cell membrane permeability and inducing a group of defensive enzymes [30]. However, some processes performed by this steroid on heavy metal stress still need further research; because of this, we hypothesized that EBR mitigates the toxic effects of heavy metals, especially Pb. Therefore, this study aimed to verify whether the exogenous application of EBR can preserve tomato plants from oxidative damages caused by excess Pb, evaluating the responses associated with leaf anatomy, antioxidant metabolism, photosynthetic apparatus, and biomass.

2. Materials and Methods

2.1. Location and Growth Conditions

The experiment was performed at the Universidade Federal Rural da Amazônia, Paragominas, Brazil (2°55′ S, 47°34′ W). The study was conducted in a greenhouse illuminated with sunlight, but with temperature and humidity controlled. The minimum, maximum, and median temperatures were 23.3, 29.2, and 25.5 °C, respectively. The relative humidity during the experimental period varied between 60% and 80% and photoperiod of 12/12.

2.2. Plants, Containers and Acclimation

Seeds of *Solanum lycopersicum* L. cv Santa Clara were germinated using vegetable substrate and transplanted on the 14th day into 1.2-L pots filled with a mixed substrate of sand and vermiculite at a ratio of 3:1. Plants were cultivated under semi-hydroponic conditions containing 500 mL of nutritive solution. A modified Hoagland and Arnon [31] solution was used as a source of nutrients; the ionic strength started at 50% and was modified to 100% after 2 days.

2.3. Experimental Design

The experiment was randomized with four treatments, two lead concentrations (0 and 200 μM Pb, described as −Pb and +Pb, respectively) and two EBR (0 and 100 nM EBR, described as −EBR and +EBR, respectively). Five replicates for each one of the four treatments were conducted and used in the experiment in a total of 20 experimental units, with three plants in each unit. Pb and EBR concentrations were chosen according to studies by Guedes et al. [32] and Maia et al. [33], respectively.

2.4. 24-Epibrassinolide (EBR) Preparation and Application

Fifteen-day-old seedlings were sprayed with 24-epibrassinolide (EBR) or Milli-Q water (containing a proportion of ethanol that was equally used in the preparation of the EBR solution) for 20 days (days 10−30 after the start of the experiment), being applied this steroid with intervals of 5 days. 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] [34].

2.5. Plant Nutrition and Pb Treatment

The 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 $MgSO_4{\cdot}7H_2O$, 62.50 μM KCl, 31.25 μM H_3BO_3, 2.50 μM $MnSO_4{\cdot}H_2O$, 2.50 μM $ZnSO_4{\cdot}7H_2O$, 0.63 μM $CuSO_4{\cdot}5H_2O$, 0.63 μM $NaMoO_4{\cdot}5H_2O$, and 250.0 μM $NaEDTAFe{\cdot}3H_2O$. To induce Pb stress, $PbCl_2$ was used at concentrations of 0 and 200 μM Pb and was applied over 10 days (days 20−30 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 tissues were harvested for anatomical, biochemical, and nutritional analyses.

2.6. Determination of Pb 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 was added and transferred to a Teflon digestion vessel in agreement with Paniz et al. [35]. Determination of Pb, Mg, K, Ca, Cu, Zn, and Mn was performed using an inductively coupled plasma mass spectrometer (model ICP-MS 7900; Agilent).

2.7. Measurements of Root and Leaf Anatomical Variables

Samples were collected from the middle region of the leaf limb of fully expanded leaves and roots 5 cm from the root apex. Subsequently, all collected botanical material was fixed in FAA 70 for 24 h, dehydrated in ethanol, and embedded in historesin Leica™ (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 [36]. For stomatal characterization, the epidermal impression method was used according to Segatto et al. [37]. 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 Motic plus 2.0, previously calibrated with a micrometer slide from the manufacturer. The leaf anatomical variables evaluated were the polar diameter of the stomata (PDS), equatorial diameter of the stomata (EDS), epidermis thickness from adaxial leaf side (ETAd), epidermis thickness from abaxial leaf side (ETAb), mesophyll thickness (MT), leaf aerenchyma area (LAA), bulliform cell diameter (BCD), trichome density (TD), and trichome size (TS). In both leaf faces, the stomatal density (SD) was calculated as the number of stomata per unit area and the stomatal functionality (SF) as the ratio PDS/EDS according to Castro et al. [38]. In root samples, the root epidermis thickness (RET), root exodermis thickness (RXT), root endodermis thickness (RDT), root cortex thickness (RCT), root aerenchyma area (RAA), vascular cylinder diameter (VCD), and root metaxylem diameter (RMD) were measured.

2.8. Determination of Photosynthetic Pigments

The chlorophyll and carotenoid levels were determined using 40 mg of leaf tissue. The samples were homogenized in the dark with 8 mL of 90% methanol (Sigma-Aldrich™). The homogenate was centrifuged at 6000× *g* for 10 min at 5 °C. The supernatant was removed, and the chlorophyll *a* (Chl *a*) and *b* (Chl *b*), carotenoid (Car), and total chlorophyll (total Chl) levels were quantified using a spectrophotometer (model UV-M51; Bel Photonics) according to the methodology of Lichtenthaler and Buschmann [39].

2.9. Measurement of Chlorophyll Fluorescence

The minimal fluorescence yield of the dark-adapted state (F_0), maximal fluorescence yield of the dark-adapted state (F_m), variable fluorescence (F_v), maximal quantum yield of PSII photochemistry (F_v/F_m), effective quantum yield of PSII photochemistry (Φ_{PSII}), photochemical quenching coefficient (q_P), nonphotochemical quenching (NPQ), electron transport rate (ETR), relative energy excess at the PSII level (EXC), and the ratio between the electron transport rate and net photosynthetic rate (ETR/P_N) were determined using a modulated chlorophyll fluorometer (model OS5p; Opti-Sciences). The chlorophyll fluorescence was measured in fully expanded leaves under light. Preliminary tests determined that the acropetal third of leaves in the middle third of the plant and that adapted to the dark for 30 min yielded the greatest F_v/F_m ratio. Therefore, this part of the plant was used for measurements. The intensity and duration of the saturation light pulse were 7500 μmol $m^{-2}.s^{-1}$ and 0.7 s, respectively.

2.10. Evaluation of Gas Exchange

The net photosynthetic rate (P_N), transpiration rate (E), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) were evaluated using an infrared gas analyzer (model LCPro+; ADC BioScientific). These parameters were measured at the adaxial surface of fully expanded leaves that were collected from the middle region of the plant. The water-use efficiency (WUE) was estimated according to Ma et al. [40], and the instantaneous carboxylation efficiency (P_N/C_i) was calculated using the formula described by Aragão et al. [41]. Gas exchange was evaluated in all plants under a constant CO_2 concentration (390 μmol mol^{-1} CO_2), photosynthetically active radiation (800 μmol photons m^{-2} s^{-1}), air-

flow rate (300 μmol s^{-1}), and temperature (28 °C) in the test chamber between 10:00 and 12:00 h.

2.11. Determination of Antioxidant Enzymes, Superoxide and Soluble Proteins Level

Antioxidant enzymes (SOD, CAT, APX, and POX), superoxide, and soluble proteins were extracted from leaf tissues [42]. The extraction mixture was prepared by homogenizing 500 mg of fresh plant material in 5 mL of extraction buffer, which consisted of 50 mM phosphate buffer (pH 7.6), 1.0 mM ascorbate, and 1.0 mM EDTA. Samples were centrifuged at 14,000× *g* for 4 min at 3 °C, and the supernatant was collected. Quantifying the total soluble proteins was performed using the method described by Bradford [43]. Absorbance was measured at 595 nm, using bovine albumin as a standard.

