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**24-EPIBRASSINOLIDE RELIEVES DAMAGES CAUSED BY INADEQUATE
SUPPLEMENTATION OF BORON AND ZINC IN PLANTS**

BELÉM
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Thesis submitted to the Universidade Federal Rural da
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Agronomy, to obtain the title of Doctor in Agronomy.
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Advisor: Prof. Dr. Allan Klynger da Silva Lobato

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I dedicate this accomplishment to both of my parents, siblings and friends for their continuous support.

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ABSTRACT

Boron (B) and zinc (Zn) are essential micronutrient for plant growth. B regulates the structural and membrane integrity of the cell wall and plasma membrane, ion mobility across the membrane, cell division and elongation, reproductive growth, biomolecule synthesis. Zn it plays important roles in plant development, reproduction and signaling due to its structural, catalytic and activating functions. Brassinosteroids (BRs) are a class of steroid hormones that regulate a number of physiological processes. EBR is the most active form of BRs, and exogenous application of EBR can ameliorate the effects of abiotic stresses. Therefore, the objective of this research was to investigate whether application of EBR can alleviate the effects of inadequate boron and zinc supplementation in soybean and tomato seedlings. Two experiments were carried out, the experiment 1 was with soybean plants under three levels of boron supplementation (control, low, and high B) and two EBR conditions (0 and 100 nM), and the experiment 2 was with tomato plants under three levels of zinc (control, low, and high Zn)) and two EBR conditions (0 and 100 nM). Both experiments was performed at the Campus of Paragominas of the Universidade Federal Rural da Amazônia, Paragominas, Brazil (2°55' S, 47°34' W). The study was conducted in a greenhouse with the temperature and humidity controlled. This research has shown that the application of EBR relieved the stress caused by B in soybean plant and Zn in tomato plants, synergistically stimulating leaf and root structures, photosynthesis, and growth. Therefore, our results show that EBR application could help improve plants' tolerance to boron and zinc stress.

KEYWORDS: Brassinosteroids. *Glycine max*. Nutritional stress. *Solanum lycopersicum*

RESUMO

Boro (B) e zinco (Zn) são micronutrientes essenciais para o crescimento das plantas. B regula a integridade estrutural da parede celular e da membrana plasmática, mobilidade iônica através da membrana, divisão e alongamento celular, crescimento reprodutivo e síntese de biomoléculas. O Zn desempenha papéis importantes no desenvolvimento, reprodução e sinalização das plantas devido às suas funções estruturais, catalíticas e ativadoras. Brassinosteróides (BRs) são uma classe de hormônios esteróides que regulam uma série de processos fisiológicos. O EBR é a forma mais ativa de BRs, e a aplicação exógena de EBR pode melhorar os efeitos de estresses abióticos. Portanto, o objetivo desta pesquisa foi investigar se a aplicação de EBR pode amenizar os efeitos da suplementação inadequada de boro e zinco em mudas de soja e tomate, respectivamente. Foram realizados dois experimentos, o experimento 1 foi com plantas de soja sob três níveis de suplementação de boro (controle, baixo e alto B) e duas condições EBR (0 e 100 nM), e o experimento 2 foi com plantas de tomate sob três níveis de zinco (controle, baixo e alto Zn) e duas condições EBR (0 e 100 nM). Ambos os experimentos foram realizados na Universidade Federal Rural da Amazônia, no campus de Paragominas, Brasil (2°55' S, 47°34' W). Os estudos foram conduzidos em casa de vegetação com temperatura e umidade controladas. Esta pesquisa mostrou que a aplicação de EBR aliviou o estresse causado por B na planta de soja e Zn em plantas de tomate, estimulando sinergicamente as estruturas foliares e radiculares, a fotossíntese e o crescimento. Portanto, nossos resultados mostram que a aplicação de EBR pode ajudar a melhorar a tolerância das plantas ao estresse de boro e zinco.

PALAVRAS-CHAVE: Brassinosteróides. Crescimento. Estresse nutricional. *Glycine max.* *Solanum lycopersicum*

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LIST OF ABBREVIATIONS

APX	Ascorbate peroxidase
BRs	Brassinosteroids
CAR	Carotenoids
CAT	Catalase
Chl <i>a</i>	Chlorophyll a
Chl <i>b</i>	Chlorophyll b
C_i	Intercellular CO ₂ concentration
CO ₂	Carbon dioxide
<i>E</i>	Transpiration rate
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
F_m	Maximal fluorescence yield of the dark-adapted state
F_v	Variable fluorescence
F_v/F_m	Maximal quantum yield of PSII photochemistry
g_s	Stomatal conductance
H ₂ O ₂	Hydrogen peroxide
K	Potassium
LDM	Leaf dry matter
MDA	Malondialdehyde
NPQ	Nonphotochemical quenching
O ₂ ⁻	Superoxide
PDS	Polar diameter of the stomata
P_N	Net photosynthetic rate
P_N/C_i	Instantaneous carboxylation efficiency

POX	Peroxidase
PPT	Palisade parenchyma thickness
PQ	Plastoquinone
PSII	Photosystem II
q _p	Photochemical quenching
RCD	Root cortex diameter
RDM	Root dry matter
RMD	Root metaxylem diameter
RDT	Root endodermis thickness
RET	Root epidermis thickness
ROS	Reactive oxygen species
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
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
Φ _{PSII}	Effective quantum yield of PSII photochemistry

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1 CONTEXTUALIZATION

Boron (B) is an essential micronutrient for plant growth (MATTHES; ROBI; MCSTEEN, 2020), regulates the structural and membrane integrity of the cell wall and plasma membrane, ion mobility across the membrane, cell division and elongation, reproductive growth, biomolecule synthesis (KOHLI et al., 2022). This nutrient has an extremely narrow range between deficiency and toxicity, and inadequate boron supply exhibits a detrimental effect on crop plant growth and yield. (BRDAR-JOKANOVIĆ et al., 2020). To Takada et al. (2014) B deficiency is an agricultural problem that causes significant losses in crop productivity worldwide.

Zinc (Zn) is a vital element for plant growth, as it plays important roles in plant development, reproduction and signaling due to its structural, catalytic and activating functions (KAUR et al., 2021). Zn also acts as a cofactor for many enzymes, such as carbonic anhydrase, carboxypeptidase, and Zn-superoxide dismutase (FARIDUDDIN et al., 2022). Most agricultural soils are unable to supply the Zn needs of cultivated plants, making Zn deficiency a widespread nutritional disorder, particularly in calcareous soils ($\text{pH} > 7$) (HACISALIHOGU, 2020). On the other hand, high amounts of Zn, mainly due to human activities, can be toxic to plants, causing morphological, biochemical and physiological disturbances (BALAFREJ et al., 2020).

Brassinosteroids (BRs) are a class of steroid hormones that regulate a number of physiological processes (AN et al., 2023), control the division, elongation, and differentiation of various cell types throughout the entire life cycle of the plant (PLANAS-RIVEROLA et al., 2019). EBR is the most active form of BRs, and exogenous application of EBR can ameliorate the effects of abiotic stresses such as drought (PEREIRA et al. 2019), heavy metal contamination (MAIA et al. 2022), waterlogging (PEREIRA et al. 2020), salinity (SOUSA et al., 2021) and nutritional deficiency (SANTOS et al. 2021).

Our hypothesis considered that the inadequate supply of boron and zinc, whether due to deficiency or toxicity, affects plant growth (PLANAS-RIVEROLA et al., 2019). On the other hand, studies have shown the ability of EBR to promote photosynthesis and alleviate oxidative damage in plants under abiotic stress (RODRIGUES et al. 2020; MAIA et al. 2022). Therefore, the objective of this research was to investigate whether application of EBR can alleviate the effects of inadequate boron and zinc supplementation in soybean and tomato seedlings.

2 LITERATURE REVIEW

2.1 Soybean

Soybean (*Glycine max* L.) is one of the main food products in the world (KUN and GANG, 2017) and the main commodity exported by the Brazilian agricultural market and, corresponding to half of all soy consumed globally (RAMOS et al., 2020). In the year 2021, according to the Ministério da Agricultura, Pecuária e Abastecimento, soy exports moved more than 47 billion dollars, which represents a 39.82% share of the Brazilian export market and, in 2022, this value jumped to more than 60 billion dollars, representing 38.28% due to the growth of other commodities (AGROSTAT, 2021).

It is a plant originating in East Asia, more precisely in the region known as Manchuria, located in northeast China, although it has not been disseminated to other places for almost two millennia after its appearance (HYMOWITZ, 1970; SILVA et al., 2022). After being cultivated in several countries in Asia, Europe and North America, soybeans were brought to Brazil and cultivated in Bahia, according to D'Utra's report, in 1882, where they did not perform satisfactorily due to the conditions under the latitude of 12° south (SEDIYAMA; TEIXEIRA; BARROS, 2009; SILVA et al., 2022). It was in Rio Grande do Sul that soybean found favorable conditions for development, since they were closer to the temperate climate conditions of origin of the cultivar brought from the United States (SANTOS, 1988; SILVA et al., 2022)

Soybean is a herbaceous and annual leguminous plant belonging to the Fabaceae Family (CHATURVEDI et al., 2011). It has taproots with lateral roots that grow deep to absorb water and nutrients in the upper soil layers (KASPAR, 2022), the stem is erect, cylindrical and branched with trifoliate and alternate leaves (MÜLLER, 1981; SEDIYAMA; TEIXEIRA; BARROS, 2009). Has high levels of protein (36-40%) and oil (18-20%) in your beans (LIMA et al., 2015) and has a high demand for nitrogen (N) throughout the development period due to the high protein content in the grains, requiring up to 80 kg of N per ton of grains produced (KASCHUK et al., 2016). This amount is usually supplied by Biological Nitrogen Fixation (BNF), carried out by symbiosis between the soybean plant and strains of *Bradyrhizobium* spp. together with the absorption of N from the soil (ZUFFO et al., 2018; ZILLI et al., 2021).

In addition, despite having a good ability to adapt to different conditions, soybean needs minimum conditions such as good water availability, adequate photoperiod and spacing, and minimum amounts of nutrients in the soil (CRUZ et al., 2016). It is common for soybean crops to present a deficiency in Zinc (Zn) and Boron (B) in Brazilian soils that are, in general, poor in relation to these micronutrients which, despite being required in small amounts, can cause a

reduction in crop performance when insufficiently supplied (EPSTEIN; BLOOM, 2004; FAQUIN, 2005; OLIVEIRA et al., 2017).

2.2 Tomato

Tomato (*Lycopersicon esculentum* Mill.) it is one of the most widespread and appreciated vegetables in the world and its fruits can be consumed in natura or in the form of sauces or pastes, which guarantees it a large market around the world (NUNES et al., 2019). In 2021, tomato production in Brazil was 3.679,160 tons and generated revenue of almost 6.5 billion reais, 6.6% higher than the previous year in an area of only 51 thousand hectares, which demonstrates the high productivity of Brazilian production (IBGE, 2021).

The tomato plant is native to the Andes and has a wide variety of genera and wide adaptability in different regions (NUNES et al., 2019). It is a dicotyledonous flowering plant belonging to the Solanaceae family (DUSI et al., 1993), it has a pivoting root and herbaceous and erect stem, which can reach 2 meters in height (CONTRERAS-MAGANÃ et al., 2013). The existence of numerous different species and cultivars available on the market shows their different responses to light, CO₂, temperature, water and nutrient absorption conditions. Due to this ability, these plants can thrive in climates ranging from high altitude tropical to temperate conditions (FERREIRA et al., 2017). Adjusting cultural practices, such as staking, plant management and planting density, is essential to guarantee the quality and appearance of tomato fruits, which increases their value and, consequently, profitability (MARIN et al., 2005; SHIRAIGE et al., 2010; ALMEIDA et al., 2015).

The tomato plant has a wide range of climatic conditions in which it can develop, however, for optimal productivity, this vegetable crop requires some more specific conditions, found mainly in the south and southeast regions, with low temperature and precipitation at an altitude of approximately , 600 meters (FERREIRA et al., 2003; ALMEIDA et al., 2015). Regarding nutrition, like most vegetables, tomato responds very well to increased N concentration, which causes an increase in dry matter in all parts of the plant, an increase in leaf area, flowering, fruiting and productivity (SINGH; SHARMA, 1999; FERREIRA et al., 2003).

In tomatoes, the deficiency of micronutrients such as Zinc is characterized by a reduction in the growth and development of the youngest leaves, which become small and deformed, and may also present a slight chlorosis that, finally, considerably reduce the capacity for flowering and fruiting of the leaves. plants (EPSTEIN; BLOOM, 2004; OLIVEIRA et al., 2009; ALMEIDA et al., 2015).

2.3 Boron

Boron (B) is an essential micronutrient for the plant, it is absorbed by the roots mainly in the form of boric acid (BRDAR-JOKANOVIĆ et al., 2020). Since its discovery in the mid-1920s, Boron has been a problem for researchers as its regulatory and biochemical roles in plants were uncertain and it was believed that much still needed to be learned (NIELSEN, 1991). It is known that its concentration in dicots is significantly higher than in monocots and it is present in the soil over a wide pH range as a neutral molecule $B(OH)_3$ at pH below 7 and $B(OH)_4^-$ at higher pH, similar to Silicon, albeit in substantially higher concentrations (EPSTEIN; BLOOM, 2006; FERREIRA VARANDA et al., 2018).

In the plant organism, B performs several functions such as, acting as an enzyme regulator, contributing to the process of structuring and functioning of membranes (BROADLEY et al., 2012; BHATLA et al. 2018). In addition to participating in cell wall formation, carbohydrate synthesis and transport, protein synthesis, nitrogen fixation, photosynthesis and growth (FERNANDES, 2006; DA SILVA et al., 2017). Boron reaches the roots of plants by mass flow and is passively absorbed by the difference in concentration between the media (PAULL; NABLE; RATHJEN, 1992; PRINCI et al. 2016). Within the plant, boron transport occurs by facilitated diffusion, high-affinity transporters, and channel proteins (DORDA; CHRISPEELS; BROWN, 2000; OZTURK, 2010).

Since it is a little mobile element in the soil, the absorption of B depends, to a large extent, on the availability of water in the soil (TANAKA; FUJIWARA, 2008; DA SILVA et al., 2017). Under conditions of adequate water availability in the soil, fertilization with B in soybean caused accumulation of the element in the leaves and increased plant performance in relation to the number of pods and grain yield (GALERIANI et al., 2022).

B deficiency mainly affects plant growth tissues such as apical buds which are often damaged and may die (WIMMER; EICHERT, 2013). Tissues affected by deficiency, such as stems, leaves and fruits, become hard, dry and brittle, sometimes with spots (GUPTA; SOLANKI, 2013). Flowering is also strongly affected and the fruits, when they manage to form, show the symptoms and appearance observed on the stem (EPSTEIN; BLOOM, 2006; YAU; RYAN, 2008; MIWA; FUJIWARA, 2010; ALMEIDA et al., 2015).

On the other hand, B toxicity is a problem in several places in the world such as Australia and China and is usually caused by the high concentration of this element in the soil or by irrigation with B-rich water (NABLE et al. 1997; CAMACHO-CRISTÓBAL et al., 2008). Studies indicate that the ideal levels of B in the soil for good productivity are between 0.7 and

1.0 mg dm³ (DA SILVA, 2017), which may be linked to other factors, as some studies indicate optimal doses of up to 3.0 mg (SOMAVILA et al., 2022).

2.4 Zinc

Zinc is a heavy metal that, unlike copper, iron and manganese, is a divalent cation that does not have redox activity because it does not undergo valence changes (KUMAR, et al. 2022). This element is present in a large amount of proteins, whether in the active site, such as in carbonic anhydrase; either as an integral component of proteins (SINGH et al. 2019; JOMOVA, 2022). Some research suggests Zn also plays a role in the metabolism of reactive oxygen species and Zn-finger proteins, which regulate defense and signaling responses in plants under stress (STANTON et al., 2022).

In the soil, Zn has low mobility, and this is a factor to be considered when deciding on the fertilization of crops, since it has a low leaching rate, which can contribute to its residual effect in the soil (VALLADARES et al., 2009; HAN et al., 2011). Studies indicate that the critical levels of zinc in the soil are between 0.8 and 1.0 mg dm⁻³ (Fageria, 2000; Galvão, 1986; 2004). In a study with foliar fertilization with Zn, Oliveira et al. (2017), reported that the agronomic characteristics of soybeans were positively affected up to the dosage of 12 kg ha⁻¹, with an increase in productivity and plant growth.