2.12. Superoxide Dismutase Assay

For the SOD assay (EC 1.15.1.1), 2.8 mL of a reaction mixture containing 50 mM phosphate buffer (pH 7.6), 0.1 mM EDTA, 13 mM methionine (pH 7.6), 75 μM NBT, and 4 μM riboflavin was mixed with 0.2 mL of supernatant. The absorbance was then measured at 560 nm [44]. One SOD unit was defined as the amount of enzyme required to inhibit 50% of the NBT photoreduction. The SOD activity was expressed in unit mg^{-1} protein.

2.13. Catalase Assay

For the CAT assay (EC 1.11.1.6), 0.2 mL of supernatant and 1.8 mL of a reaction mixture containing 50 mM phosphate buffer (pH 7.0) and 12.5 mM hydrogen peroxide were mixed, and the absorbance was measured at 240 nm [45]. The CAT activity was expressed in μmol H_2O_2 mg^{-1} protein min^{-1}.

2.14. Ascorbate Peroxidase Assay

For the APX assay (EC 1.11.1.11), 1.8 mL of a reaction mixture containing 50 mM phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM EDTA, and 1.0 mM hydrogen peroxide was mixed with 0.2 mL of supernatant, and the absorbance was measured at 290 nm [46]. The APX activity was expressed in μmol AsA mg^{-1} protein min^{-1}.

2.15. Peroxidase Assay

For the POX assay (EC 1.11.1.7), 1.78 mL of a reaction mixture containing 50 mM phosphate buffer (pH 7.0) and 0.05% guaiacol was mixed with 0.2 mL of supernatant, followed by the addition of 20 μL of 10 mM hydrogen peroxide. The absorbance was then measured at 470 nm [47]. The POX activity was expressed in μmol tetraguaiacol mg^{-1} protein min^{-1}.

2.16. Determination of Superoxide Concentration

To determine O_2^-, 1 mL of extract was incubated with 30 mM phosphate buffer [pH 7.6] and 0.51 mM hydroxylamine hydrochloride for 20 min at 25 °C. Sulphanilamide (17 mM) and 7 mM α-naphthylamine were added to the incubation mixture for 20 min at 25 °C. After the reaction, ethyl ether was added in an identical volume and centrifuged at 3000× *g* for 5 min. The absorbance was measured at 530 nm [48].

2.17. Extraction of Nonenzymatic Compounds

Nonenzymatic compounds (H_2O_2 and MDA) were extracted as described by Wu et al. [49]. Briefly, a mixture was prepared to extract H_2O_2 and MDA by homogenizing 500 mg of fresh leaf material in 5 mL of 5% (*w*/*v*) trichloroacetic acid. Samples were centrifuged at 15,000× *g* for 15 min at 3 °C to collect the supernatant.

2.18. Determination of Hydrogen Peroxide Concentration

To measure H_2O_2, 200 µL of supernatant and 1800 µL of the reaction mixture (2.5 mM potassium phosphate buffer [pH 7.0] and 500 mM potassium iodide) were mixed, and the absorbance was measured at 390 nm [50].

2.19. Quantification of Malondialdehyde Concentration

MDA was determined by mixing 500 µL of supernatant with 1 mL of the reaction mixture, which contained 0.5% (*w*/*v*) thiobarbituric acid in 20% trichloroacetic acid. The mixture was incubated in boiling water at 95 °C for 20 min, with the reaction terminated by placing the reaction container in an ice bath. The samples were centrifuged at 10,000× *g* for 10 min, and the absorbance was measured at 532 nm. The nonspecific absorption at 600 nm was subtracted from the absorbance data. The amount of MDA−TBA complex (red pigment) was calculated [51], with minor modifications and using an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.20. Determination of Electrolyte Leakage

Electrolyte leakage was measured according to the method of Gong et al. [52] with minor modifications. Fresh tissue (200 mg) was cut into pieces 1 cm in length and placed in containers with 8 mL of distilled deionized water. The containers were incubated in a water bath at 40 °C for 30 min, and the initial electrical conductivity of the medium (EC_1) was measured. The samples were then boiled at 95 °C for 20 min to release the electrolytes. After cooling, the final electrical conductivity (EC_2) was measured. The percentage of electrolyte leakage was calculated using the formula EL (%) = (EC_1/EC_2) × 100.

2.21. Measurements of Biomass

The biomass of roots and leaves was measured based on constant dry weights (g) after drying in a forced-air ventilation oven at 65 °C.

2.22. 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% [53]. Standard deviations were calculated for each treatment. Statistical analysis of the data was done using R® software [54].

3. Results

3.1. Pb Content Was Minimized by EBR in Plants Exposed to Toxicity

The treatment with 200 µM of Pb caused a significant increase in the content of this metal on the analyzed tissues (Table 1). However, plants treated with Pb and EBR showed reductions of 55%, 65%, and 45% in the content of Pb in the tissues of the root, stem, and leaf, respectively, concerning the same treatment without the steroid.

Table 1. Lead contents in tomato plants treated with EBR and subjected to Pb toxicity.

Pb	EBR	Pb in Root (µg g DM^{-1})	Pb in Stem (µg g DM^{-1})	Pb in Leaf (µg g DM^{-1})
−	−	0.20 ± 0.02 ^{c}	0.00 ± 0.00 ^{d}	0.00 ± 0.00 ^{c}
−	+	0.51 ± 0.04 ^{c}	0.07 ± 0.00 ^{c}	0.07 ± 0.01 ^{c}
+	−	115.58 ± 8.33 ^{a}	0.91 ± 0.07 ^{a}	1.25 ± 0.10 ^{a}
+	+	51.64 ± 3.93 ^{b}	0.32 ± 0.02 ^{b}	0.69 ± 0.04 ^{b}

Pb = lead; EBR = 24-epibrassinolide. Columns with different letters indicate significant differences from the Scott–Knott test ($p < 0.05$). Means ± SD, $n = 5$.

3.2. EBR Positively Modulated the Root and Leaf Structures

Toxicity caused by Pb caused reductions in RET, RDT, RCT, VCD, and RMD (Table 2 and Figure 1). Plants exposed to Pb and treated with EBR increased in RET, RDT, and RMD by 23%, 24%, and 20%, respectively. To leaf structures, Pb stress also caused decreases (Table 2 and Figure 1). However, the application of EBR in plants submitted to 200 µM Pb showed increases of 10%, 21%, 9%, and 10% in ETAd, ETAb, PPT, and SPT, respectively, when compared to equal treatment without EBR.

Table 2. Root and leaf structures in tomato plants treated with EBR and subjected to Pb toxicity.

Pb	EBR	RET (µm)	RDT (µm)	RCT (µm)	VCD (µm)	RMD (µm)
−	−	21.1 ± 1.5 [a]	18.8 ± 1.3 [a]	208 ± 08 [a]	155 ± 10 [a]	37.7 ± 2.9 [a]
−	+	20.9 ± 1.4 [a]	19.7 ± 1.2 [a]	209 ± 10 [a]	156 ± 10 [a]	39.1 ± 2.2 [a]
+	−	15.5 ± 1.4 [c]	13.3 ± 0.9 [c]	166 ± 11 [b]	142 ± 11 [a]	25.4 ± 1.9 [c]
+	+	19.1 ± 1.5 [b]	16.4 ± 0.9 [b]	169 ± 11 [b]	148 ± 11 [a]	30.4 ± 2.3 [b]
Pb	EBR	ETAd (µm)	ETAb (µm)	PPT (µm)	SPT (µm)	Ratio PPT/SPT
−	−	19.02 ± 0.60 [a]	12.82 ± 1.04 [a]	67.21 ± 1.21 [a]	73.42 ± 1.57 [a]	0.91 ± 0.02 [a]
−	+	19.99 ± 0.78 [a]	13.27 ± 1.01 [a]	67.37 ± 1.08 [a]	73.78 ± 1.39 [a]	0.91 ± 0.03 [a]
+	−	16.01 ± 0.89 [c]	8.08 ± 0.58 [c]	59.36 ± 1.49 [c]	63.86 ± 1.76 [c]	0.93 ± 0.04 [a]
+	+	17.65 ± 0.71 [b]	9.80 ± 0.67 [b]	64.73 ± 1.17 [b]	70.28 ± 1.45 [b]	0.92 ± 0.02 [a]

Pb = lead; EBR = 24-epibrassinolide; RET = root epidermis thickness; RDT = root endodermis thickness; RCT = root cortex thickness; VCD = vascular cylinder diameter; RMD = root metaxylem diameter; 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 ($p < 0.05$). Means ± SD, n = 5.

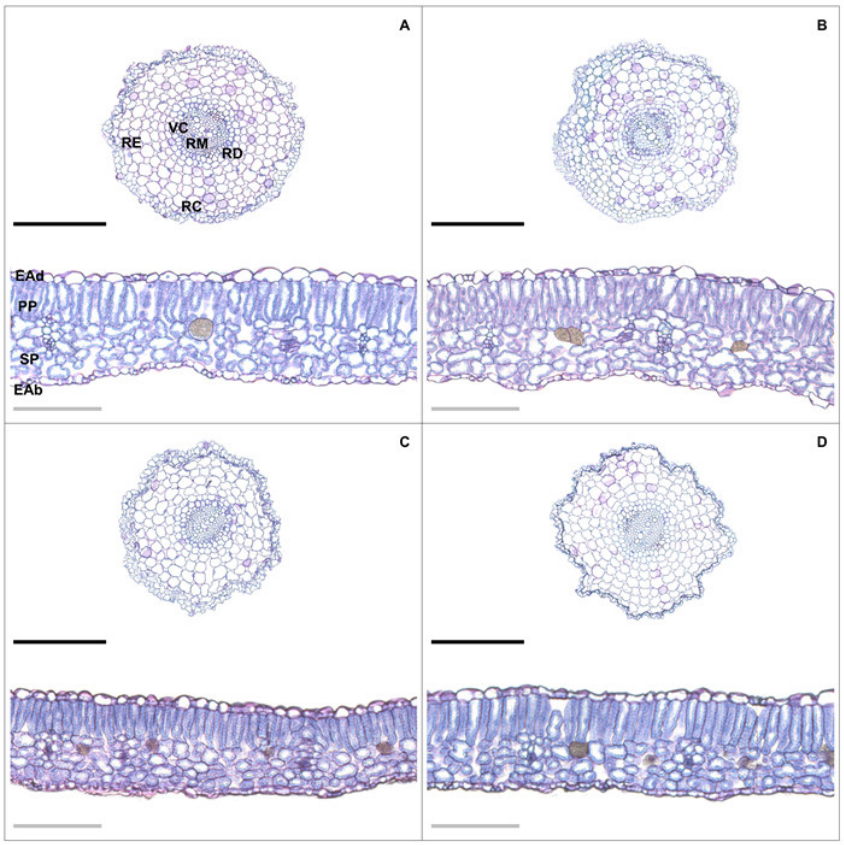

Figure 1. Root and leaf cross sections in tomato plants treated with 24-epibrassinolide (EBR) and subjected to lead (Pb) toxicity. −Pb/−EBR (**A**), −Pb/+EBR (**B**), +Pb/−EBR (**C**), and +Pb/+EBR (**D**).

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 = 300 μm and gray bars: 150 μm.

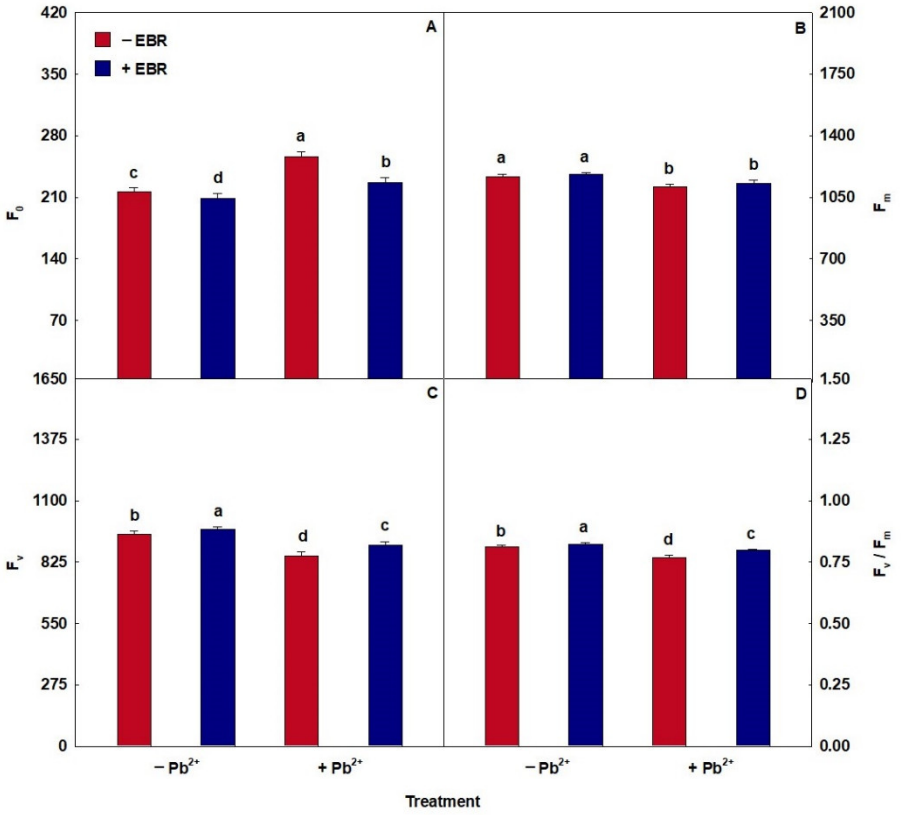

Figure 2. Minimal fluorescence yield of the dark-adapted state (F_0), maximal fluorescence yield of the dark-adapted state (F_m), variable fluorescence (F_v), and maximal quantum yield of PSII photochemistry (F_v/F_m) in tomato plants treated with 24-epibrassinolide (EBR) and subjected to lead (Pb) toxicity. Columns with different letters indicate significant differences from the Scott–Knott test ($p < 0.05$). Means ± SD, $n = 5$.