Boron and Zinc are the micronutrients that most frequently cause deficiency in plants due to their low concentration in Brazilian soils, especially in Cerrado soils (FAQUIN, 2005; LOPES; GUILHERME, 2016). Practices that seek to reduce soil acidity can also negatively influence the availability of zinc in the soil (GONÇALVES et al., 2018). Thus, the symptoms caused by the deficiency of this micronutrient, which are related to plant growth, are often due to a dysfunction in the synthesis of “zinc fingers” proteins, which are essential in DNA transcription (BOWEN, 1979; EPSTEIN; BLOOM, 2006), generating an effect on various plant functions that depend on specific proteins such as photosynthesis and respiration (MARSCHNER, 2012).

On the other hand, excess zinc in the environment can cause problems for the development of higher plants (MARSCHNER, 1995). In general, zinc toxicity causes symptoms similar to those of iron deficiency such as chlorosis and reduced growth (MCCAULEY; JONES; JACOBSEN, 2009). This led the researchers to relate the symptoms to the inhibition of Fe activity due to excess zinc in the soil, which may be due to the composition of that soil or the mining activity that can release zinc into the soil (WOOLHOUSE, 1983; SOARES et al., 2001; SILVA et al., 2016).

Investigating the effects of high Zn concentration on sugar beet plants (*Beta vulgaris* L.), Sagardoy et al. (2009) observed that plants treated with high concentrations of Zn sulfate

in the nutrient solution (50, 100 e 300 μm) showed a reduction in the fresh and dry mass of the root and shoot, in addition to a decrease in the levels of N, Mg, K and Mn in all parts of the plant.

2.5 Brassinosteroids

Brassinosteroids (BRs) are a group of polyhydroxylated steroid hormones found in almost all plant species, and are involved in several physiological processes of plants throughout their life cycle (CHUNG; CHOE, 2013). BRs were first isolated and characterized in the 1970s, discovered in *Brassica napus* pollen based on their ability to promote growth (MITCHELL et al., 1970; PLANAS-RIVEROLA et al., 2019). And they are currently considered on the same level as classic plant hormones such as auxin, cytokinin, ethylene, gibberellic acid and abscisic acid (OH et al., 2020).

BRs regulate many important agronomic traits including plant height, leaf angle, panicle architecture, seed size and germination, tillering, photosynthesis, senescence, flowering (BAJGUZ et al., 2020; SUN et al., 2021). They also influence root growth and control both cell division and cell elongation by establishing an increasing signaling gradient along the longitudinal axis of the root (VUKALINOVIĆ et al., 2021). Rozhon et al. (2019) BRs are steroids that have strong growth-promoting capabilities, are crucial for all stages of plant development, and participate in adaptive growth processes and stress response reactions.

BRs are considered derivatives of 5 α -cholesterol, but vary in structure due to carbon side chains (KOUR et al., 2021). These steroids are widely distributed throughout the plant kingdom and exhibit great structural diversity (BARTWAL; ARORA, 2020). The most active form of BR is 24-epibrassinolide, it is produced by the addition of many oxygen atoms to campesterol by various cytochrome monooxygenases (CYPs) (FUJIYAMA et al., 2019). To

Exogenous application of BRs benefits plant growth (WEI et al., 2020). Additionally, BRs are non-toxic and ecological, they can be used as abiotic and biotic stress mitigators without disturbing the balance of the ecosystem (HUSSAIN et al., 2020). Investigating the effects of brassinosteroids application on soybean plants under water deficit, Pereira et al. (2019) reported that the application of 100 nM of EBR mitigated the effects of water stress and improved photosynthesis. In soybean plants under inadequate manganese supplementation, Rodrigues et al. (2020) observed that EBR mitigated the damage caused by both deficiency and Mn toxicity.

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PAPER I

(Journal of Soil Science and Plant Nutrition)



Anatomical Modifications Modulated by Pretreatment with 24-Epibrassinolide Alleviate Boron Stress in Soybean Plants: Valuable Repercussions on Nutrient Contents, Photosynthesis, and Biomass

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Abstract

Boron (B) is an essential micronutrient for vascular plants, and its availability in the soil represents a limiting factor for world agricultural production. 24-Epibrassinolide (EBR) is a biodegradable and eco-friendly plant steroid hormone, with multiple benefits connected to growth and development. We hypothesized that inadequate B supplementation could limit plant development, causing damage to leaf and root structures. Therefore, the objective of this research was to verify the possible contributions of EBR in root and leaf structures and biomass accumulation in soybean plants under inadequate B supplies (deficiency or toxicity). The experiment followed a completely randomized factorial design with two concentrations of 24-epibrassinolide (0 and 100 nM EBR, described as –EBR and +EBR, respectively) combined with three B supplies (0.6, 30, and 1500 μM B, described as low, control, and high supply of B). EBR alleviated the damages occasioned by the inadequate B supplies on root tissues, specifically maximizing the vascular cylinder, metaxylem, and epidermis, improving the nutritional status. This steroid also minimized the harmful effects of B stress on leaf anatomy, stimulating the epidermis on both leaf sides, palisade parenchyma, and spongy parenchyma; structures intrinsically related to protection and carbon dioxide availability to the photosynthetic process. Concomitantly, this steroid had a positive impact on biomass accumulation. These results are explained by beneficial actions on leaf structures and photosynthetic machinery. Therefore, our results demonstrate that the EBR application can improve soybean plants' tolerance under inadequate B supplementation.

Keywords Boron supplies · Brassinosteroids · *Glycine max* · Mesophyll · Metaxylem

1 Introduction

Soybean (*Glycine max*) is a leguminous species with high nutritional value, rich in proteins (Bamji and Corbitt 2017; Kim et al. 2006; Wang et al. 2008), being a commodity with broad importance in human and animal nutrition and industrial products (Goldsmith 2008; Nishinari et al. 2014). In the 2017/2018 harvest, world soybean production was around 336 million tons, with Brazil, the USA, and Argentina accounting for around 82% of world production. The root is the organ responsible for water and nutrient uptake, fixing the plant in the soil (Seago and Fernando 2013; Zeng et al. 2019), and is covered by the root epidermis. This tissue has direct contact with the soil solution, contributing to fluxes of ions (Du and Wei 2018; Javelle et al. 2011; Thomas et al.

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2007). The cortex is adjacent to the epidermis, consisting of parenchymal cells with spaces between them, working as storage tissue (Enstone et al. 2003; Pérez Chaca et al. 2014). The endodermis corresponds to the last layer of the cortex, acting as a barrier aiming to modulate the water and nutrient supplies due to the presence of Casparian strips (Barberon et al. 2016; Lux et al. 2011; Purushothaman et al. 2013).

The leaf is the main organ responsible for photosynthesis, frequently presenting stomata in abaxial and adaxial faces (Maia et al. 2018), and is covered by the epidermis that protects this organ against abiotic stresses (Gao et al. 2019; Nemeskéri and Helyes 2019; Wyka et al. 2019). Stomata modulate essential processes, such as CO₂ absorption, transpiration, and thermal regulation (Veza et al. 2018). In soybean plants, the mesophyll is formed by the palisade and spongy parenchyma (Gonçalves et al. 2017; Lansing and Franceschi 2000) being found in chloroplasts and other organelles that act during the photosynthesis process (Ju et al. 2017).

Boron (B) is an essential micronutrient for vascular plants, and its availability in the soil represents a limiting factor for world agricultural production (Kobayashi et al. 2018). B deficiency can be triggered by inadequate soil conditions, such as high pH and low organic matter content, and environmental factors, such as drought, high temperature, and light (Atique-ur-Rehman et al. 2018). Another problem is that B is susceptible to leaching in soil because it is highly soluble and mobile, which can easily lead to plant deficiency after excessive rainfall (Goli et al. 2019). On the other hand, in arid and semi-arid regions, there can be problems related to the toxicity of this micronutrient (Nawaz et al. 2020), where B can be taken to the surface layers of the soil due to the combined actions linked to the evaporation process of the soil water and capillary process (Yau and Ryan 2008).

In plants, B has structural and metabolic functions, being intrinsically related to calcium in cell wall development, acting in protein synthesis, sugar transport, respiration, and carbohydrate metabolism (Reid 2014; Shireen et al. 2018). Additionally, the mobility and permeability of this nutrient can vary by species (Wimmer and Eichert 2013). The B deficiency reflects mainly on meristematic tissues (Hänsch and Mendel 2009), affecting the root growth and the development of seeds, flowers, and fruits (Herrera-Rodríguez et al. 2010; Oiwa et al. 2013), also limiting absorption and transport of water and nutrients, due to damages to the root structures, including the xylem vessels (Li et al. 2017). On the other hand, B excess is associated with oxidative stress, in which B toxicity causes the overproduction of reactive oxygen species (ROS), such as superoxide (O₂⁻) and hydrogen peroxide (H₂O₂), causing an imbalance in metabolism, affecting the division and expansion of the plant cells (Farhaly et al. 2021; Sakamoto et al. 2011).

Brassinosteroids (BRs) are steroidal phytohormones that have multiple benefits on plant metabolism, including regulation of ion channels in the plasma membrane (Zhang et al. 2005), improvements in photosynthetic apparatus performance (Shahbaz et al. 2008), antioxidant metabolism (Zhang et al. 2008), and regulation of flowering and cell expansion (Clouse 2002). These steroids induce positive repercussions on root and leaf tissues (Maia et al. 2018; Oliveira et al. 2019; Ribeiro et al. 2019), being detected in plants exposed to nutritional stress (deficiency and/or excess) (Lima et al. 2018; Vriet et al. 2012; Zhang et al. 2008). Among the different types of BRs, 24-epibrassinolide (EBR) is the most used BR because it is considered the natural bioactive form and is biodegradable and highly efficient (Khrupach 2000; Maia et al. 2018).

We hypothesize that inadequate B supplementation can limit plant development, causing damage to leaf and root structures (Huang et al. 2014; Li et al. 2017; Mei et al. 2016). Recent studies have demonstrated that EBR can play an important role in plant development, especially under stressful conditions, because this steroid triggers benefits in root metaxylem (Santos et al. 2020), in nutritional status (Lima et al. 2018), also favoring the maintenance of chloroplastic pigments (Rodrigues et al. 2020) and improving the growth (Ribeiro et al. 2020). Therefore, the objective of this research was to verify the possible contributions of EBR in root and leaf structures and biomass accumulation in soybean plants under inadequate B supplies.

2 Materials and Methods

2.1 Location and Growth Conditions

The experiment was performed at the Campus of Paragominas of the Universidade Federal Rural da Amazônia, Paragominas, Brazil (2°55' S, 47°34' W). The study was conducted in a greenhouse with temperature and humidity-controlled. The minimum, maximum, and median temperatures were 24, 33, and 25.1 °C, respectively. The relative humidity during the experimental period varied between 60 and 80%.

2.2 Plants, Containers, and Acclimation

Seeds of *Glycine max* (L.) Merr. var. M8644RR Monsoy™ were germinated and grown in 1.2-L pots filled with a mixed substrate of sand and vermiculite at a ratio of 3:1. The plants were cultivated under semi-hydroponic conditions containing 500 mL of distilled water for 4 days. A nutritional solution described by Pereira et al. (2019) was used for plant nutrition, with ionic strength beginning at 50% (5th day) and later modified to 100% after 2 days (7th day). After

this period, the nutritional solution remained at total ionic strength.

2.3 Experimental Design

The experiment followed a completely randomized factorial design with two concentrations of 24-epibrassinolide (0 and 100 nM EBR, described as – EBR and + EBR, respectively) and three B supplies (0.6, 30, and 1500 μM B, described as low, control, and high supply of B). With five replicates for each of the six treatments, a total of 30 experimental units were used in the experiment, with one plant in each unit.

2.4 24-epibrassinolide Preparation and Application

Eight-day-old seedlings were sprayed with EBR or Milli-Q water (containing a proportion of ethanol equal to that used to prepare the EBR solution; described as – EBR) at 5-day intervals until day 28. Solution of EBR (Sigma-Aldrich, USA) (100 nM) was prepared by dissolving the solute in ethanol followed by dilution with Milli-Q water [ethanol: water (v/v) = 1:10000] (Ahammed et al. 2013a).

2.5 Plant Conduction and Boron Supplies

Plants received the following macro- and micronutrients contained in the nutrient solution in agreement with Pereira et al. (2019). For B treatments, H_3BO_3 was used at concentrations of 0.6 μM (low), 30 μM (control), and 1500 μM (high) applied over 12 days (days 16–28 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 28 of the experiment, physiological and morphological parameters were measured for all plants, and leaf tissues were harvested for anatomical, biochemical, and nutritional analyses.

2.6 Chlorophyll Fluorescence, Gas Exchange, and Anatomical Variables

Chlorophyll fluorescence was measured as described by Maia et al. (2018). Gas exchange was evaluated following the calibration procedures described by Pereira et al. (2019). Samples were collected following the methodology of Oliveira et al. (2019) and O'Brien et al. (1964). Stomatal characterization was carried out according to Segatto et al. (2004).

2.7 Determination of Antioxidant Enzymes, Superoxide, and Soluble Proteins

Antioxidant enzymes (SOD, CAT, APX, and POX), superoxide, and soluble proteins were extracted from root tissues

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

2.8 Quantification of Hydrogen Peroxide, Malondialdehyde, and Electrolyte Leakage

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

2.9 Determination of Photosynthetic Pigments, Nutrient Contents, and Biomass

The chlorophyll and carotenoid determinations were performed using a spectrophotometer (model UV-M51; Bel Photonics), according to the methodology of Lichtenthaler and Buschmann (2001). The determination of B, K, Ca, S, Cu, Mn, and Mo was carried out using an inductively coupled plasma mass spectrometer (model ICP-MS 7900; Agilent). The growth of roots, stems, and leaves was measured based on constant dry weights (g) after drying in a forced-air ventilation oven at 65°C .

2.10 Data Analysis

The normality of residues was checked using Shapiro–Wilk test. Data were subjected to two-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). All statistical procedures used the Assisat 7.7 software.

3 Results

Steroid application increased B contents in treatments control and with low B supply

The low and high B supplies promoted changes in B contents in the root, stem, and leaf tissues of soybean plants (Table 1). Plants sprayed with EBR and submitted to low B presented increases in B contents of 56%, 2%, and 19% in root, stem, and leaf, respectively, compared with the same treatment without EBR. The control + EBR treatment had increases of 36%, 26%, and 4% in root, stem, and leaf, respectively. However, plants submitted to high B and sprayed with EBR presented reductions of 26%, 12%, and 6% in root, stem, and leaf, respectively.

EBR maximized the protection of roots and CO₂ availability in leaves under B stress.

Low and high B supplies occasioned decreases in root anatomy (Table 2 and Fig. 1). Plants sprayed with EBR and exposed to low B treatment had increased RET (root epidermis thickness), RDT (root endodermis thickness), RCD (root cortex diameter), VCD (vascular cylinder diameter),

and RMD (root metaxylem diameter) by 6%, 5%, 29%, 14%, and 4%, respectively when compared to the same treatment without EBR. While control + EBR treatment presented increases of 12% (RET), 15% (RDT), 21% (RCD), 6% (VCD), and 4% (RMD). To high B with EBR, the variables RET, RDT, RCD, VCD, and RMD had increases of 21%, 34%, 8%, 10%, and 12%, respectively. To leaf structures (Table 2 and Fig. 1), low and high B supplies promoted decreases, except for the PPT/SPT ratio. For ETAd, ETAb, PPT, and SPT plants sprayed with EBR under the low B treatment, we detected increases of 14%, 18%, 27%, and 32%, respectively, while the control treatment had increases of 3%, 18%, 6%, and 10%, respectively. High B presented increases of 20%, 8%, 14%, and 17% respectively. While for PPT/SPT, there were reductions of 4% (low B), 4% (control), and 4% (high B) in plants treated with EBR, if compared to equal treatment without EBR.

Table 1 Boron contents in soybean plants sprayed with EBR and exposed to different B supplies

EBR	B supply	B in root ($\mu\text{g g DM}^{-1}$)	B in stem ($\mu\text{g g DM}^{-1}$)	B in leaf ($\mu\text{g g DM}^{-1}$)
–	Low	14.34 ± 0.96 ^{Cb}	12.54 ± 0.64 ^{Ca}	13.85 ± 0.75 ^{Cb}
–	Control	17.77 ± 0.59 ^{Bb}	16.78 ± 0.70 ^{Bb}	24.45 ± 1.89 ^{Ba}
–	High	77.95 ± 3.79 ^{Aa}	40.71 ± 2.09 ^{Aa}	219.08 ± 2.06 ^{Aa}
+	Low	22.36 ± 1.00 ^{Ba}	12.74 ± 0.75 ^{Ca}	16.54 ± 0.53 ^{Ca}
+	Control	24.13 ± 1.03 ^{Ba}	21.18 ± 1.35 ^{Ba}	25.44 ± 0.63 ^{Ba}
+	High	57.71 ± 1.11 ^{Ab}	35.91 ± 1.40 ^{Ab}	204.92 ± 2.82 ^{Ab}

B = boron; EBR = 24-epibrassinolide. Columns with different uppercase letters between B supplies (low, control, and high B supply under equal EBR level) and lowercase letters between EBR level (with and without EBR under equal B supply) indicate significant differences from the Scott-Knott test ($P < 0.05$). Means ± SD, $n = 5$

Table 2 Root and leaf structures in soybean plants sprayed with EBR and exposed to different B supplies

EBR	B supply	RET (μm)	RDT (μm)	RCD (μm)	VCD (μm)	RMD (μm)
–	Low	9.3 ± 0.2 ^{Bb}	15.5 ± 0.7 ^{Ba}	282 ± 12 ^{Bb}	223 ± 8 ^{Bb}	54.2 ± 0.5 ^{Cb}
–	Control	10.6 ± 0.6 ^{Ab}	18.7 ± 0.7 ^{Ab}	358 ± 24 ^{Ab}	258 ± 14 ^{Aa}	64.1 ± 0.3 ^{Ab}
–	High	8.1 ± 0.3 ^{Cb}	12.5 ± 0.2 ^{Cb}	289 ± 7 ^{Bb}	237 ± 6 ^{Bb}	55.8 ± 0.3 ^{Bb}
+	Low	9.9 ± 0.3 ^{Ba}	16.3 ± 0.6 ^{Ba}	363 ± 20 ^{Ba}	255 ± 15 ^{Ba}	56.1 ± 0.7 ^{Ca}
+	Control	11.9 ± 0.7 ^{Aa}	21.5 ± 0.8 ^{Aa}	432 ± 10 ^{Aa}	273 ± 9 ^{Aa}	66.6 ± 0.6 ^{Aa}
+	High	9.8 ± 0.6 ^{Ba}	16.7 ± 0.4 ^{Ba}	313 ± 17 ^{Ca}	261 ± 9 ^{Ba}	62.7 ± 0.9 ^{Ba}
EBR	B supply	ETAd (μm)	ETAb (μm)	PPT (μm)	SPT (μm)	Ratio PPT/SPT
–	Low	16.1 ± 1.1 ^{Bb}	13.7 ± 0.9 ^{Bb}	74 ± 4 ^{Bb}	47 ± 2 ^{Bb}	1.57 ± 0.04 ^{Aa}
–	Control	20.4 ± 1.1 ^{Aa}	15.8 ± 0.6 ^{Ab}	107 ± 3 ^{Ab}	71 ± 4 ^{Ab}	1.51 ± 0.07 ^{Aa}
–	High	15.3 ± 1.1 ^{Bb}	14.2 ± 0.9 ^{Bb}	74 ± 3 ^{Bb}	47 ± 1 ^{Bb}	1.58 ± 0.09 ^{Aa}
+	Low	18.4 ± 0.9 ^{Ba}	16.1 ± 0.8 ^{Ba}	94 ± 4 ^{Ba}	62 ± 2 ^{Ba}	1.50 ± 0.06 ^{Aa}
+	Control	21.1 ± 1.4 ^{Aa}	18.6 ± 0.8 ^{Aa}	113 ± 4 ^{Aa}	78 ± 2 ^{Aa}	1.45 ± 0.08 ^{Aa}
+	High	18.4 ± 1.1 ^{Ba}	15.3 ± 0.7 ^{Ba}	84 ± 6 ^{Ca}	55 ± 3 ^{Ca}	1.52 ± 0.06 ^{Aa}

B, boron; EBR, 24-epibrassinolide; RET, root epidermis thickness; RDT, root endodermis thickness; RCD, root cortex diameter; 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 uppercase letters between B supplies (low, control, and high B supply under equal EBR level) and lowercase letters between EBR level (with and without EBR under equal B supply) indicate significant differences from the Scott-Knott test ($P < 0.05$). Means ± SD, $n = 5$

3.1 Nutritional Status was Improved after EBR Pretreatment

The low and high B supplies promoted reductions in nutrient contents in root, stem, and leaf tissues (Table 3). Plants sprayed with EBR and exposed to low B presented increases in K, Ca, S, Cu, Mn, and Mo contents of 21%, 1%, 16%, 33%, 17%, and 3% in the root; 6%, 2%, 21%, 25%, 1%, and 21% in the stem; and 9%, 14%, 10%, 13%, 7%, and 10% in leaf, respectively, when compared to the same treatment without EBR. The control treatment with EBR also had increases in K, Ca, S, Cu, Mn, and Mo of 21%, 14%, 24%, 9%, 7%, and 7% (root); 4%, 1%, 5%, 12%, 33%, and 9% (stem); and 8%, 6%, 11%, 11%, 14%, and 18% (leaf) in this order, when compared to the same treatment without EBR. Plants submitted to high B with EBR presented increments in K, S, Cu, Mn, and Mo of 8%, 16%, 5%, 25%, and 5%, but Ca suffered a reduction of 2% in the root. In the stem, there were verified increases in K, Ca, S, Cu, Mn, and Mo of 5%, 7%, 9%, 14%, 8%, and 11%. To leaf, we detected increases of 11%, 9%, 7%, 9%, 5%, and 14%, respectively, when compared to the same treatment without EBR.

3.2 B Stressed Plants Suffered Minor Oxidative Damage on Photosynthetic Machinery

The low and high supplementation with B reduced the photosynthetic pigments considering Chl *a*, Chl *b*, Total Chl, and Car variables (Table 4), causing increases in Chl *a*/Chl *b* and Chl/Car when compared to the control treatment. Plants sprayed with EBR and exposed to low B treatment had increased Chl *b*, Total Chl, and Car (by 8%, 2%, and 6%, respectively) compared to the same treatment without EBR; the control treatment showed increases of 26%, 33%, 28%, and 27%, respectively; and in the high B supply, there were increases of 5%, 24%, 9%, and 16% in Chl *a*, Chl *b*, Total Chl, and Car, respectively. In plants sprayed with EBR, the Chl *a*/Chl *b*, and Chl/Car ratios showed reductions compared to non-pulverized plants. Concerning chlorophyll fluorescence (Fig. 2), low and high B treatments promoted reductions, except F_0 . For F_0 , plants sprayed with EBR had reductions of 11%, 13%, and 2% for the low, control, and high B treatments, respectively, compared to the same treatment without EBR (Fig. 2A). In relation to F_m (Fig. 2B), F_v (Fig. 2C), and F_v/F_m (Fig. 2D) under the application of EBR, the low B supplement had increases of 1%, 4%, and 3%, respectively; in the control B + EBR treatment there were increases of 1%, 4%, and 4%, respectively; and the high B with EBR treatment presented increments of 4%, 6%, and 2%, respectively. On photosystem II (Table 4), low and high B supplies induced reductions in Φ_{PSII} , q_p , and ETR, while increases in NPQ, EXC, and ETR/ P_N presented increases compared to the control treatment. Plants sprayed

with EBR and exposed to low treatment had increased Φ_{PSII} , q_p , and ETR by 21%, 6%, and 19%, respectively, whereas for NPQ, EXC, and ETR/ P_N decreased by 9%, 10%, and 32%, respectively. In the control treatment + EBR, Φ_{PSII} , q_p , and ETR presented increases of 12%, 10%, and 13%, respectively, whereas NPQ, EXC, and ETR/ P_N presented reductions of 5%, 5%, and 7%, respectively. The high B supply + EBR treatment showed increases of 13%, 14%, and 2% for the variables Φ_{PSII} , ETR, and NPQ, respectively, but for q_p , EXC, and ETR/ P_N , there were reductions of 7%, 6%, and 9%, respectively. Concerning gas exchange (Table 4), low and high B supplies caused reductions. Plants sprayed with EBR and exposed to low treatment resulted in increases for E , g_s , P_N , and P_N/C_i of 77%, 133%, 79%, and 123%, respectively, whereas for C_i , there was a reduction of 20%. Control plants with EBR, P_N , WUE, and P_N/C_i presented increases of 21%, 55%, and 24%, respectively, while C_i , E , and g_s had reductions of 3%, 21%, and 18%, respectively. The treatment with high B + EBR presented increases of 24%, 38%, and 25% in P_N , WUE, and P_N/C_i , respectively, and for C_i , E , and g_s , there were reductions of 2%, 4%, and 8%, respectively.

3.3 Stomatal Performance was Upregulated after EBR Application

The low and high B supplies reduced stomatal characteristics, except for PDS and EDS (Table 5). Plants sprayed with EBR under the low treatment B on the adaxial face presented increases for SD, SF, and SI of 30%, 6%, and 42%, respectively, compared to the same treatment without EBR. Under the control treatment, the adaxial face had increased for SD and SI by 9% and 18%, respectively; in high B treatment, there were increases of 23%, 5%, and 28% in SD, SF, and SI, respectively. For PDS and EDS in the abaxial face, EBR + low B induced decreases of 13% and 12%, respectively. Under control treatment combined with EBR suffered decreases of 2% (PDS) and 6% (EDS) and under the high B with EBR had reductions of 13% in PDS and 12% in EDS.

3.4 EBR Spray Increased the Activities of Antioxidant Enzymes in Plants Exposed to B Stress

Low and high B supplies increased enzyme activities (Fig. 3). For SOD, plants sprayed with EBR had increases of 176%, 115%, and 73% in supplements low, control, and high B, respectively, relative to the same treatment without EBR (Fig. 3A). Concerning CAT, plants exposed to EBR presented increases of 9%, 17%, and 27% in the low, control, and high B treatments, respectively (Fig. 3B). To APX, plants pretreated with EBR presented elevations of 21%, 13%, and 42% in the low, control, and high B treatments, respectively (Fig. 3C). In POX, plants with EBR had increases of 21%

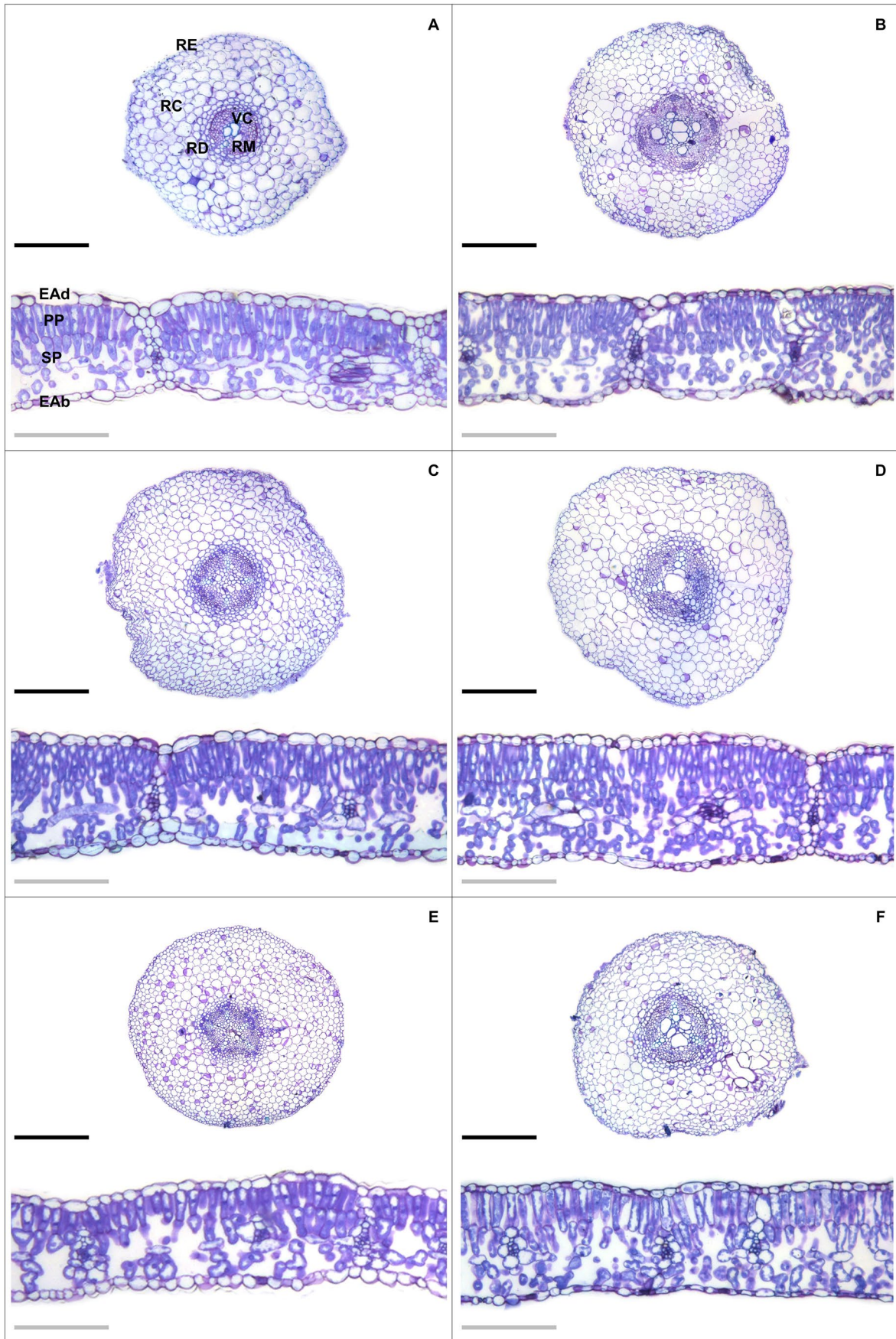


Fig. 1 Root and leaf cross sections in soybean plants sprayed with 24-epibrassinolide (EBR) and exposed to different boron (B) supplies. B low/−EBR (A), B low/+EBR (B), B control/−EBR (C), B control/+EBR (D), B high/−EBR (E), and B high/+EBR (F). RE root epidermis, RC root cortex, RD root endodermis, VC vascular cylinder, RM root metaxylem, EAd adaxial epidermis, EAb abaxial epidermis, PP palisade parenchyma, SP spongy parenchyma. Black bars, 300 μm; grey bars, 150 μm

(low B), 48% (control), and 48% (high B) (Fig. 3D). On stress indicators (Fig. 4), supplies with low and high B levels caused increases. For O_2^- , plants sprayed with EBR had reductions of 1%, 2%, and 26% in low, control, and high B, respectively, compared to the same treatment without EBR (Fig. 4A). Concerning H_2O_2 , plants sprayed with EBR had decreases of 2%, 14%, and 10% in the low, control, and high B treatments, respectively (Fig. 4B). To MDA, plants pre-treated with EBR presented reductions of 9%, 3%, and 33% in treatments using low, control, and high B, respectively (Fig. 4C). EL (plants with EBR) was decreased by 1%, 19%, and 5% under low, control, and high B supplies, respectively (Fig. 4D).

3.5 EBR Mitigated the B Stress-Induced Effects on Biomass

The low and high B supplies promoted significant reductions in growth compared to the control treatment (Fig. 5), except for RDM. For LDM, plants sprayed with EBR and exposed to the control treatment had a 6% increase compared with equal treatment without EBR (Fig. 5A). Concerning RDM, the treatments under low, control, and high B + EBR had increases of 15%, 8%, and 61%, in this order (Fig. 5B). To SDM (Fig. 5C) and TDM (Fig. 5D), the low B supply + EBR promoted increases of 7% and 1%, respectively; whereas the control supply with EBR presented increases of 1% and 5%, respectively; and under high B + EBR, increases of 5% and 10%, respectively.