3.3. Nutrient Contents and Metal Homeostasis Were Up-Regulated by the Steroid

Plants submitted to Pb stress showed negative changes in nutrient contents (Table 3). On the other hand, the application of EBR in plants treated with the metal promoted beneficial effects on the content of Mg, K, Cu, Zn, and Mn, with increases in the root of 30%, 19%, 41%, 16%, and 7%, respectively; stem increases of 6%, 3%, 34%, 12%, and 7%; and increases in the leaf of 14%, 19%, 41%, 18%, and 15%, compared to treatment with Pb only. The Ca content in the root and leaf was increased by 29% and 11%, respectively.

Table 3. Nutrient contents in tomato plants treated with EBR and subjected to Pb toxicity.

Pb	EBR	Mg (mg g DM^{-1})	K (mg g DM^{-1})	Ca (mg g DM^{-1})	Cu (μg g DM^{-1})	Zn (μg g DM^{-1})	Mn (μg g DM^{-1})
Contents in root							
−	−	8.62 ± 0.26 ^{a}	27.72 ± 0.44 ^{b}	3.55 ± 0.15 ^{a}	8.37 ± 0.33 ^{a}	25.90 ± 0.32 ^{b}	315.11 ± 7.82 ^{a}
−	+	8.85 ± 0.43 ^{a}	29.99 ± 0.56 ^{a}	3.68 ± 0.19 ^{a}	8.55 ± 0.26 ^{a}	26.65 ± 0.26 ^{a}	314.78 ± 9.27 ^{a}
+	−	5.80 ± 0.29 ^{c}	20.09 ± 0.94 ^{d}	2.48 ± 0.15 ^{c}	5.44 ± 0.24 ^{c}	19.89 ± 0.53 ^{d}	272.14 ± 8.21 ^{c}
+	+	7.54 ± 0.19 ^{b}	23.86 ± 0.81 ^{c}	3.20 ± 0.11 ^{b}	7.66 ± 0.20 ^{b}	23.04 ± 0.75 ^{c}	290.44 ± 9.54 ^{b}
Contents in stem							
−	−	5.66 ± 0.21 ^{a}	124.47 ± 0.71 ^{a}	7.42 ± 0.18 ^{a}	4.51 ± 0.18 ^{a}	21.02 ± 0.33 ^{b}	32.00 ± 0.66 ^{a}
−	+	5.74 ± 0.25 ^{a}	124.87 ± 0.58 ^{a}	7.68 ± 0.31 ^{a}	4.77 ± 0.18 ^{a}	22.19 ± 0.57 ^{a}	31.70 ± 0.48 ^{a}

+	−	4.95 ± 0.14 c	118.59 ± 0.58 c	7.05 ± 0.52 a	2.99 ± 0.20 c	17.74 ± 0.46 d	29.48 ± 0.79 b
+	+	5.26 ± 0.15 b	122.07 ± 0.75 b	7.29 ± 0.15 a	4.02 ± 0.16 b	19.92 ± 0.54 c	30.69 ± 0.53 b
				Contents in leaf			
−	−	7.57 ± 0.27 a	29.36 ± 0.61 a	17.54 ± 0.30 a	7.22 ± 0.20 a	16.51 ± 0.23 b	76.73 ± 0.99 a
−	+	7.44 ± 0.22 a	30.13 ± 0.69 a	17.84 ± 0.52 a	7.40 ± 0.30 a	17.30 ± 0.26 a	75.90 ± 0.99 a
+	−	5.77 ± 0.37 c	23.76 ± 0.92 c	14.96 ± 0.77 c	4.59 ± 0.36 c	12.87 ± 0.28 d	64.02 ± 1.07 c
+	+	6.58 ± 0.28 b	28.19 ± 0.52 b	16.68 ± 0.53 b	6.46 ± 0.30 b	15.13 ± 0.33 c	73.36 ± 1.11 b

Pb = lead; EBR = 24-epibrassinolide; Mg = magnesium; K = potassium; Ca = calcium; Cu = copper; Zn = zinc; Mn = manganese. Columns with different letters indicate significant differences from the Scott–Knott test ($p < 0.05$). Means ± SD, $n = 5$.

3.4. An Antioxidant System Modulated by EBR

The toxicity of Pb caused an increase in the enzymatic activity of SOD, CAT, APX, and POX (Figure 3). However, the combined effect of Pb + EBR increased the performance of SOD, CAT, APX, and POX by 26%, 18%, 25%, and 20%, respectively, compared to the same treatment without the steroid.

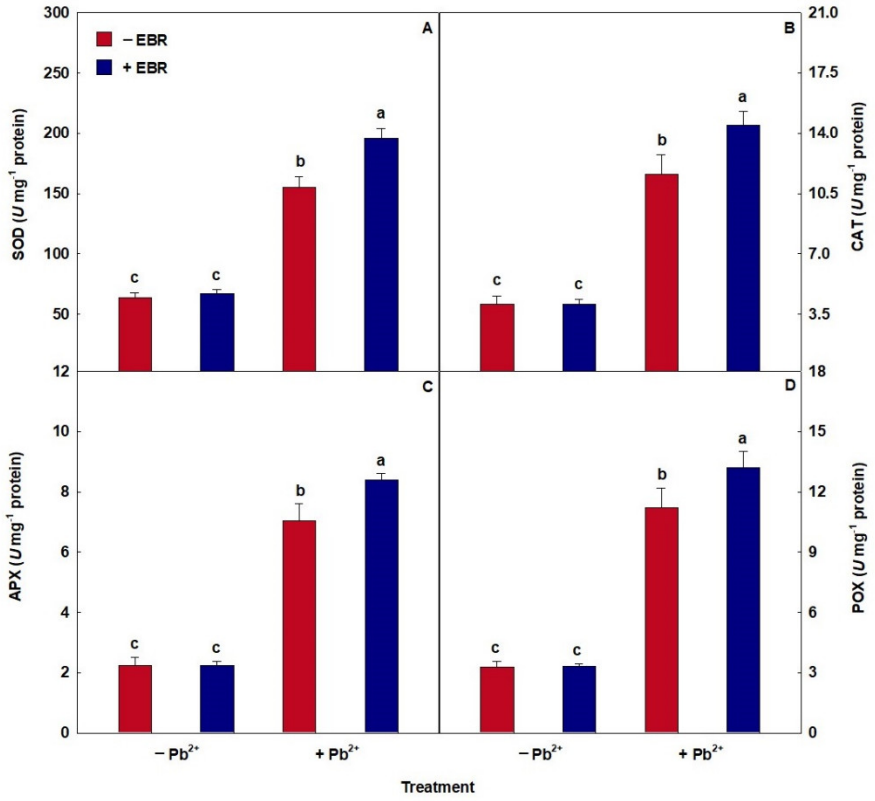

Figure 3. Activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and peroxidase (POX) in tomato plants treated with 24-epibrassinolide (EBR) and subjected to lead (Pb) toxicity. Columns with different letters indicate significant differences from the Scott–Knott test ($p < 0.05$). Means ± SD, $n = 5$.

3.5. Steroid Stimulates Oxidative Damage Reduction

Treatment with Pb induced increases in oxidative compounds (Figure 4). On the other hand, the application of EBR in plants subjected to stress by Pb showed decreases of 23%, 36%, 37%, and 8% in compounds O_2^-, H_2O_2, MDA, and EL, respectively, when compared with the same treatment without EBR.