4 Discussion

Our findings provide evidence supporting the ability of EBR to alleviate the negative effects triggered by B stress (deficiency or toxicity) in soybean plants. The EBR application reduced B contents in plants subjected to toxicity and increased B contents in control and low B treatments. The positive effects detected in the control and low B can be attributed to improvements in the transport mechanisms of this element in the plant, especially to inflow channels and transporters BOR1, BOR2, and NIP5. The BOR2 transporter promotes the cross-linking of the pectin polysaccharide rhamnogalacturonan II (RG-II) and root elongation, maximizing nutrient uptake, including B (Takada et al. 2014).

The NIP5 influx channels are located in the epidermis, cortex, and endoderm cells, while NIP6 is required for transport between the xylem and phloem (Robert and Friml 2009; Takano et al. 2008; Tanaka et al. 2008).

On the other hand, under B toxicity, EBR probably stimulated the BOR4 transporter, which is located in the endoderm and pericycle, being responsible for the detoxification mechanism of B in the plant via secretion (Miwa et al. 2007; Takano et al. 2008). Concomitantly, EBR-induced benefits on antioxidant metabolism, contributing to homeostasis and reducing reactive oxygen species (ROS) often found in plants exposed to stress conditions, including B toxicity (Bajguz and Hayat 2009). B is a micronutrient with high plant mobility (Takano et al. 2008), and multiple B transport mechanisms have been described in the literature (Robert and Friml 2009). This element is a small molecule presenting high permeability relative to other nutrients, with uptake linked to several transporters and broad distribution in plant tissues (Reid 2014). Landi et al. (2012) reported that B toxicity causes oxidative stress due to B accumulation in leaf cell walls, causing imbalances of cytoplasmic metabolism. Devi et al. (2012), evaluating soybean responses under different concentrations of B (0.5, 1.0, 1.5, and 2.0 kg ha⁻¹), observed increases in B contents in leaves and stems.

Soybean plants exposed to deficiency and toxicity of B suffered reductions related to root anatomy variables, but the use of EBR mitigated these effects, reflected in increases in RET, RDT, RCD, VCD, and RMD. The epidermis, endodermis, and cortex are specialized tissues linked to the protection and ionic transport of the apoplast in the direction of the symplast (Scheres et al. 2002). The endoderm still acts as an apoplastic barrier controlling water and nutrient uptake (Enstone et al. 2003; Lux et al. 2004). Increases in RET, RDT, and RCD indicate that EBR spray reduced the damages caused by the B stresses, promoting the increase of the cell expansion rate in these tissues and stimulating the mitotic cycle to maintain cell proliferation in the meristem (Hacham et al. 2011). The effects of EBR on RMD indicate that it promotes benefits on root protection, with gradual increase and consequent improvement in hydraulic conductivity (Hameed et al. 2009). With the thickening of the root, higher absorption of water and nutrients can occur due to positive repercussions on vascularization (Meyer et al. 2011). Ghanati et al. (2005) demonstrated that soybean seedlings exposed to B toxicity (5 mM) had inhibition of root growth due to hypodermis formation and suberin deposition in cortical cell walls corroborated by our research. A study conducted by Aquea et al. (2012) investigating the effects of B toxicity on *Arabidopsis thaliana* demonstrated that there were cellular changes in the root meristem, inhibiting root growth.

EBR had positive effects on leaf anatomy (ETAd, ETAb, PPT, and SPT). These results evidenced the ability of this

Table 3 Nutrient contents in soybean plants sprayed with EBR and exposed to different B supplies

EBR	B supply	K (mg g DM ⁻¹)	Ca (mg g DM ⁻¹)	S (mg g DM ⁻¹)	Cu (μg g DM ⁻¹)	Mn (μg g DM ⁻¹)	Mo (μg g DM ⁻¹)
Contents in root							
-	Low	24.26 ± 0.72 ^{Cb}	8.38 ± 0.09 ^{Ba}	2.25 ± 0.08 ^{Cb}	5.70 ± 0.37 ^{Cb}	281.30 ± 8.59 ^{Bb}	4.93 ± 0.18 ^{Ca}
-	Control	29.86 ± 0.93 ^{Ab}	11.01 ± 0.68 ^{Ab}	3.00 ± 0.13 ^{Ab}	8.80 ± 0.06 ^{Ab}	326.67 ± 5.67 ^{Ab}	5.38 ± 0.19 ^{Ab}
-	High	26.81 ± 0.51 ^{Bb}	10.29 ± 0.20 ^{Aa}	2.41 ± 0.07 ^{Bb}	8.28 ± 0.07 ^{Bb}	259.66 ± 12.83 ^{Cb}	5.15 ± 0.10 ^{Bb}
+	Low	29.43 ± 0.64 ^{Ba}	8.44 ± 0.31 ^{Ca}	2.60 ± 0.06 ^{Ca}	7.58 ± 0.17 ^{Ca}	328.21 ± 11.96 ^{Ba}	5.09 ± 0.12 ^{Ca}
+	Control	34.90 ± 1.14 ^{Aa}	12.53 ± 0.73 ^{Aa}	3.71 ± 0.11 ^{Aa}	9.58 ± 0.11 ^{Aa}	349.21 ± 6.68 ^{Aa}	5.75 ± 0.11 ^{Aa}
+	High	28.94 ± 1.12 ^{Ba}	10.10 ± 0.84 ^{Ba}	2.79 ± 0.11 ^{Ba}	8.68 ± 0.19 ^{Ba}	323.95 ± 14.56 ^{Ba}	5.41 ± 0.19 ^{Ba}
Contents in stem							
-	Low	46.33 ± 0.74 ^{Cb}	8.50 ± 0.09 ^{Ca}	1.23 ± 0.20 ^{Bb}	0.84 ± 0.03 ^{Cb}	9.97 ± 0.51 ^{Ba}	4.44 ± 0.20 ^{Cb}
-	Control	55.12 ± 0.54 ^{Ab}	10.41 ± 0.34 ^{Aa}	1.63 ± 0.08 ^{Aa}	1.21 ± 0.06 ^{Ab}	14.53 ± 0.77 ^{Ab}	5.56 ± 0.11 ^{Ab}
-	High	52.30 ± 1.38 ^{Bb}	8.97 ± 0.27 ^{Bb}	1.37 ± 0.05 ^{Ba}	1.11 ± 0.04 ^{Bb}	13.81 ± 0.46 ^{Ab}	5.13 ± 0.10 ^{Bb}
+	Low	48.93 ± 1.11 ^{Ca}	8.71 ± 0.15 ^{Ca}	1.49 ± 0.08 ^{Ba}	1.05 ± 0.05 ^{Ca}	10.05 ± 0.34 ^{Ca}	5.39 ± 0.14 ^{Ca}
+	Control	57.41 ± 1.83 ^{Aa}	10.54 ± 0.10 ^{Aa}	1.71 ± 0.09 ^{Aa}	1.35 ± 0.05 ^{Aa}	19.34 ± 0.83 ^{Aa}	6.07 ± 0.14 ^{Aa}
+	High	55.15 ± 1.32 ^{Ba}	9.59 ± 0.13 ^{Ba}	1.49 ± 0.03 ^{Ba}	1.27 ± 0.05 ^{Ba}	14.86 ± 0.46 ^{Ba}	5.69 ± 0.06 ^{Ba}
Contents in leaf							
-	Low	22.05 ± 0.85 ^{Cb}	8.09 ± 0.16 ^{Cb}	2.37 ± 0.09 ^{Cb}	0.88 ± 0.04 ^{Bb}	25.04 ± 0.43 ^{Cb}	2.92 ± 0.07 ^{Cb}
-	Control	27.23 ± 1.38 ^{Ab}	10.06 ± 0.45 ^{Ab}	2.81 ± 0.12 ^{Ab}	1.27 ± 0.08 ^{Ab}	32.83 ± 0.83 ^{Ab}	4.04 ± 0.11 ^{Ab}
-	High	24.94 ± 0.63 ^{Bb}	9.56 ± 0.25 ^{Bb}	2.62 ± 0.08 ^{Bb}	1.19 ± 0.08 ^{Ab}	31.53 ± 0.48 ^{Bb}	3.85 ± 0.10 ^{Bb}
+	Low	23.94 ± 0.63 ^{Ca}	9.20 ± 0.95 ^{Ba}	2.60 ± 0.07 ^{Ca}	0.99 ± 0.06 ^{Ca}	26.80 ± 1.17 ^{Ca}	3.21 ± 0.14 ^{Ca}
+	Control	29.30 ± 0.68 ^{Aa}	10.70 ± 0.21 ^{Aa}	3.11 ± 0.18 ^{Aa}	1.41 ± 0.05 ^{Aa}	37.36 ± 0.74 ^{Aa}	4.76 ± 0.19 ^{Aa}
+	High	27.62 ± 1.31 ^{Ba}	10.44 ± 0.17 ^{Aa}	2.81 ± 0.12 ^{Ba}	1.30 ± 0.04 ^{Ba}	32.97 ± 0.99 ^{Ba}	4.40 ± 0.12 ^{Ba}

B, boron; EBR, 24-epibrassinolide; K, potassium; Ca, calcium; S, sulfur; Cu, copper; Mn, manganese; Mo, molybdenum. Columns with different uppercase letters between boron (B) supplies (low, control, and high B supply under equal 24-epibrassinolide (EBR) level) and lowercase letters between EBR level (with and without EBR under equal B supply) indicate significant differences from the Scott-Knott test ($P < 0.05$). Means ± SD, $n = 5$

steroid to mitigate the damage to leaf structures exposed to B toxicity. On the other hand, B deficiency interferes negatively with cell wall structures (Meriño-Gergichevich et al. 2017), causing imbalances in water relations and reductions in leaf elongation rate (Wimmer and Eichert 2013). EBR spray on plants with B deficiency, or toxicity promoted leaf integrity and anatomy (Shahbaz and Ashraf 2007). Sotiropoulos et al. (2002) studied *Actinidia deliciosa* and *Actinidia arguta* species submitted to five B treatments (20, 50, 100, 200, and 500 μM B) and observed a reduced thickness of the leaf cross-section due to the reduction of PPT and SPT.

In general, EBR treatment causes increases in macro-nutrient and micronutrient contents in low, adequate, and high B supplies because EBR is efficient in regulating the absorption of ions in the plant cell (Khrpach 2000; Shahbaz and Ashraf 2007). B modulates the secretory activities in the membranes, exerting influence on proton extrusion and electric potential generation, which is essential for ATPase enzyme activities, causing membrane hyperpolarization and consequent stimulation in the absorption of K and Ca ions (Ahmad et al. 2009; Brown et al. 2002). In plants under B deficiency and toxicity, the membrane properties linked to nutrient uptake often modified, explaining the reductions in nutritional contents (K, Ca, S, Cu, Mn, and Mo). Davis et al.

(2003), evaluating the responses of tomato plants under the soil and leaf application of B, observed increases in K and Ca contents in the shoot, similar to our results.

EBR spray in plants under B stress (low and high supplies) increased the photosynthetic pigments (Chl *a*, Chl *b*, Total Chl, and Car), suggesting that the EBR helped to pigment membrane damages, which was confirmed by the reductions of MDA and EL. In other words, ROS accumulation causes oxidative damage and lipid peroxidation, producing MDA with subsequent increments of EL, signaling deleterious effects on membrane integrity (Genisel et al. 2013; Li et al. 2018; Mito et al. 2019; Sun et al. 2015; Yao et al. 2017). Similar results were found by Zhang et al. (2014) and Dong et al. (2017), where EBR spraying increased the levels of Chl *a*, Chl *b*, and Total Chl in melon plants subjected to high temperature and in wheat plants under saline stress (120 mM NaCl).

EBR application in plants exposed to different B concentrations promoted better results concerning chlorophyll fluorescence. Low and high B supplies caused inhibition of the processes linked to the electron transport chain. However, our results revealed that the EBR treatment was able to increase F_m , F_v , and F_v/F_m values and reduce F_0 , demonstrating beneficial effects on photochemical reactions of

Table 4 Photosynthetic pigments, chlorophyll fluorescence, and gas exchange in soybean plants sprayed with EBR and exposed to different B supplies

EBR	B supply	Chl <i>a</i> (mg g ⁻¹ FM)	Chl <i>b</i> (mg g ⁻¹ FM)	Total Chl (mg g ⁻¹ FM)	Car (mg g ⁻¹ FM)	Ratio Chl <i>a</i> /Chl <i>b</i>	Ratio Total Chl/Car
-	Low	4.99 ± 0.33 ^{Aa}	1.58 ± 0.09 ^{Ba}	6.57 ± 0.31 ^{Ba}	1.27 ± 0.08 ^{Ba}	3.16 ± 0.18 ^{Ba}	5.17 ± 0.09 ^{Ba}
-	Control	5.40 ± 0.45 ^{Ab}	1.87 ± 0.15 ^{Ab}	7.27 ± 0.42 ^{Ab}	1.45 ± 0.06 ^{Ab}	2.89 ± 0.26 ^{Ba}	5.01 ± 0.45 ^{Ba}
-	High	5.23 ± 0.50 ^{Aa}	1.47 ± 0.07 ^{Bb}	6.70 ± 0.50 ^{Bb}	1.05 ± 0.03 ^{Cb}	3.56 ± 0.20 ^{Aa}	6.38 ± 0.56 ^{Aa}
+	Low	5.00 ± 0.31 ^{Ba}	1.71 ± 0.10 ^{Ba}	6.71 ± 0.40 ^{Ca}	1.34 ± 0.07 ^{Ba}	2.92 ± 0.12 ^{Aa}	5.00 ± 0.33 ^{Ba}
+	Control	6.82 ± 0.36 ^{Aa}	2.48 ± 0.13 ^{Aa}	9.30 ± 0.28 ^{Aa}	1.84 ± 0.14 ^{Aa}	2.75 ± 0.27 ^{Aa}	5.05 ± 0.37 ^{Ba}
+	High	5.51 ± 0.32 ^{Ba}	1.82 ± 0.02 ^{Ba}	7.33 ± 0.33 ^{Ba}	1.22 ± 0.10 ^{Ca}	3.03 ± 0.17 ^{Ab}	6.01 ± 0.36 ^{Aa}
EBR	B supply	Φ _{PSII}	q _p	NPQ	ETR (μmol m ⁻² s ⁻¹)	EXC (μmol m ⁻² s ⁻¹)	ETR/P _N
-	Low	0.29 ± 0.00 ^{Bb}	0.69 ± 0.05 ^{Ba}	1.07 ± 0.07 ^{Aa}	42.9 ± 0.6 ^{Bb}	0.62 ± 0.01 ^{Aa}	10.78 ± 0.63 ^{Aa}
-	Control	0.33 ± 0.01 ^{Ab}	0.77 ± 0.02 ^{Ab}	0.97 ± 0.06 ^{Ba}	48.5 ± 1.6 ^{Ab}	0.57 ± 0.02 ^{Ba}	5.16 ± 0.26 ^{Ba}
-	High	0.24 ± 0.01 ^{Cb}	0.74 ± 0.03 ^{Aa}	0.98 ± 0.06 ^{Ba}	35.5 ± 1.2 ^{Cb}	0.64 ± 0.02 ^{Aa}	5.37 ± 0.29 ^{Ba}
+	Low	0.35 ± 0.01 ^{Ba}	0.73 ± 0.03 ^{Ba}	0.97 ± 0.04 ^{Ab}	51.1 ± 2.0 ^{Ba}	0.56 ± 0.02 ^{Bb}	7.30 ± 0.49 ^{Ab}
+	Control	0.37 ± 0.01 ^{Aa}	0.85 ± 0.06 ^{Aa}	0.92 ± 0.06 ^{Aa}	54.8 ± 1.3 ^{Aa}	0.54 ± 0.01 ^{Bb}	4.79 ± 0.28 ^{Ba}
+	High	0.27 ± 0.01 ^{Ca}	0.69 ± 0.04 ^{Ba}	1.00 ± 0.04 ^{Aa}	40.3 ± 1.8 ^{Ca}	0.60 ± 0.03 ^{Ab}	4.91 ± 0.22 ^{Ba}
EBR	B supply	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 (μmolmmol ⁻¹)	P _N /C _i (μmol m ⁻² s ⁻¹ Pa ⁻¹)
-	Low	3.9 ± 0.2 ^{Cb}	1.01 ± 0.05 ^{Cb}	0.03 ± 0.01 ^{Bb}	306 ± 25 ^{Aa}	3.9 ± 0.3 ^{Aa}	0.013 ± 0.001 ^{Cb}
-	Control	9.4 ± 0.7 ^{Ab}	2.32 ± 0.14 ^{Ba}	0.11 ± 0.01 ^{Aa}	227 ± 12 ^{Ba}	4.0 ± 0.2 ^{Ab}	0.042 ± 0.002 ^{Ab}
-	High	6.6 ± 0.4 ^{Bb}	2.71 ± 0.17 ^{Aa}	0.12 ± 0.01 ^{Aa}	241 ± 14 ^{Ba}	2.4 ± 0.1 ^{Bb}	0.028 ± 0.001 ^{Bb}
+	Low	7.0 ± 0.5 ^{Ca}	1.79 ± 0.07 ^{Ba}	0.07 ± 0.01 ^{Ca}	246 ± 18 ^{Ab}	3.9 ± 0.2 ^{Ba}	0.029 ± 0.001 ^{Ca}
+	Control	11.4 ± 0.5 ^{Aa}	1.83 ± 0.08 ^{Bb}	0.09 ± 0.01 ^{Bb}	220 ± 16 ^{Aa}	6.2 ± 0.4 ^{Aa}	0.052 ± 0.002 ^{Aa}
+	High	8.2 ± 0.4 ^{Ba}	2.61 ± 0.66 ^{Aa}	0.11 ± 0.01 ^{Aa}	235 ± 7 ^{Aa}	3.3 ± 0.2 ^{Ba}	0.035 ± 0.001 ^{Ba}