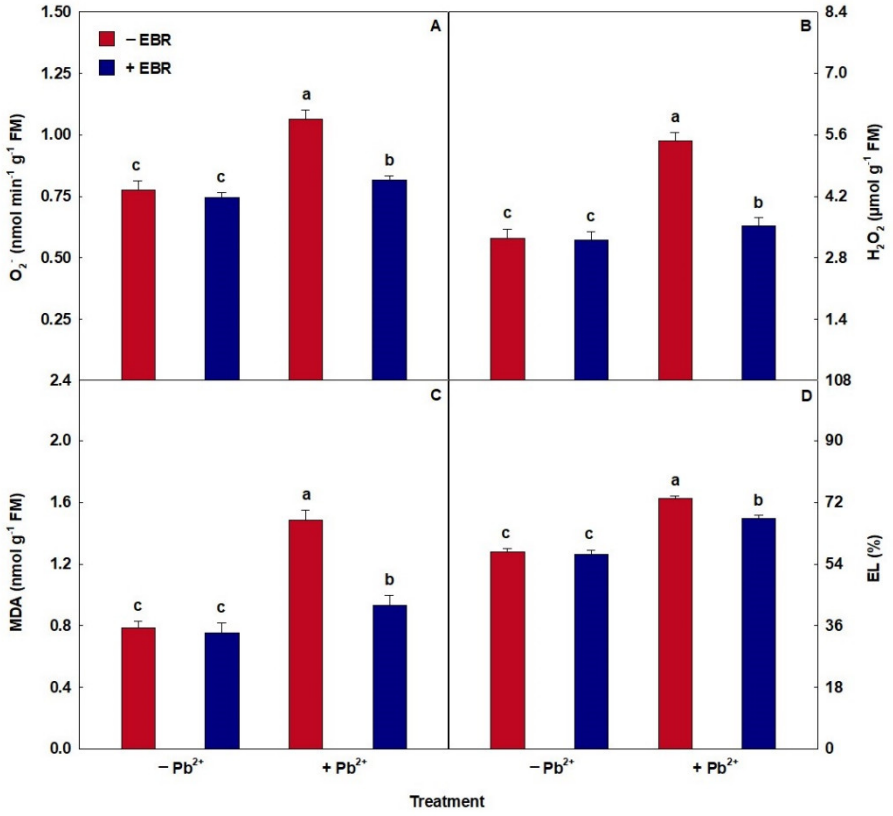

Figure 4. Superoxide (O_2^-), hydrogen peroxide (H_2O_2), malondialdehyde (MDA), and electrolyte leakage (EL) in tomato plants treated with 24-epibrassinolide (EBR) and subjected to lead (Pb) toxicity. Columns with different letters indicate significant differences from the Scott–Knott test ($p < 0.05$). Means ± SD, $n = 5$.

3.6. EBR Benefited Maintenance of Membrane Integrity of Chloroplasts and Alleviated the Pb Interference on Light Capture and Gas Exchange

Plants exposed to Pb toxicity showed decreases in the values of Chl *a*, Chl *b*, Chl Total, and Car (Table 4). However, the combined treatment of Pb + 100 nM EBR promoted increases in Chl *a*, Chl *b*, Chl total, and Car of 21%, 25%, 22%, and 29%, respectively, compared to the Pb treatment. EBR instigated improvements in the capture of light energy. In chlorophyll fluorescence (Figure 2), Pb toxicity caused an increase in F_0 and a reduction in F_v and F_v/F_m. However, plants exposed to Pb + EBR showed a decrease of 11% in F_0 and increases of 6% and 4% in F_v and F_v/F_m, respectively, concerning the same treatment without EBR. Treatment with 200 µM Pb showed no difference in F_m in the absence or presence of EBR. Plants exposed to Pb also showed decreases in Φ_{PSII}, q_P, and ETR and addition in NPQ, EXC, and ETR/P_N (Table 4). However, the Pb + EBR treatment promoted increases in Φ_{PSII}, q_P, and ETR of 24%, 11%, and 24%, respectively, and reductions of 23%, 7%, and 4% in NPQ, EXC, and ETR/P_N, respectively, compared to the similar treatment without the steroid. Concerning gas exchange (Table 4), Pb caused negative effects. However, the Pb + EBR treatment stimulated increases of 29%, 18%, 26%, and 32%, respectively, in P_N, g_s, WUE, and P_N/Ci and a decrease (5%) in Ci, when compared to plants exposed to Pb toxicity without EBR.

Table 4. Photosynthetic pigments, chlorophyll fluorescence, gas exchange in tomato plants treated with EBR and subjected to Pb toxicity.

Pb	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
−	−	4.84 ± 0.14 ^{b}	2.05 ± 0.12 ^{b}	6.89 ± 0.17 ^{b}	0.67 ± 0.04 ^{b}	2.37 ± 0.16 ^{b}	10.27 ± 0.80 ^{a}
−	+	5.16 ± 0.16 ^{a}	2.38 ± 0.14 ^{a}	7.54 ± 0.12 ^{a}	0.78 ± 0.05 ^{a}	2.18 ± 0.18 ^{b}	9.70 ± 0.67 ^{a}
+	−	3.60 ± 0.19 ^{d}	1.29 ± 0.11 ^{d}	4.89 ± 0.22 ^{d}	0.45 ± 0.03 ^{d}	2.79 ± 0.21 ^{a}	10.80 ± 0.84 ^{a}
+	+	4.35 ± 0.17 ^{c}	1.61 ± 0.09 ^{c}	5.96 ± 0.21 ^{c}	0.58 ± 0.04 ^{c}	2.70 ± 0.14 ^{a}	10.29 ± 0.91 ^{a}
Pb	EBR	Φ_{PSII}	q_P	NPQ	ETR (μmol m^{-2} s^{-1})	EXC (μmol m^{-2} s^{-1})	ETR/P_N
−	−	0.271 ± 0.006 ^{b}	0.378 ± 0.008 ^{b}	0.72 ± 0.02 ^{c}	39.86 ± 0.87 ^{b}	0.667 ± 0.008 ^{c}	2.75 ± 0.04 ^{c}
−	+	0.312 ± 0.005 ^{a}	0.417 ± 0.009 ^{a}	0.57 ± 0.03 ^{d}	45.81 ± 0.75 ^{a}	0.621 ± 0.008 ^{d}	2.73 ± 0.05 ^{c}
+	−	0.200 ± 0.003 ^{d}	0.324 ± 0.007 ^{d}	1.06 ± 0.05 ^{a}	29.41 ± 0.47 ^{d}	0.740 ± 0.005 ^{a}	2.98 ± 0.04 ^{a}
+	+	0.248 ± 0.006 ^{c}	0.361 ± 0.007 ^{c}	0.82 ± 0.03 ^{b}	36.39 ± 0.93 ^{c}	0.690 ± 0.009 ^{b}	2.86 ± 0.06 ^{b}
Pb	EBR	P_N (μmol m^{-2} s^{-1})	E (mmol m^{-2} s^{-1})	g_s (mol m^{-2} s^{-1})	C_i (μmol mol^{-1})	WUE (μmol $mmol^{-1}$)	P_N/C_i (μmol m^{-2} s^{-1} Pa^{-1})
−	−	14.48 ± 0.43 ^{b}	2.88 ± 0.05 ^{a}	0.306 ± 0.021 ^{a}	275 ± 4 ^{b}	5.03 ± 0.23 ^{b}	0.053 ± 0.002 ^{b}
−	+	16.78 ± 0.41 ^{a}	2.94 ± 0.09 ^{a}	0.330 ± 0.022 ^{a}	262 ± 4 ^{c}	5.71 ± 0.18 ^{a}	0.064 ± 0.003 ^{a}
+	−	9.87 ± 0.21 ^{d}	2.72 ± 0.07 ^{b}	0.222 ± 0.019 ^{c}	293 ± 6 ^{a}	3.63 ± 0.16 ^{d}	0.034 ± 0.001 ^{d}
+	+	12.72 ± 0.19 ^{c}	2.78 ± 0.04 ^{b}	0.262 ± 0.020 ^{b}	280 ± 5 ^{b}	4.58 ± 0.20 ^{c}	0.045 ± 0.002 ^{c}

Pb = lead; EBR = 24-epibrassinolide; Chl *a* = chlorophyll *a*; Chl *b* = chlorophyll *b*; Total chl = total chlorophyll; Car = carotenoids; 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. Columns with different letters indicate significant differences from the Scott–Knott test ($p < 0.05$). Means ± SD, $n = 5$.

3.7. Limitations on Stomatal Performance Were Attenuated by EBR

Pb excess provoked negative impacts on the characteristics of the stomata (Table 5). Plants exposed to Pb toxicity and treated with 100 nM EBR showed, however, increases in SD and SI, on the adaxial face of 13% and 13%; and in abaxial, 18% and 9%, respectively. In EDS, there was a 7% and 6% reduction in the adaxial and abaxial faces, respectively, compared to the equal treatment without EBR.

Table 5. Stomatal characteristics in tomato plants treated with EBR and subjected to Pb toxicity.

Pb	EBR	SD (Stomata Per mm^2)	PDS (μm)	EDS (μm)	SF	SI (%)
			Adaxial face			
−	−	14.37 ± 0.50 ^{b}	12.09 ± 0.38 ^{b}	21.08 ± 0.58 ^{c}	0.58 ± 0.05 ^{a}	11.96 ± 0.61 ^{a}
−	+	16.30 ± 0.98 ^{a}	11.73 ± 0.44 ^{b}	19.86 ± 0.55 ^{d}	0.59 ± 0.04 ^{a}	12.73 ± 0.89 ^{a}
+	−	11.72 ± 0.77 ^{d}	13.46 ± 0.31 ^{a}	24.16 ± 0.81 ^{a}	0.56 ± 0.04 ^{a}	9.39 ± 0.53 ^{c}
+	+	13.27 ± 0.59 ^{c}	12.86 ± 0.34 ^{a}	22.50 ± 0.74 ^{b}	0.57 ± 0.04 ^{a}	10.60 ± 0.64 ^{b}
			Abaxial face			
−	−	32.21 ± 1.17 ^{b}	10.43 ± 0.35 ^{b}	19.75 ± 0.52 ^{c}	0.53 ± 0.04 ^{a}	21.71 ± 0.98 ^{b}
−	+	36.09 ± 1.26 ^{a}	10.14 ± 0.30 ^{b}	18.65 ± 0.49 ^{d}	0.54 ± 0.04 ^{a}	25.34 ± 1.07 ^{a}
+	−	19.37 ± 1.02 ^{d}	11.51 ± 0.51 ^{a}	22.98 ± 0.61 ^{a}	0.50 ± 0.03 ^{a}	17.28 ± 0.62 ^{d}
+	+	22.94 ± 1.05 ^{c}	11.25 ± 0.43 ^{a}	21.57 ± 0.74 ^{b}	0.52 ± 0.03 ^{a}	18.80 ± 0.70 ^{c}

Pb = lead; EBR = 24-epibrassinolide; 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 ($p < 0.05$). Means ± SD, $n = 5$. All authors have read and agreed to the published version of the manuscript.

3.8. EBR Promoted Higher Biomass Accumulation

The stress caused by Pb caused a reduction in the amount of dry matter (Figure 5). In contrast, the application of EBR in plants stressed by Pb increased the values of LDM, RDM, SDM, and TDM by 95%, 115%, 74%, and 92%, respectively, relative to the same treatment in the absence of EBR.

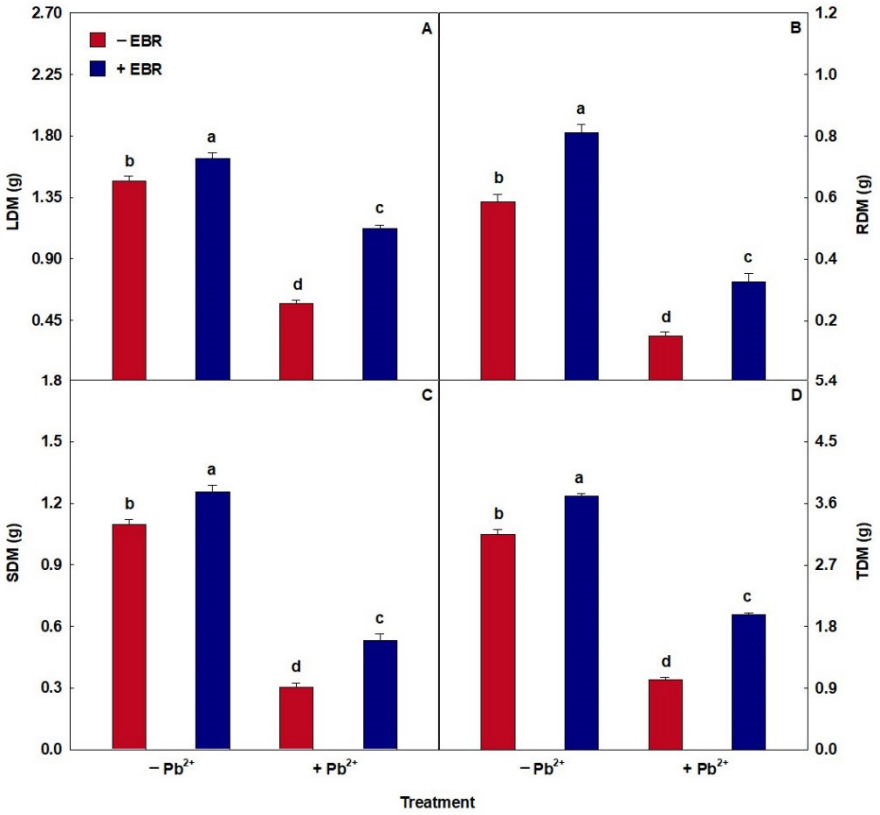

Figure 5. Leaf dry matter (LDM), root dry matter (RDM), stem dry matter (SDM), and total dry matter (TDM) in tomato plants treated with 24-epibrassinolide (EBR) and subjected to lead (Pb) toxicity. Columns with different letters indicate significant differences from the Scott–Knott test ($p < 0.05$). Means ± SD, $n = 5$.