B, boron; 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 CO₂ concentration; WUE, water-use efficiency; P_N/C_i, carboxylation instantaneous efficiency. Columns with different uppercase letters between B supplies (low, control, and high B supply under equal EBR level) and lowercase letters between EBR level (with and without EBR under equal B supply) indicate significant differences from the Scott-Knott test (*P* < 0.05). Means ± SD, *n* = 5

PSII, with greater protection to the photosynthetic apparatus (Zhang et al. 2013). Additionally, lower *F*₀ values associated with increased *F*_m in plants exposed to EBR application indicate a positive action of this steroid over the light-harvesting complex (Melo et al. 2017). Wang et al. (2009), evaluating maize plants exposed to 550 mg Mn kg⁻¹ soil combined with 0.1 mg L⁻¹ EBR found increases in *F*_v/*F*_m, compared with equal treatment without EBR. Lima and Lobato (2017), investigating cowpea plants sprayed with 100 nM EBR and submitted to water deficit, reported an increase in *F*_m (32%) and reduction in *F*₀ (15%), respectively, similar to results found in this research.

EBR promoted increases in Φ_{PSII}, q_p, and ETR values and reductions in NPQ, EXC, and ETR/P_N in plants exposed to high and low B supplies. Increases in Φ_{PSII} values indicate that EBR application improved energy capture efficiency and increased the proportion of open reaction centers in the PSII (q_p) (Yu et al. 2004). Higher q_p values are associated with increased capture capacity of the PSII electron-acceptor

molecule (plastoquinone) frequently associated with reducing power and ATP consumption and avoiding photoinhibition (Khamsuk et al. 2018). Additionally, reductions in NPQ values in low B + EBR-treated plants demonstrate the protective effect of this steroid on PSII to excess energy, alleviating damage to thylakoid membranes (Wu et al. 2014). Thussagunpanit et al. (2015) studied rice plants under high-temperature conditions, and the application of EBR (1 nM) also promoted increases in Φ_{PSII}, q_p, and ETR, in agreement with our results. Shu et al. (2016), evaluating tomato seedlings under low irradiance (180 μmol m⁻² s⁻¹) and low temperature (15 °C day/7 °C night), observed beneficial effects of exogenous EBR application (0.1 μmol L⁻¹) on the parameters of Φ_{PSII} and q_p, indicating that EBR alleviated the photoinhibition caused by the simulated stress.

An increase in ETR and a decrease in EXC in response to EBR application indicated that this steroid improved the electron transport in PSII. Reductions in ETR/P_N of EBR-treated plants indicate an increase in carboxylation

Fig. 2 Minimal fluorescence yield of the dark-adapted state F_0 (A), maximal fluorescence yield of the dark-adapted state F_m (B), variable fluorescence F_v (C), and maximal quantum yield of PSII photochemistry F_v/F_m (D) in soybean plants sprayed with 24-epibrassinolide (EBR) and exposed to different boron (B) supplies. Columns with different uppercase letters between B supplies (low, control, and high B supply under equal EBR level) and lowercase letters between EBR level (with and without EBR under equal B supply) indicate significant differences from the Scott-Knott test ($P < 0.05$). Means \pm SD, $n = 5$

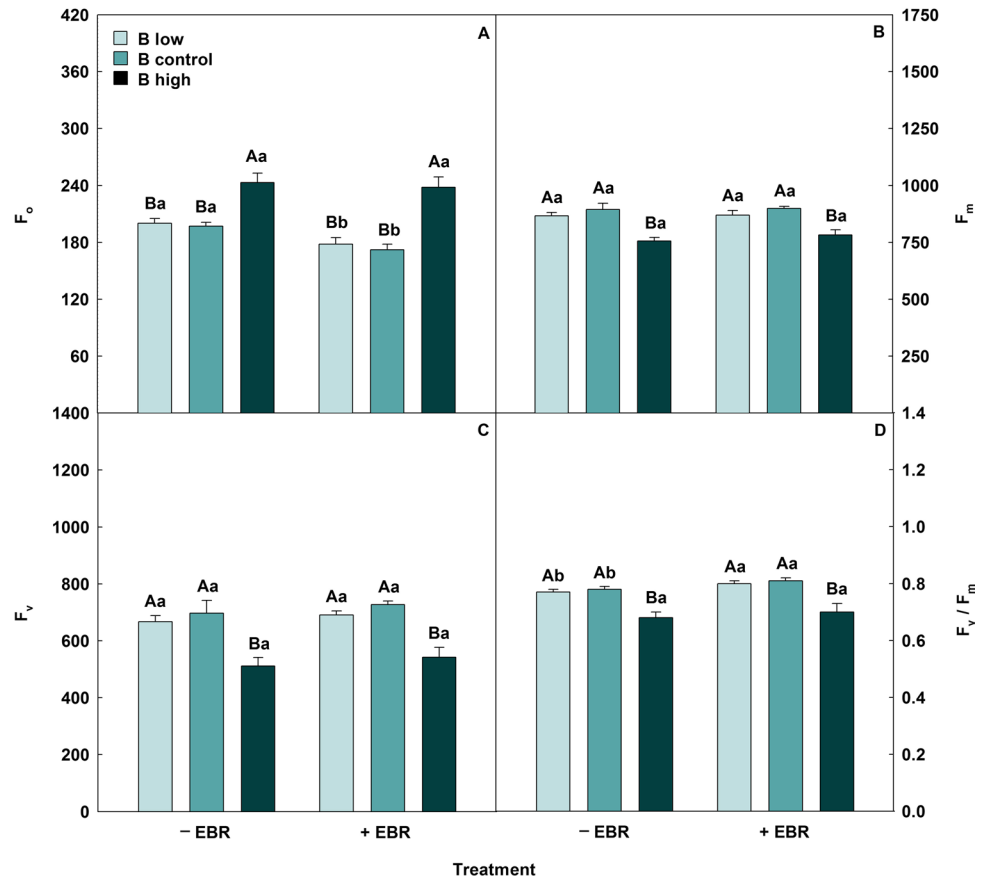


Table 5 Stomatal characteristics in soybean plants sprayed with EBR and exposed to different B supplies

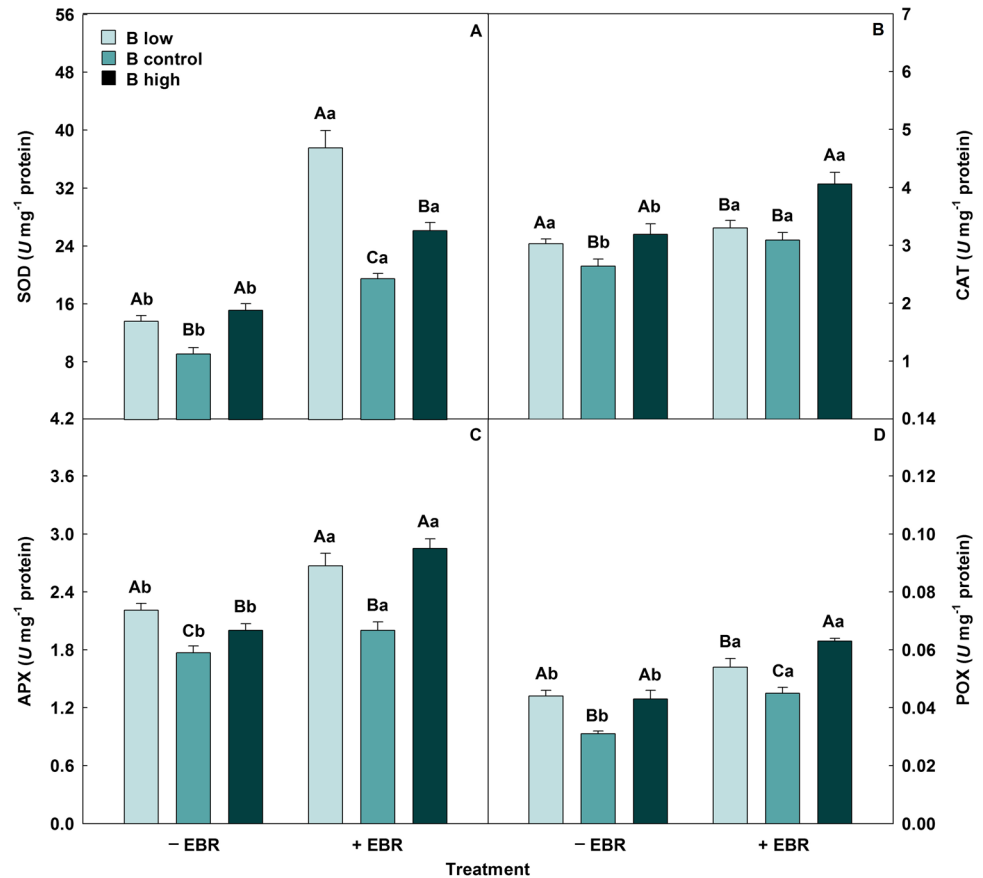
EBR	B supply	SD (stomata per mm ²)	PDS (μ m)	EDS (μ m)	SF	SI (%)
Adaxial face						
-	Low	71 \pm 1 ^{Cb}	8.9 \pm 0.5 ^{Ba}	20.9 \pm 1.5 ^{Ba}	0.43 \pm 0.03 ^{Cb}	3.6 \pm 0.2 ^{Cb}
-	Control	92 \pm 3 ^{Ab}	8.6 \pm 0.5 ^{Ba}	18.1 \pm 1.1 ^{Ca}	0.47 \pm 0.02 ^{Aa}	4.9 \pm 0.2 ^{Ab}
-	High	78 \pm 3 ^{Bb}	9.9 \pm 0.4 ^{Aa}	22.3 \pm 1.5 ^{Aa}	0.44 \pm 0.02 ^{Bb}	4.0 \pm 0.1 ^{Bb}
+	Low	92 \pm 3 ^{Ca}	8.4 \pm 0.3 ^{Bb}	18.9 \pm 0.8 ^{Ab}	0.44 \pm 0.02 ^{Ba}	5.1 \pm 0.3 ^{Ba}
+	Control	100 \pm 4 ^{Aa}	8.3 \pm 0.1 ^{Ba}	17.5 \pm 1.1 ^{Ba}	0.47 \pm 0.02 ^{Aa}	5.8 \pm 0.3 ^{Aa}
+	High	96 \pm 3 ^{Ba}	8.8 \pm 0.3 ^{Ab}	18.9 \pm 0.7 ^{Ab}	0.46 \pm 0.02 ^{Aa}	5.1 \pm 0.4 ^{Ba}
Abaxial face						
-	Low	257 \pm 15 ^{Bb}	11.2 \pm 0.7 ^{Aa}	21.3 \pm 1.6 ^{Aa}	0.53 \pm 0.03 ^{Aa}	10.2 \pm 0.7 ^{Bb}
-	Control	292 \pm 8 ^{Ab}	9.7 \pm 0.5 ^{Ba}	18.1 \pm 0.9 ^{Ba}	0.54 \pm 0.03 ^{Aa}	12.7 \pm 0.8 ^{Ab}
-	High	242 \pm 13 ^{Cb}	11.0 \pm 0.5 ^{Aa}	20.3 \pm 1.4 ^{Aa}	0.54 \pm 0.03 ^{Aa}	9.7 \pm 0.5 ^{Bb}
+	Low	292 \pm 8 ^{Ba}	9.7 \pm 0.4 ^{Ab}	18.8 \pm 1.0 ^{Ab}	0.51 \pm 0.03 ^{Ba}	12.8 \pm 0.5 ^{Ba}
+	Control	307 \pm 20 ^{Aa}	9.5 \pm 0.5 ^{Aa}	17.1 \pm 0.8 ^{Ba}	0.55 \pm 0.02 ^{Aa}	13.7 \pm 1.0 ^{Aa}
+	High	278 \pm 15 ^{Ca}	9.6 \pm 0.4 ^{Ab}	17.8 \pm 0.8 ^{Bb}	0.54 \pm 0.03 ^{Aa}	13.1 \pm 0.8 ^{Ba}

B, boron; 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 uppercase letters between B supplies (low, control, and high B supply under equal EBR level) and lowercase letters between EBR level (with and without EBR under equal B supply) indicate significant differences from the Scott-Knott test ($P < 0.05$). Means \pm SD, $n = 5$

efficiency and probably lower carbon losses by photorespiration (Ahmed et al. 2013a; Pereira et al. 2019). Increases in ETR and Φ_{PSII} were found by Dobrikova et al. (2014),

investigating the effects of three EBR concentrations (0.01, 0.1, and 1 mg L⁻¹) on the membrane structures of thylakoids in pea plants, obtaining increases of 25% and 35% in ETR

Fig. 3 Activities of superoxide dismutase SOD (A), catalase CAT (B), ascorbate peroxidase APX (C), and peroxidase POX (D) in soybean plants sprayed with 24-epibrassinolide (EBR) and exposed to different boron (B) supplies. Columns with different uppercase letters between B supplies (low, control, and high B supply under equal EBR level) and lowercase letters between EBR level (with and without EBR under equal B supply) indicate significant differences from the Scott-Knott test ($P < 0.05$). Means \pm SD, $n = 5$

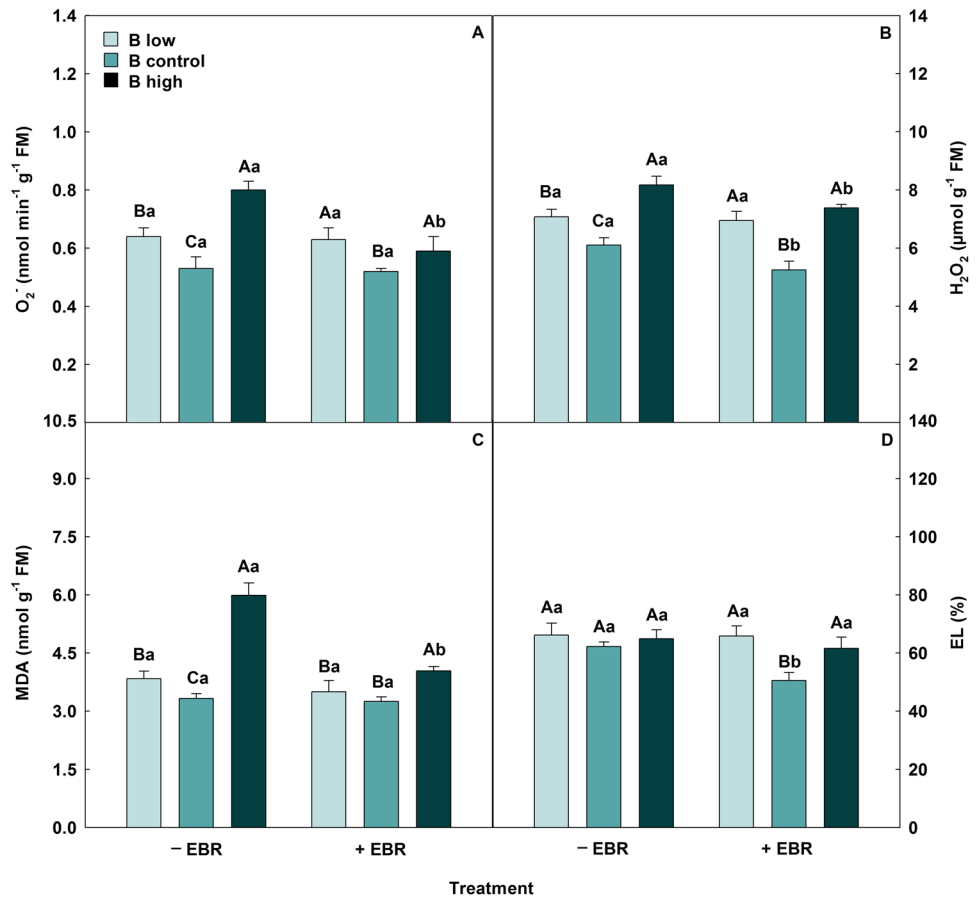


under 0.01 and 0.1 mg L⁻¹ EBR, respectively, when compared to the control treatment.

EBR application mitigated the effects of low and high B treatments on gas exchange. Higher values of P_N , E , g_s , and P_N/C_i under low B conditions and increases in P_N , WUE, and P_N/C_i in high B treatment are associated with positive actions of the EBR on photochemical and diffusional aspects found in this study. Additionally, there is a linear relationship between the ability to capture, utilize, and dissipate light and CO₂ uptake (Wong et al. 2012). We detected increases in F_v/F_m , Φ_{PSII} , and ETR values proving higher photosynthetic efficiency despite low and high B concentrations. Parallely, increases in P_N can be associated with lower C_i values, resulting in higher P_N/C_i , suggesting a higher activity of Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and, consequently, increases in CO₂ uptake in the Calvin-Benson cycle. Increases in g_s values combined with higher SD, SI, and SF and lower PDS and EDS suggested higher photosynthetic capacity in EBR-treated plants due to more uniform CO₂ diffusion to Rubisco sites (Tanaka et al. 2013). Our results are consistent with the findings by Hu et al. (2013), evaluating pepper plants, being found significant increases in P_N , g_s , and E and a decrease in C_i in plants under water deficit and treated with EBR (0.01 mg L⁻¹), compared to plants without steroid.

Plants submitted to low and high B in association with the application of EBR presented a better performance concerning stomatal characteristics than plants not treated with EBR. Increases in stomatal density (SD), stomatal functionality (SF), and stomatal index (SI) on the adaxial face of leaves under the influence of EBR demonstrate that this steroid acts on stomatal development, activating proteins involved in the signaling pathway of the stomatal mechanism (Lin et al. 2013). Changes in stomatal, such as shape, size, and quantity, are efficient strategies of plants to cope with stressful conditions because the increase in the density and reduction in the size of the stomata contribute to a higher diffusion of CO₂ to the carboxylation sites and increases in WUE (Devi and Reddy 2018; Franks and Beerling 2009). Our results show that EBR played an important role against the effects of low and high B, contributing to the increases in stomata quantity and efficiency (SD and SI) while reducing the size (smaller PDS and EDS) and positively reflecting on the P_N values (Zhao et al. 2015). Increases in SD were observed in grape leaves subjected to water deficit and exogenous EBR applications (0.05, 0.10, and 0.20 mg L⁻¹), promoting increases of 43%, 15%, and 53%, respectively, compared to plants without EBR (Wang et al. 2015). Oliveira et al. (2018) observed positive effects of 50 nM EBR against saline

Fig. 4 Superoxide O_2^- (A), hydrogen peroxide H_2O_2 (B), malondialdehyde MDA (C), and electrolyte leakage EL (D) in soybean plants sprayed with 24-epibrassinolide (EBR) and exposed to different boron (B) supplies. Columns with different uppercase letters between B supplies (low, control, and high B supply under equal EBR level) and lowercase letters between EBR level (with and without EBR under equal B supply) indicate significant differences from the Scott-Knott test ($P < 0.05$). Means \pm SD, $n = 5$



stress in young *Eucalyptus urophylla* plants, with increases of 23%, 7%, and 24% in SD, SF, and SI, respectively, and reductions of 8% and 10% in PDS and EDS, in this order, compared to stress treatment without EBR, being similar to our results.

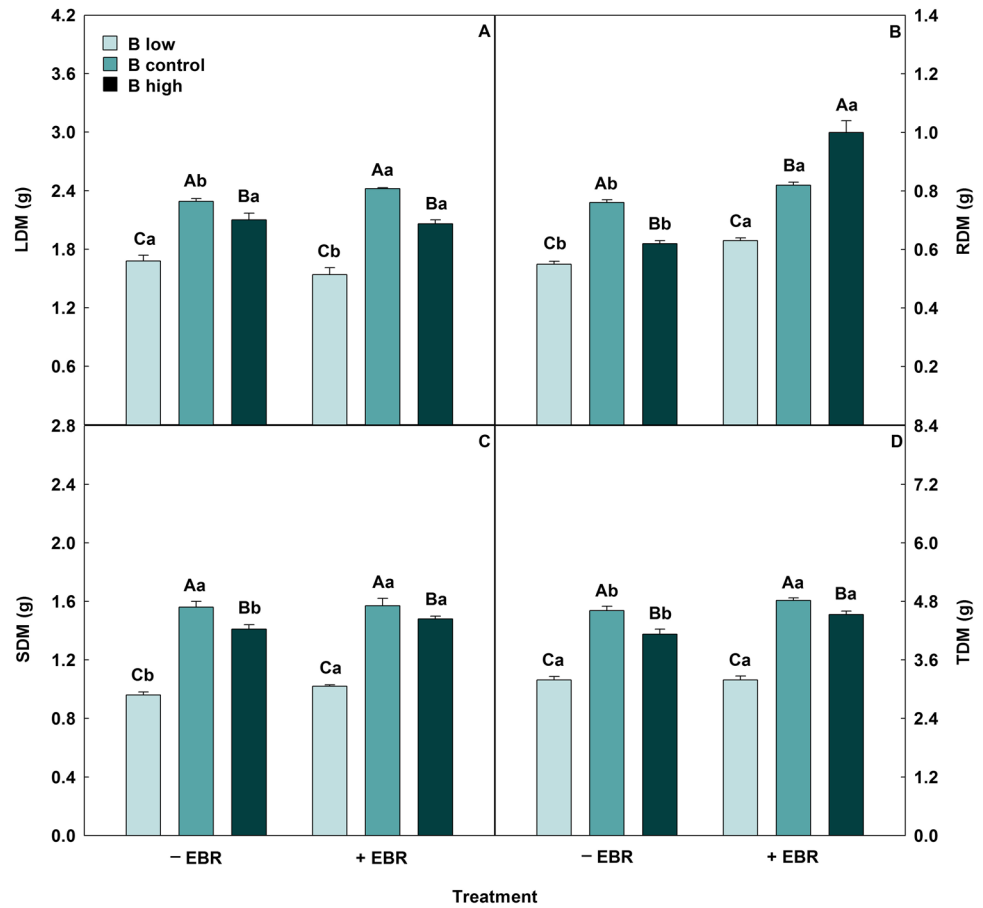
Plants treated with EBR under low and high B supplies had increases in the activities of antioxidant enzymes (SOD, CAT, APX, and POX), indicating beneficial interference of the EBR on the antioxidant defense system. These results are related to the increases in F_v/F_m and ETR because the maximization of the photosynthetic potential reduces the availability of NADPH and ATP (Zhou et al. 2004), which are consumed in the Calvin cycle, facilitating the transport of electrons and reducing the possibility of ROS formation (Ogweno et al. 2008). Li et al. (2015) studying the antioxidant system in pepper seedlings submitted to two thermal regimes (28/18 °C and 15/5 °C) and spray with EBR (0.1 μ M) showed significant increases of 29%, 31%, 25%, and 9% after the EBR application for SOD, POX, CAT, and APX, respectively. Surgun et al. (2016) analyzed the EBR effects on the B-tolerance mechanisms in *Arabidopsis thaliana* plants submitted to three B concentrations (0, 0.80, and 1.60 mM) combined with two EBR levels (0.01, and 1 mM) and described that the application of EBR maximized the

activities linked to antioxidant enzymes (SOD, CAT, APX, and POX).

The stress promoted by the high B stress-induced increases in O_2^- , H_2O_2 , MDA, and EL, but these effects were reduced after the application of EBR. EBR spray increased the activities of antioxidant enzymes, as previously detected. The antioxidant enzymes act in the neutralization of the ROS (Dalyan et al. 2018; Zhang et al. 2008), with the SOD enzyme acting at the beginning of the defense process, catalyzing the conversion of O_2^- to H_2O_2 , which is subsequently degraded by the CAT, APX, and POX enzymes (Zhou et al. 2018). Ahammed et al. (2013b), working with tomato plants exposed to EBR and three concentrations of polychlorinated biphenyls (0.4, 2.0, and 10.0 μ g L $^{-1}$), reported decreases in O_2^- , H_2O_2 , and MDA levels, similar to the results found in this research. Ogwenno et al. (2008) investigated the photosynthetic efficiency and oxidative stress in tomato plants under two temperature conditions, normal (25/18 °C) and high (40/30 °C), and three EBR concentrations (0.01, 0.1, 1.0 mg L $^{-1}$), reporting reductions in H_2O_2 and MDA levels in plants treated with EBR.

Plants subjected to deficiency or toxicity of B when sprayed with EBR had increased related to growth (LDM, RDM, SDM, and TDM). These responses can be attributed

Fig. 5 Leaf dry matter LDM (A), root dry matter RDM (B), stem dry matter SDM (C), and total dry matter TDM (D) in soybean plants sprayed with 24-epibrassinolide (EBR) and exposed to different boron (B) supplies. Columns with different uppercase letters between B supplies (low, control, and high B supply under equal EBR level) and lowercase letters between EBR level (with and without EBR under equal B supply) indicate significant differences from the Scott-Knott test ($P < 0.05$). Means \pm SD, $n = 5$



to the beneficial roles of EBR on anatomical responses, chlorophyll fluorescence, and gas exchange, evidenced by the increases in mesophyll cells (palisade parenchyma and spongy parenchyma), Φ_{PSII} , ETR, and P_N , as presented in this study. The application of EBR also favored increases in photosynthetic pigments and gas exchange, positively influencing the biomass (Xie et al. 2011; Naz et al. (2015); Hayat et al. (2011) studied tomato plants under the three concentrations of EBR (10^{-6} , 10^{-8} , 10^{-10} M) and the three concentrations of HBL (10^{-6} , 10^{-8} , 10^{-10} M) and reported that plants treated with EBR had more intense effects on length, fresh matter, dry matter, and foliar area when compared to plants treated with HBL. A study conducted by Liu et al. (2018) analyzing the B toxicity in *Puccinellia tenuiflora* seedlings submitted to three different B levels described reductions of 69% and 40% in root dry matter and shoot dry matter, respectively, in plants treated with high B.

5 Conclusions

EBR pretreatment promoted improvements in leaf and root structures, also inducing increases in biomass accumulation. EBR alleviated the damages occasioned by the inadequate

B supplies on root tissues, more specifically maximizing the vascular cylinder, metaxylem, and epidermis, improving the nutritional status. This steroid also minimized the harmful effects of B stress on leaf anatomy, stimulating the epidermis on both leaf sides, palisade parenchyma, and spongy parenchyma; both structures are intrinsically related to protection and CO_2 availability to the photosynthetic process. Concomitantly, this steroid had a positive impact on biomass accumulation. These results are explained by beneficial actions on leaf structures and photosynthetic machinery. Therefore, our results demonstrate that the EBR application can improve soybean plants' tolerance under inadequate B supplementation.

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Author Contribution AKSL was the advisor for this project, planned all phases of the research, and critically revised the manuscript. YCP, ANSN, BTSA, BRSS, and MAMB conducted the experiments,

performed physiological, biochemical, anatomical, and morphological determinations, and wrote and edited the manuscript. BLB carried out the nutritional determinations and critically revised the manuscript. AB critically revised the manuscript. All authors read and approved the final version of the manuscript.

Declarations

Conflict of Interest The authors declare no competing interests.

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Exogenous 24-epibrassinolide reverses disturbances in zinc-stressed tomato by synergistically stimulating leaf structures, photosynthesis and growth



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ABSTRACT

Zinc (Zn) is the most abundant and important transition metal in plants, involved in the metabolism of nucleic acids, carbohydrates, proteins, and lipids. Inadequate Zn supplementation negatively affects leaf development and photosynthetic performance, impairing plant growth. However, recent research has demonstrated the potential of 24-epibrassinolide (EBR) to mitigate the effects of abiotic stresses. This study aims to verify whether exogenous application of EBR can alleviate stress caused by Zn in tomato plants, evaluating the possible repercussions on leaf structures, photosynthesis, and growth. The experiment followed a completely randomized factorial design with 100 nM EBR and Zn supplies (0.006, 3, and 1500 μM Zn, described as low, control, and high supply of Zn). Exogenous EBR promoted benefits in leaf anatomy, with increments in epidermis from the adaxial leaf side, palisade parenchyma and spongy parenchyma of 28%, 12%, and 38% (low Zn) and 12%, 11% and 30% (high Zn), respectively. This organic molecule increased the stomatal density and effective quantum yield of photosystem II photochemistry, resulting in a higher net photosynthetic rate. EBR attenuated the harmful effects of low and high Zn supplies on biomass. Therefore, this research proves that EBR application could partially reverse disturbances in zinc-stressed plants, synergistically stimulating leaf structures, photosynthesis and growth.

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1. Introduction

Zinc (Zn) is the most abundant and important transition metal in plants (Zlobin et al., 2019). Zn is an essential micronutrient in the metabolism of nucleic acids, carbohydrates, proteins, and lipids (Bashir et al., 2012; Yaghoobian et al., 2021). Additionally, Zn plays a central role in all living cells as a cofactor for enzymes and structural element that allows the proper folding of proteins (Cabot et al., 2019; Kühnlenz et al., 2016; Lanquar et al., 2014). This element also constitutes several enzymes, including RNA polymerase, alkaline phosphatase, alcohol dehydrogenase, Cu–Zn superoxide dismutase, and carbonic anhydrase (Gupta et al., 2016). In addition, Zn finger proteins (ZFPs) are also involved in plant growth regulation and responses to biotic and abiotic stresses (Mapodzeke et al., 2021). The

optimal level of Zn in the soil required for adequate plant growth varies between 25 and 150 mg kg^{-1} (Eisalou et al., 2021). Most plants contain between 30 and 100 $\mu\text{g Zn g}^{-1}$ dry matter, and concentrations above 300 $\mu\text{g Zn g}^{-1}$ DM are generally toxic (Lin et al., 2016). In soil, Zn can be present in different forms, mainly as a free ion (Zn^{2+} e ZnOH^+) or complexed in organic matter and a colloidal fraction (ZnSO_4 and ZnHPO_4) (Balafrej et al., 2020). Zn availability can be influenced by CaCO_3 content, pH, phosphate status, general Zn content, organic matter, Fe/Mn oxide content, phosphorus availability, sodium content, and high exchangeable magnesium/calcium ratio (Duplay et al., 2014; Zeng et al., 2021).