4. Discussion

Here, the various values we recorded in the Pb-treated plants demonstrate that the application of 200 µM of $PbCl_2$ results in the uptake and accumulation of this metal in plants, mainly in the roots. However, the application of 100 nM EBR reduced the Pb content because this steroid is involved in regulating the electrical characteristics of membranes, cell membrane permeability, and ion transport [55], possibly inhibiting uptake and accumulation by stimulating processes that act on immobilization, sequestration, and precipitation of this heavy metal [56]. Rossato et al. [57], testing progressive Pb concentrations in *Pluchea sagittalis* plants, observed increases in the content of this metal in the root, stem, and leaf. Evaluating Pb stress in *Acutodesmus obliquus*, Talarek-Karwel et al. [58] identified a reduction in Pb content after EBR application.

EBR application alleviated the loss caused by Pb toxicity in the nutritional content, increasing the values of macronutrients (Mg, K, and Ca) and micronutrients (Cu, Zn, and Mn). These elements play essential roles in plant metabolism, being required as constituents of crucial molecules or as cofactors of various enzymatic processes. A high concentration of Pb can cause an imbalance in the absorption, assimilation, and translocation of

nutrients through changes in the permeability of the plasma membrane and influence the processes involved in the transfer of these elements across the root membrane [59], which is consistent with the reduction in the root structures of plants exposed only to Pb we detected in our study. After absorption, Pb binds to the membrane and cell wall, which induces rigidity to these components and reduces cell division, inhibiting the growth of root tissues [55]. On the other hand, EBR plays an important role in signalization, regulation, and differentiation of the root tissue, inducing increments in root structures after the application of this steroid, especially in RET, RDT, and RMD, being these results clearly connected to the protection of this organ against the deleterious effects occasioned by the Pb [60,61], as well as benefits on nutritional status confirmed by the increase in the macro and micronutrient contents. Yuan et al. [62] found that the application of EBR helped to maintain the ionic balance in *Cucumis sativus* plants under Ca stress. *Pisum sativum* plants exposed to Cd stress [63] also demonstrated beneficial effects on nutritional status with EBR treatment. Santos et al. [64] researching Zn-induced stress in *Glycine max*, detected increases in the root structures after applying 100 nM of EBR.

Plants treated with EBR after exposure to Pb stress had increased enzyme activities (SOD, CAT, APX, and POX). The enhanced activities of the SOD, POX, and CAT enzymes are probably associated with the upregulation of gene expression of the SOD, POX, and CAT encoding genes [65]. BRs participate in the processes of gene expression, transcription, and translation in normal and stressed plants, stimulating increased activity of antioxidant enzymes in scavenging excess ROS, probably up-regulating the mRNA expression levels of Cu/Zn-SOD, Mn-SOD, CAT, APX, or specific genes, such as *RBOH* (respiratory burst oxidase homolog), *MAPK1* (mitogen-activated protein kinase 1), and *MAPK3* (mitogen-activated protein kinase 3) [66,67]. These findings are also connected with a positive balance in the nutritional status promoted by EBR since nutrients such as Zn, Cu, and Mn, which had their contents elevated after steroid application in this study, act as cofactors of SOD isozymes [68]. Zhou et al. [69] studying *Vitis vinifera* plants under Cu stress, detected enhanced enzyme activities with the EBR pretreatment. Similar results were described by Kohli et al. [70] with *Brassica juncea* seedlings under Pb stress had increased SOD, CAT, APX, and POX activities in response to EBR supplementation.

Pb toxicity induces molecular damage through the formation of ROS, such as O_2^- and H_2O_2, implying modifications to membrane structure and function, starting lipid peroxidation, and altered cell biochemical processes [71], consequently resulting in oxidative damages to membrane lipids, proteins, chloroplast pigments, enzymes, and nucleic acids [72], which was identified in this research by increases in oxidative compounds found in plants exposed to 200 μM Pb. Plants respond to excess ROS by inducing enzymatic antioxidants like SOD, CAT, APX, and POX that operate synergistically to repress metal toxicity [73]. Simultaneously, EBR works by eliminating these ROS in cells by regulating a complex antioxidant mechanism, stimulating the activity of these enzymes [74], where, in a simplified way, SOD acts as the first line of defense to counter the O_2^- and catalyzes the conversion of O_2 to H_2O_2, then H_2O_2 can be eliminated by CAT, POX, and APX, and broken into H_2O and O_2 [68]. This effect of EBR was observed in our study through the reductions of O_2^-, H_2O_2, MDA, and EL triggered by the increases found in the enzymes SOD, CAT, APX, and POX. *Brassica napus* plants presented increases in oxidative compounds under Pb stress conditions [75]. On the other hand, in research by Kohli et al. [76] studying *Brassica juncea* plants exposed to Pb had reductions in O_2^-, H_2O_2, and MDA after EBR application.

Treatment with EBR in plants alleviated Pb stress on photosynthetic pigment content (Chl *a*, Chl *b*, total Chl, and Car). Photosynthetic pigments have high sensitivity to toxic metals, such as Pb, because its excess is related to the peroxidation of chloroplast membranes, which can provoke a deterioration of light-harvesting pigments and induces harm to the reaction center pigments [18,77]. Moreover, the disturbance caused by Pb on Chl content may be ascribed to the inhibition of its biosynthesis, the impaired uptake of essential ions such as Mg, Cu, and Mn, and the increased chlorophyllase (*CHLASE*) activity

that catalyzes chlorophyll degradation [19,78]. However, EBR promotes an increase in chlorophyll content because it is directly or indirectly involved in the up-regulating Chl biosynthesis enzyme activity or the stimulation of gene expression levels related, for example, to Chl *a*/*b* binding protein-encoding [79]. In addition, this steroid attenuated these toxic effects of Pb on chlorophyll molecules through the improvements promoted in oxidative compounds and nutritional status, identified in this study by the reduction of MDA and EL and the increases in the contents of Mg, Zn, and Cu. In research by Kohli et al. [65], working with *Brassica juncea* plants under Pb toxicity obtained increased photosynthetic pigment content and decreased *CHLASE* gene expression after treatment with EBR. Similar to this study, EBR also showed positive effects on Chl *a*, Chl *b*, total Chl, and Car in a study conducted by Zhong et al. [24] with *Festuca arundinacea* plants subjected to Pb stress.

EBR application relieved the damage generated by Pb excess in F_0, F_v, and F_v/F_m. Pb harms the efficiency of PSII photochemical reaction and electron transport chain, resulting in decreases in Fv/Fm, which may also be connected to impaired Q_A oxidation, further causing a decrease in electron transport from PSII to PSI [80]. The variable F_v/F_m is related to the functional state of the oxygen evolution complex and can be used as a sensitive indicator of photosynthetic performance, and when found at low levels, together with higher levels of F_0, it indicates extensive photoinhibition due to environmental stresses [81]. In contrast, EBR can increase F_v and F_v/F_m and reduce F_0 values, diminishing the photoinhibition effects and decreasing the dissipation of excitation energy in the antennas of photosystem II [61]. Similar to our research, Guedes et al. [32] describe increases in F_v, F_v/F_m, and decrease in F_0 after application of 100 nM EBR in *Oryza sativa* plants exposed to 200 μM Pb. Tadaiesky et al. [82] working with *Oryza sativa* plants under Fe stress, reported that EBR also promoted improvements in F_0, F_v, and F_v/F_m.