System agriculture with a deficiency in Zn supply to crops and negligent management can lead to Zn scarcity in soils, which could induce Zn deficits in staple foods and cause human Zn malnutrition (Liedschulte et al., 2021). Low concentrations of bioavailable Zn in soil limit agricultural production to about 40% of cultivated land (Sinclair et al., 2018). Zn deficiency has several negative effects on

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plants, such as reduced internode length, branch death, and intervening chlorosis and reduced biomass, mainly in a shoot (Sadeghzadeh, 2013). Low Zn causes oxidative stress due to the excessive production of reactive oxygen species (ROS), such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), damaging chloroplast and mitochondria (Tewari et al., 2019). On the other hand, toxic levels of Zn in soils are associated with anthropogenic action, more specifically the environmental pollution or heavy use of agricultural inputs, such as fertilizers and pesticides (Kühnlenz et al., 2016). In plants, Zn excess causes morphological, biochemical, and physiological disturbances, impairing plant growth and development (Balafrej et al., 2020). At the cellular level, Zn toxicity can trigger nucleolar stress, such as morphological changes and reduction in the nucleolar area (Carvalho et al., 2019). Parallely, excessive contents of this element in leaf often result in inhibition of photosystem I and II activities, lower ribulose-1,5-biphosphate-carboxylase/oxygenase (RuBisCO) activity during carbon fixation, ROS accumulation, and nutritional imbalance (Ghori et al., 2019; Kaur and Garg, 2021; Malandrakis et al., 2021; Oliveira et al., 2021). Eisalou et al. (2021) detected that the damage caused by Zn toxicity in roots is more intense than in other plant organs, described as the roots being the first organ to have contact with Zn. Castro et al. (2021), evaluating different varieties of *Vitis vinifera* under high Zn supplementation, reported that excess Zn resulted in osmotic stress and inhibition of root elongation, combined with negative changes in root and leaf nucleolar activity.

Inadequate Zn supplementation negatively impacts leaf development and photosynthetic performance, impairing plant growth (Feigl et al., 2015; Sadeghzadeh, 2013). In other words, Zn deficiency reduces photosynthesis by causing a decrease in chloroplastic pigments, inhibiting PS II light-gathering activity, damaging the photosynthetic apparatus, and causing chloroplast disintegration (Fu et al., 2015). Zn plays an important role in the development and function of the chloroplast, as in the Zn-dependent activity of SPP peptidase and the photosystem II repair process, turning into a photodamaged D1 protein (Hänsch and Mendel, 2009). To Hafeez et al. (2013), low Zn supplementation stunts plant growth, decreasing the number of tillers and favoring the presence of chlorosis and smaller leaves.

At the same time, Zn toxicity induces the inactivation of part of the photosystem II reaction centers and impairs electron transport (Paunov et al., 2018). Todeschini et al. (2011) reported that excess zinc also alters the morphology and ultrastructure of the leaves and negatively affects PSII activity. The level of Zn toxicity in plants varies depending on the plant genotype and various climatic and soil factors (Song et al., 2011). For Andrejić et al. (2018), plants with high amounts of Zn in the leaves show significant reductions in photosynthetic rate, transpiration rate, stomatal conductance, intercellular CO_2 concentrations, and chlorophyll *a* and *b* levels. In parallel, studies show that plants with high Zn content have lower chlorophyll and biomass accumulation (Kaya et al., 2009).

Brassinosteroids (BRs) are involved in several plant development processes, such as flowering, germination, cell division and growth, vascular development, photosynthesis, and senescence (Sharma and Bhardwaj 2007; Rajewska et al. 2016; Siddiqi and Husen 2021). BRs are widely distributed in the plant kingdom and are active in very low concentrations, ranging from nanomolar to micromolar (Arora et al., 2010). 24-Epibrassinolide (EBR) is the most active form of BRs, known for its high capacity to modulate the plant antioxidant system (Guedes et al., 2021). EBR application increases the activities of catalase, ascorbate peroxidase, superoxide dismutase, glutathione reductase, and the content of ascorbate and glutathione (Talarek-Karwel et al., 2019). Recent studies emphasize the multiple EBR roles, alleviating the negative impacts of abiotic and biotic stresses in plant metabolism, including extreme temperature (Chen et al., 2019; Yang et al., 2019), salinity (Oliveira et al., 2019), water deficit (Pereira et al., 2019), waterlogging (Pereira et al., 2020), fungal infection (Bibi et al., 2017), heavy metal toxicity (Talarek-Karwel et al. 2019; Saraiva et al.

2021; Yu et al. 2021), and the nutritional deficiency (Rodrigues et al., 2020).

This study aims to answer whether exogenous EBR application can alleviate stress caused by Zn in tomato plants, evaluating the possible repercussions on leaf structures, photosynthesis and growth.

2. Materials and methods

2.1. Location and climatic conditions

The experiment was performed at the Campus of Paragominas of the Universidade Federal Rural da Amazônia, Paragominas, Brazil ($2^{\circ} 55' S$, $47^{\circ} 34' W$). The study was conducted in a greenhouse with the temperature and humidity controlled. The minimum, maximum, and median temperatures were 25.5, 32.5, and 26.6 $^{\circ}C$, respectively. The relative humidity during the experimental period varied between 60% and 80%. The average maximum photosynthetic photon flux density was $890 \mu mol m^{-2} s^{-1}$, measured in the plant canopy, and photoperiod was 12 h.

2.2. Plants, containers and plant nutrition

Seeds of *Solanum lycopersicum* L. cv. Caline IPA-7 HortivaleTM were germinated using PlantmaxTM substrate. Sixteen-day-old seedlings with similar aspects and sizes were selected and placed in 1.2 L containers (0.15 m in height and 0.10 m in diameter) filled with a mixed substrate of sand and vermiculite in a 3:1 ratio. A nutritional solution described by Pereira et al. (2019) was used for plant nutrition, with ionic strength beginning at 50% (16th day) and later modified to 100% after 2 days (18th day). After this period, the nutritional solution remained at total ionic strength.

2.3. Experimental design

The experiment followed a completely randomized factorial design with two concentrations of 24-epibrassinolide (0 and 100 nM EBR, described as - EBR and + EBR, respectively) and three Zn supplies (0.006, 3 and 1500 μM Zn, described as low, control and high supply of Zn). With five replicates for each of the six treatments, 30 experimental units were used in the experiment, with one plant in each unit. EBR level was defined in agreement with Maia et al. (2018) and Zn concentrations were chosen based on research of Kaur and Garg (2021).

2.4. 24-epibrassinolide (EBR) preparation and application

Twenty-day-old young plants were sprayed with 24-epibrassinolide (EBR) or Milli-Q water (containing a proportion of ethanol equal to that used to prepare the EBR solution) at 5-d intervals until day 45. 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] (Ahamed et al., 2013).

2.5. Nutrient solution and Zn supplies

Plants received the following macro- and micronutrients contained in the nutrient solution in agreement with Pereira et al. (2019). For Zn treatments, $ZnCl_2$ was used at concentrations of 0.006 μM (low), 3 μM (control) and 1500 μM (high) applied over 12 days (days 33–45 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 45 of the experiment, physiological and morphological parameters were measured for all plants, with leaf tissue harvested for anatomical and biochemical determinations and nutritional analyses were performed using leaf, stem and root tissues.

2.6. Measurement of chlorophyll fluorescence and gas exchange

Chlorophyll fluorescence was measured in dark-adapted leaves for 30 min, 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 analyzer (model LCPro⁺; ADC BioScientific) in a chamber under constant CO₂ (twelve-g CO₂ cylinder), photosynthetically active radiation, air-flow rate and temperature conditions at 450 $\mu\text{mol mol}^{-1}\text{CO}_2$, 800 $\mu\text{mol photons m}^{-2}\text{ s}^{-1}$, 300 $\mu\text{mol s}^{-1}$ and 28 °C, respectively, between 10:00 and 12:00 h. 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).

2.7. Anatomical measurements

Samples were collected from the middle region of the 2nd leaflet inserted in the 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 LeicaTM (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 analyzed 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 concerning total epidermal cells by area. In the root samples, the root epidermis thickness (RET), root endodermis thickness (RDT), root cortex diameter (RCD), vascular cylinder diameter (VCD), and root metaxylem diameter (RMD) were measured.

2.8. Enzymatic assays and superoxide anion

Extraction was performed using 500 mg plant material (expanded leaves harvested in middle region of the plant) 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 $\times g$ 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 $\text{mg}^{-1}\text{ protein}$ (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 $\mu\text{mol H}_2\text{O}_2\text{ mg}^{-1}\text{ protein min}^{-1}$ (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 $\mu\text{mol AsA mg}^{-1}\text{ protein min}^{-1}$ (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 $\mu\text{mol tetraguaiacol mg}^{-1}\text{ protein min}^{-1}$ (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] (Eltner and Heupel, 1976). Total soluble proteins were analyzed using the methodology described by Bradford (1976).

2.9. Determining of Zn and nutrients

Milled samples (100 mg) of root, stem and leaf tissues (tissue harvested in middle region of the organ) were predigested using conical tubes (50 ml) with 2 ml of sub-boiled HNO₃. Subsequently, 8 ml of a solution containing 4 ml of H₂O₂ (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). Zn, Mg, K, Ca, Mn, Fe and Cu were determined using an inductively coupled plasma mass spectrometer (model ICP-MS 7900; Agilent). All found values agreed with certified values (NIST 1570a and NIST 1577c).

2.10. Stress indicators, chloroplastic pigments and biomass

Stress indicators were determined using 500 mg of fresh material (expanded leaves harvested in middle region of the plant) homogenized with 5 ml of 5% (w/v) trichloroacetic acid and subsequently centrifuged at 15,000 $\times g$ for 15 min at 3 °C to collect the supernatant (Wu et al., 2006). Hydrogen peroxide (H₂O₂) 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.

2.11. Data analysis

The normality of residues was verified with the Shapiro-Wilk test. Data were subjected to two-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 data analysis was performed using RTM software.

3. Results

3.1. Beneficial interaction between steroid and Zn

Low and high Zn supplies promoted an imbalance in Zn contents in the root, stem, and leaf tissues in tomato plants (Table 1). Significant interaction effects linked to EBR \times Zn supply were detected to all variables presented in Table 1. Plants under low Zn and sprayed with EBR had increases in Zn levels of 48% (leaf), 10% (stem), and 6% (root) compared with low Zn without EBR application. The increases for the control treatment and EBR spray were 16%, 29%, and 3% for

Table 1
Zn contents in tomato plants sprayed with EBR and exposed to different Zn supplies.

EBR	Zn supply	Zn in root ($\mu\text{g g DM}^{-1}$)	Zn in stem ($\mu\text{g g DM}^{-1}$)	Zn in leaf ($\mu\text{g g DM}^{-1}$)
–	Low	26.79 \pm 1.80 ^{Ba}	8.25 \pm 0.65 ^{Ba}	14.16 \pm 1.06 ^{Cb}
–	Control	28.06 \pm 0.74 ^{Ba}	8.39 \pm 0.35 ^{Bb}	18.97 \pm 0.77 ^{Bb}
–	High	3,901.35 \pm 86.28 ^{Aa}	1,099.29 \pm 40.11 ^{Aa}	1,117.70 \pm 72.53 ^{Aa}
+	Low	28.50 \pm 0.84 ^{Ba}	9.10 \pm 0.46 ^{Ca}	21.00 \pm 0.29 ^{Ba}
+	Control	28.91 \pm 0.65 ^{Ba}	10.85 \pm 0.48 ^{Ba}	22.03 \pm 0.92 ^{Ba}
+	High	2,511.17 \pm 131.79 ^{Ab}	891.88 \pm 17.85 ^{Ab}	907.05 \pm 13.25 ^{Ab}
Interaction effects				
EBR \times Zn supply (F-test)		*	*	*

EBR = 24-epibrassinolideo; Zn = zinc. Columns with different uppercase letters between Zn supplies (low, control and high Zn supply under equal EBR level) and lowercase letters between EBR level (with and without EBR under equal Zn supply) indicate significant differences from the Scott-Knott test ($P < 0.05$). Interaction effects linked to EBR \times Zn supply are described by **, * and ns, corresponding to significant at 1% probability level, significant at 5% probability level and not significant, in this order. Means \pm SD, $n = 5$.

leaf, stem, and root, respectively, compared with equal treatment without EBR. On the other hand, plants under high Zn + EBR had reductions in Zn contents of 19% (leaf), 19% (stem), and 36% (root) compared to the same treatment without EBR.

3.2. EBR synergistically protected root tissues and stimulated leaf structures

Low and high Zn supplies provided reductions in root anatomy (Table 2). To table 2, significant interaction effects linked to EBR \times Zn supply were confirmed to all variables, with exception to RET. Plants sprayed with EBR and exposed to the low Zn increased RET, RDT, RCD, VCD, and RMD (by 9%, 6%, 13%, 34% and 55%, respectively) compared to the same treatment without EBR. The control treatment with EBR presented increases in RET, RDT, RCD, VCD, and RMD (by 8%, 8%, 10%, 93%, and 98%, respectively) and under high Zn + EBR supply increases (by 6%, 6%, 11%, 75% and 73%, respectively) compared to the same treatment without EBR. Regarding leaf structures, plants exposed to low and high Zn supplies suffered negative interferences (Fig. 1 and Table 2). However, the application of EBR promoted increases in ETAd, ETAb, PPT, and SPT under low Zn (of 28%, 4%, 12% and 38%, respectively), in the control treatment (8%, 7%, 9%, and 28%,

respectively), and under high Zn (of 11%, 5%, 11%, and 30%, respectively). Regarding PPT/SPT, EBR induced reductions of 19%, 14%, and 12% in the plants submitted to low, control, and high Zn treatments, respectively, compared to the same treatment without EBR.

3.3. Pre-treatment with EBR in plants under low and high Zn supplies improved nutritional balance

Plants exposed to low and high Zn suffered reductions in the contents of all evaluated nutrients (root, stem and leaf) (Table 3), being found significant interaction effects linked to EBR \times Zn supply in all variables studied. However, the exogenous EBR application increased the nutrient contents in these tissues. Compared to the same treatment without EBR, plants treated with EBR + low Zn (root) presented increases of 20%, 7%, 40%, 4%, 12%, and 2% for Mg, K, Ca, Mn, Fe, and Cu, respectively (Table 3); for the control with EBR (root), the increases were 7%, 11%, 28%, 1%, 3%, and 11%, respectively; and in the high Zn + EBR (root) treatment, the increases were by 12%, 9%, 51%, 7%, 22% and 12%, respectively. Compared to equal treatment without EBR, we detected increases in Mg, K, Ca, Mn, Fe, and Cu values for the treatment with low Zn + EBR (leaf) (17%, 6%, 4%, 41%, 26%, and 21%, respectively), control with EBR (leaf) (25%, 42%, 4%, 35%, 18%,

Table 2
Root and leaf structures in tomato plants sprayed with EBR and exposed to different Zn supplies.