Plants exposed to excess Pb treated with EBR showed beneficial effects on chlorophyll fluorescence with increases in Φ_{PSII}, q_P, and ETR and decreases in NPQ, EXC, and ETR/P_N. These results are related to the improvements obtained by EBR in F_v, F_v/F_m, and F_0 described in this study. Pb stress conditions can cause damage to electron transport, absorption, and conversion of light energy, photochemical efficiency of the reaction center, and dissipation of excess energy in the photosynthesis process, especially in PSII activity [83]. Studies evaluating the toxicity caused by Pb suggest that the absorption and dissipation of energy within the PSII are high, while the capture and transport of electrons are reduced due to instability of Chl molecules or damage to the electron transfer system [84]. Despite that, EBR can stimulate the electron flux and improve the efficiency of the PSII, probably associated with the oxidation of Q_A, which receives and transfers electrons between PSII and PSI, as well as avoiding damage caused by excess energy in the reaction centers [28,85]. Alam et al. [86] related increases of 50% in Φ_{PSII} and 81% in q_P and decreases of 37% in NPQ after treatment with EBR in *Glycine max* seedlings under NaCl stress. Resembling our study, Santos et al. [87] evaluating seedlings of *Vigna unguiculata* exposed to Cd stress, found increases in Φ_{PSII}, q_P, and ETR, and reductions in NPQ, EXC, and ETR/P_N after application of 100 nM EBR.

Pb toxicity causes modifications in photosynthesis due to disruption of photosynthetic pigments synthesis, injury of chloroplast ultrastructure, changes in lipid and protein composition of the thylakoid membrane, imbalance minerals uptake, restricted electron transport, inhibited activities of Calvin cycle enzymes, besides deficiency of CO_2 in the result of stomatal closure [88,89]. On the other hand, plants treated with EBR and submitted to stress Pb-induced showed increases in P_N, g_s, WUE, and P_N/C_i and a decrease in C_i. These responses may be related to the steroid's role in promoting higher efficiency of PSII and probably better fixation of CO_2 in the Calvin–Benson cycle by the RUBISCO enzyme during photosynthesis, also suggested by the increase in P_N and reduction in C_i [90]. Zhou et al. [91] examining *Robinia pseudoacacia* seedlings under Pb stress, observed that the values of P_N and g_s decreased with the progressive increase in the metal. In a study with

Glycine max seedlings under Mn stress, increases in P_N, g_s, WUE, and P_N/C_i and decreased C_i after EBR application have been reported [9].

Reports have shown that the anatomical structure is altered by Pb toxicity through disturbance of external and internal tissues, which can lead to the collapse of parenchymal cells [92]. However, EBR spray revealed ameliorations on leaf structures with increases in ETAd, ETAb, PPT, and SPT. Increases in ETAd and ETAb are associated with the benefits promoted by EBR through stimulating cell division and expansion in the leaf as a strategy to avoid excessive water loss during the transpiration process, which was observed in the increase in WUE in our research [14,93]. As regards improvements in PPT and SPT, the increase is linked to the positive effects of EBR on gas exchange in this study, mainly in P_N and P_N/C_i, since the higher thickness of these tissues indicates better use of light energy and greater accumulation of CO_2. Santos et al. [94] investigating Fe stress, verified additions on the ETAd, ETAb, PPT, and SPT variables in *Glycine max* seedlings treated with EBR.

The positive effects promoted by EBR found in the stomatal characteristics, through the increases in SD, EDS, and SI in plants under Pb toxicity, are correlated to the role of this steroid in improving stomatal performance. Furthermore, EBR stimulates stomata production by regulating specific proteins that act in the stomatal pathway [95]. These results are also reinforced by the fact that increased stomatal density is linked to stomatal conductance and increased intercellular spaces, thus increasing CO_2 uptake and photosynthesis, which can be corroborated by increases in g_s, P_N and SPT, and decreases in C_i, obtained in our research with EBR application [96]. Ribeiro et al. [97] working with *Eucalyptus urophylla* seedlings exposed to Ni stress, described increases in SD, EDS, and SI variables after EBR treatment.

EBR positively modulates growth in Pb-stressed plants through increments in LDM, SDM, RDM, and TDM, mitigating the toxic effects of this heavy metal. Pb toxicity causes damaging effects on the growth and biomass of plants since excessive amounts in the intercellular space of this metal aggravated ultrastructural injury to leaf, often leading to cell death through disruption of chloroplasts, obstruction of chlorophyll biosynthesis, and interfering with nutrient elements transportation and photosynthetic machinery [20,98]. In contrast, as noted in the results of this work, EBR is involved in multiple roles in plants, relieving stress by restoring redox balance, stimulating pigment biosynthesis, promoting benefits in leaf anatomy, and improving chlorophyll fluorescence and gas exchange. Fariduddin et al. [99] reported increased biomass in *Brassica juncea* plants treated with EBR and exposed to Mn toxicity. Jan et al. [100] described biomass improvements by analyzing the effect of EBR on *Solanum lycopersicon* plants exposed to Cr toxicity.

5. Conclusions

This study demonstrates the interferences caused by Pb stress in tomato plants. On the other hand, our results in leaf anatomy, photosynthetic apparatus, and biomass confirm the positive action of the exogenous application of 100 nM of EBR. This steroid attenuates leaf tissue cell disruption by stimulating cell division and expansion, making this structure thicker, improving membrane selectivity, promoting greater use of light energy and, consequently, higher fixation of CO_2. Additionally, EBR induced increases in the activity of antioxidant enzymes (SOD, CAT, APX, and POX), reducing membrane oxidative damages, thus preserving the chloroplast ultrastructure, being demonstrated in the reductions of oxidative compounds (O_2^-, H_2O_2, MDA, and EL) and maintenance of photosynthetic pigments. The improvements obtained in these pigments made it possible to reduce the instability of Chl molecules caused by the excess of Pb, increasing the capture and transport of electrons, improving the efficiency of PSII, and mitigating the damage caused by photoinhibition. Simultaneously, the benefits provided by EBR in the photosynthetic apparatus resulted in increments in the accumulation of biomass. Therefore, this research shows evidence of EBR's ability to alleviate the harmful effects of Pb toxicity in tomato plants.

Author Contributions: A.K.S.L. was the advisor of this research, planned all phases of the research and critically revised the manuscript; C.F.M. conducted the experiment in the greenhouse and performed physiological, biochemical, and morphological determinations, wrote and edited the manuscript; B.R.S.S. measured anatomical parameters; B.L.B. nutritional analysis; A.B. critically revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare that they have no competing interests.

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GENERAL CONCLUSIONS

This research strengthens the importance that EBR has in the mitigation damage caused in tomato plants subjected to Ni and Pb toxicity through protecting the photosynthetic machinery, upregulating the antioxidant system and improving anatomical structure. EBR induces the maintenance of chloroplast membrane integrity, the synthesis of chlorophyll content and the photosynthetic efficiency of electron transport. This steroid alleviates the oxidative stress induced by these HMs by stimulating enzymes linked to redox metabolism, such as SOD, APX and POX, which are involved in the detoxification of reactive oxygen species, including superoxide and hydrogen peroxide. This steroid attenuates cellular degradation of leaf tissue by stimulating cell division and expansion, making this structure thicker, improving membrane selectivity, promoting greater use of light energy and fixation of CO_2, consequently also improving photosynthetic efficiency. All steroid functions converge towards improving nutritional status and increasing biomass, which was observed in our results. However, in future studies, advanced molecular approaches are necessary to better determine the mechanisms of action of EBR in plants under Ni and Pb toxicity.