EBR	Zn supply	RET (μm)	RDT (μm)	RCD (μm)	VCD (μm)	RMD (μm)
–	Low	15.30 \pm 1.28 ^{Aa}	17.95 \pm 0.51 ^{Bb}	189.32 \pm 5.02 ^{Bb}	140.35 \pm 7.89 ^{Bb}	21.68 \pm 0.59 ^{Ab}
–	Control	16.50 \pm 0.91 ^{Aa}	19.71 \pm 0.81 ^{Aa}	233.19 \pm 9.39 ^{Ab}	158.80 \pm 5.33 ^{Ab}	24.75 \pm 1.76 ^{Ab}
–	High	16.10 \pm 1.48 ^{Aa}	18.46 \pm 0.38 ^{Ba}	212.05 \pm 10.63 ^{Bb}	147.43 \pm 4.49 ^{Bb}	22.20 \pm 1.39 ^{Ab}
+	Low	16.69 \pm 1.41 ^{Aa}	19.11 \pm 0.21 ^{Ba}	214.22 \pm 3.76 ^{Ca}	187.79 \pm 3.97 ^{Ca}	33.62 \pm 1.53 ^{Ca}
+	Control	17.76 \pm 1.01 ^{Aa}	21.29 \pm 0.79 ^{Aa}	257.11 \pm 10.03 ^{Aa}	306.75 \pm 3.89 ^{Aa}	49.08 \pm 3.27 ^{Aa}
+	High	17.09 \pm 0.62 ^{Aa}	19.50 \pm 0.49 ^{Ba}	236.39 \pm 8.82 ^{Ba}	257.91 \pm 19.23 ^{Ba}	38.39 \pm 3.16 ^{Ba}
Interaction effects						
EBR \times Zn supply (F-test)		ns	*	*	*	*
EBR	Zn supply	ETAd (μm)	ETAb (μm)	PPT (μm)	SPT (μm)	Ratio PPT/SPT
–	Low	15.90 \pm 1.10 ^{Cb}	12.96 \pm 0.32 ^{Aa}	78.85 \pm 1.38 ^{Bb}	89.07 \pm 3.37 ^{Cb}	0.89 \pm 0.04 ^{Aa}
–	Control	24.27 \pm 1.42 ^{Ab}	14.16 \pm 1.10 ^{Aa}	89.63 \pm 3.16 ^{Ab}	113.58 \pm 5.56 ^{Ab}	0.79 \pm 0.01 ^{Ba}
–	High	19.41 \pm 0.51 ^{Bb}	13.50 \pm 0.69 ^{Aa}	83.80 \pm 2.98 ^{Bb}	97.92 \pm 4.42 ^{Bb}	0.83 \pm 0.02 ^{Aa}
+	Low	20.31 \pm 0.65 ^{Ba}	13.45 \pm 0.46 ^{Ba}	88.11 \pm 2.57 ^{Ba}	122.80 \pm 1.22 ^{Ca}	0.72 \pm 0.04 ^{Ab}
+	Control	26.30 \pm 1.06 ^{Aa}	15.18 \pm 0.21 ^{Aa}	98.13 \pm 1.87 ^{Aa}	145.35 \pm 6.75 ^{Aa}	0.68 \pm 0.03 ^{Ab}
+	High	21.66 \pm 1.09 ^{Ba}	14.22 \pm 0.64 ^{Ba}	93.10 \pm 2.63 ^{Ba}	127.64 \pm 3.03 ^{Ba}	0.73 \pm 0.05 ^{Ab}
Interaction effects						
EBR \times Zn supply (F-test)		*	*	*	**	*

EBR = 24-epibrassinolideo; Zn = zinc; RET = root epidermis thickness; RDT = root endodermis thickness; RCD = root cortex diameter; 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 uppercase letters between Zn supplies (low, control and high Zn supply under equal EBR level) and lowercase letters between EBR level (with and without EBR under equal Zn supply) indicate significant differences from the Scott-Knott test ($P < 0.05$). Interaction effects linked to EBR \times Zn supply are described by **, * and ns, corresponding to significant at 1% probability level, significant at 5% probability level and not significant, in this order. Means \pm SD, $n = 5$.

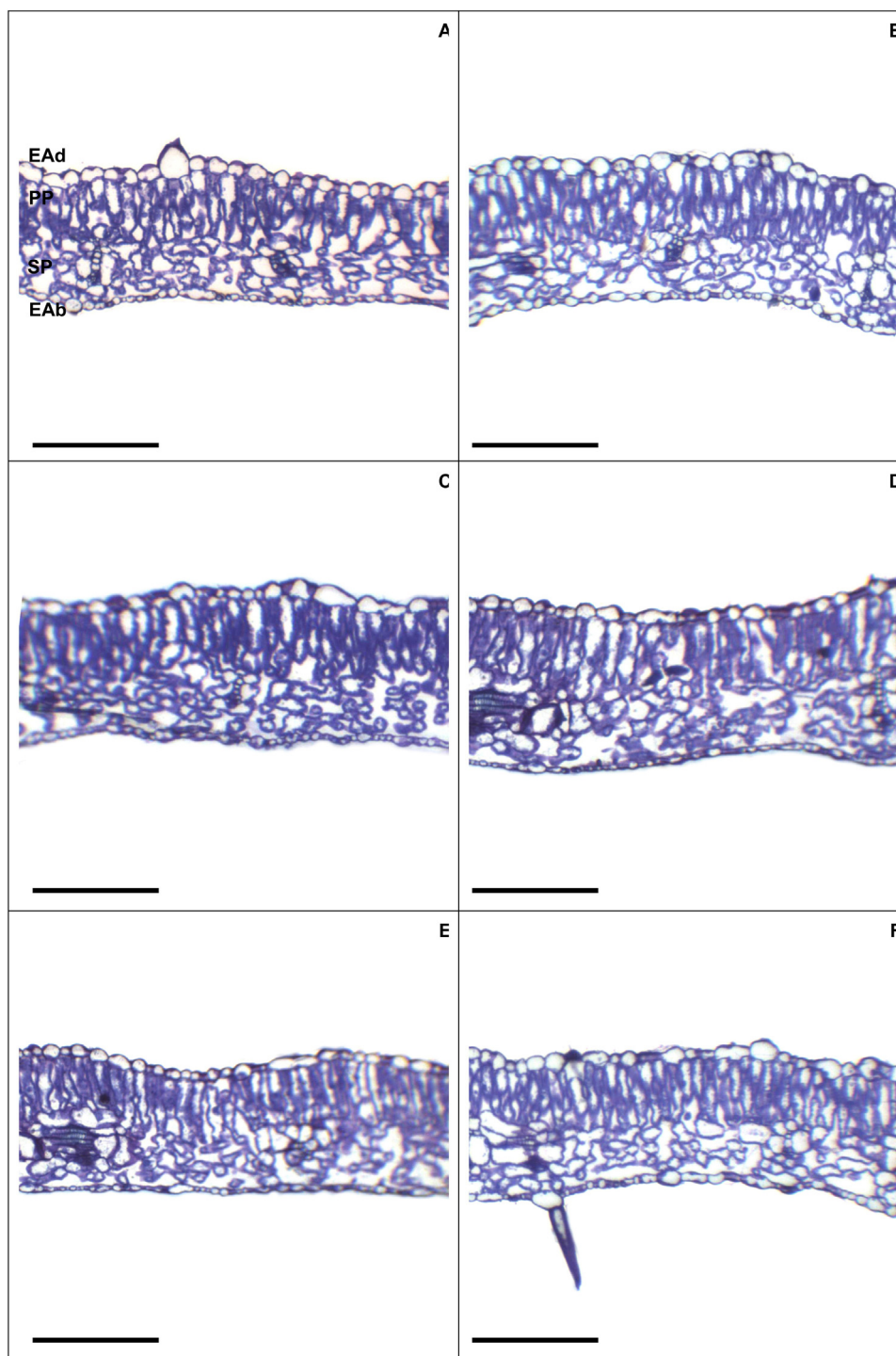


Fig. 1. Leaf cross sections in tomato plants sprayed with EBR and exposed to different Zn supplies. Zn low / -EBR (A), Zn low / +EBR (B), Zn control / -EBR (C), Zn control / +EBR (D), Zn high / -EBR (E) and Zn high / +EBR (F). Legends: EAd = adaxial epidermis; EAb = abaxial epidermis; PP = palisade parenchyma; SP = spongy parenchyma. Black bars = 200 μm .

and 27%, respectively), and high Zn supplementation and EBR (leaf) (10%, 48%, 4%, 15%, 6% and 36%, respectively).

3.4. Zn stress reduces the stomatal density, but EBR partially reverses this negative effect

The stress caused by Zn's low and high levels promoted decreases in SD, SF, and SI (Table 4). Significant interaction effects linked to EBR \times Zn supply were showed to all variables presented in Table 4. However, there were increases in PDS and EDS. On the adaxial face,

plants sprayed with EBR presented increases in SD, SF, and SI: by 16%, 7%, and 15% (low Zn); 9%, 8%, and 5% (control); and 10%, 7%, and 20% (high Zn), respectively. Plants under EBR had decreases in PDS and EDS of 8% and 15% (low treatment), 3% and 10% (control), and 7% and 13% (high Zn), respectively, compared to equal treatment without EBR. In the abaxial face, the EBR increased SD, SF, and SI in 9%, 6%, and 5% (control) and 20%, 7%, and 21% (high Zn), respectively. In the PDS and EDS, the EBR induced decreases of 7% and 12% (low Zn), 3% and 10% (control), and 10% and 15% (high Zn), respectively, when compared to the equal treatment without EBR.

Table 3
Nutrient contents in tomato plants sprayed with EBR and exposed to different Zn supplies.

EBR	Zn supply	Mg (mg g DM ⁻¹)	K (mg g DM ⁻¹)	Ca (mg g DM ⁻¹)	Mn (μg g DM ⁻¹)	Fe (μg g DM ⁻¹)	Cu (μg g DM ⁻¹)
Contents in root							
–	Low	16.04 ± 0.18 ^{Bb}	16.71 ± 0.19 ^{Bb}	6.18 ± 0.27 ^{Bb}	130.63 ± 0.92 ^{Bb}	6854.63 ± 134.06 ^{Bb}	9.95 ± 0.25 ^{Aa}
–	Control	25.06 ± 0.07 ^{Ab}	17.50 ± 0.37 ^{Ab}	7.32 ± 0.01 ^{Ab}	135.01 ± 0.96 ^{Aa}	8577.31 ± 249.36 ^{Aa}	9.46 ± 0.04 ^{Bb}
–	High	15.56 ± 0.25 ^{Cb}	16.15 ± 0.04 ^{Cb}	5.43 ± 0.32 ^{Cb}	125.14 ± 2.26 ^{Cb}	5325.21 ± 190.22 ^{Cb}	8.28 ± 0.31 ^{Cb}
+	Low	19.29 ± 0.11 ^{Ba}	17.85 ± 0.14 ^{Ba}	8.64 ± 0.20 ^{Ba}	135.98 ± 0.51 ^{Aa}	7647.03 ± 206.91 ^{Ba}	10.14 ± 0.25 ^{Ba}
+	Control	26.71 ± 0.31 ^{Aa}	19.43 ± 0.15 ^{Aa}	9.36 ± 0.22 ^{Aa}	136.88 ± 0.81 ^{Aa}	8845.79 ± 49.19 ^{Aa}	10.54 ± 0.12 ^{Aa}
+	High	17.46 ± 0.52 ^{Ca}	17.67 ± 0.34 ^{Ba}	8.22 ± 0.17 ^{Ca}	134.06 ± 0.34 ^{Ba}	6498.17 ± 344.84 ^{Ca}	9.31 ± 0.33 ^{Ca}
Interaction effects							
EBR × Zn supply (F-test)		**	*	**	*	**	**
Contents in stem							
–	Low	4.25 ± 0.17 ^{Bb}	57.84 ± 0.14 ^{Bb}	9.10 ± 0.29 ^{Bb}	11.54 ± 0.27 ^{Bb}	53.72 ± 0.62 ^{Bb}	2.67 ± 0.09 ^{Bb}
–	Control	4.80 ± 0.09 ^{Ab}	61.45 ± 0.64 ^{Ab}	9.70 ± 0.26 ^{Ab}	19.68 ± 0.07 ^{Ab}	67.21 ± 1.06 ^{Ab}	2.91 ± 0.05 ^{Aa}
–	High	4.09 ± 0.07 ^{Bb}	53.05 ± 0.44 ^{Cb}	7.55 ± 0.33 ^{Cb}	10.32 ± 0.18 ^{Cb}	28.33 ± 0.17 ^{Cb}	2.31 ± 0.08 ^{Cb}
+	Low	5.10 ± 0.05 ^{Ba}	59.14 ± 0.32 ^{Ba}	10.01 ± 0.15 ^{Ba}	12.48 ± 0.25 ^{Ba}	62.14 ± 0.70 ^{Ba}	2.96 ± 0.12 ^{Aa}
+	Control	5.95 ± 0.14 ^{Aa}	67.87 ± 0.42 ^{Aa}	10.45 ± 0.15 ^{Aa}	21.94 ± 0.24 ^{Aa}	99.36 ± 0.72 ^{Aa}	3.03 ± 0.13 ^{Aa}
+	High	4.88 ± 0.12 ^{Ca}	58.57 ± 0.42 ^{Ba}	9.51 ± 0.20 ^{Ca}	11.69 ± 0.37 ^{Ca}	37.01 ± 0.96 ^{Ca}	2.77 ± 0.04 ^{Ba}
Interaction effects							
EBR × Zn supply (F-test)		*	*	**	**	**	*
Contents in leaf							
–	Low	5.79 ± 0.09 ^{Ab}	39.07 ± 0.75 ^{Ab}	21.07 ± 0.39 ^{Bb}	18.02 ± 0.40 ^{Bb}	99.19 ± 0.22 ^{Bb}	3.55 ± 0.06 ^{Bb}
–	Control	5.94 ± 0.14 ^{Ab}	32.80 ± 0.12 ^{Bb}	21.78 ± 0.23 ^{Ab}	22.53 ± 0.31 ^{Ab}	121.57 ± 1.58 ^{Ab}	3.73 ± 0.10 ^{Ab}
–	High	5.76 ± 0.16 ^{Ab}	25.85 ± 0.68 ^{Cb}	20.43 ± 0.17 ^{Cb}	16.95 ± 0.71 ^{Cb}	73.24 ± 0.23 ^{Cb}	3.04 ± 0.11 ^{Cb}
+	Low	6.77 ± 0.29 ^{Ba}	41.58 ± 1.03 ^{Ba}	21.91 ± 0.48 ^{Ba}	25.34 ± 0.46 ^{Ba}	124.77 ± 1.44 ^{Ba}	4.28 ± 0.05 ^{Ba}
+	Control	7.45 ± 0.27 ^{Aa}	46.53 ± 0.22 ^{Aa}	22.73 ± 0.42 ^{Aa}	30.49 ± 1.11 ^{Aa}	142.85 ± 0.37 ^{Aa}	4.75 ± 0.13 ^{Aa}
+	High	6.33 ± 0.12 ^{Ca}	38.35 ± 0.73 ^{Ca}	21.16 ± 0.18 ^{Ca}	19.43 ± 0.41 ^{Ca}	77.52 ± 0.90 ^{Ca}	4.12 ± 0.08 ^{Ca}
Interaction effects							
EBR × Zn supply (F-test)		*	**	**	**	**	**

EBR = 24-epibrassinolideo; Zn = zinc; Mg = magnesium; K = potassium; Ca = calcium; Mn = manganese; Fe = iron; Cu = copper. Columns with different uppercase letters between Zn supplies (low, control and high Zn supply under equal EBR level) and lowercase letters between EBR level (with and without EBR under equal Zn supply) indicate significant differences from the Scott-Knott test ($P < 0.05$). Interaction effects linked to EBR × Zn supply are described by **, * and ns, corresponding to significant at 1% probability level, significant at 5% probability level and not significant, in this order. Means ± SD, n = 5.

3.5. Organic molecule upregulated the performance of the photosynthetic machinery in zinc-stressed plants

For chloroplastic pigments, low and high Zn supplies had negative impacts (Table 5). In Table 5, significant interaction effects linked to EBR × Zn supply were confirmed to all variables, with exception to C_i . Plants sprayed with EBR had increases in Chl *a*, Chl *b*, total Chl,

Car, and Chl total/Car under the low Zn (of 54%, 57%, 54%, and 49% and 3%, respectively) and under high Zn (of 65%, 91%, 68%, 43%, and 20%, respectively). Concerning Chl *a*/Chl *b*, there were decreases of 2%, 9%, and 13% for the low, control and high Zn treatments, respectively, when compared to the same treatment without EBR. Low and high Zn supplies promoted significant reductions in chlorophyll fluorescence (Fig. 2 and Table 5) except for F_0 . For F_0 , plants sprayed with

Table 4
Stomatal characteristics in tomato plants sprayed with EBR and exposed to different Zn supplies.

EBR	Zn supply	SD (stomata per mm ²)	PDS (μm)	EDS (μm)	SF	SI (%)
Adaxial face						
–	Low	123.0 ± 2.7 ^{Cb}	21.58 ± 0.91 ^{Aa}	31.08 ± 2.58 ^{Aa}	0.70 ± 0.01 ^{Cb}	8.93 ± 0.29 ^{Bb}
–	Control	164.3 ± 5.7 ^{Ab}	17.83 ± 0.94 ^{Ca}	23.52 ± 1.10 ^{Ca}	0.76 ± 0.01 ^{Ab}	11.47 ± 0.38 ^{Ab}
–	High	150.0 ± 7.5 ^{Bb}	19.75 ± 0.71 ^{Ba}	27.18 ± 2.06 ^{Ba}	0.73 ± 0.01 ^{Bb}	9.51 ± 0.50 ^{Bb}
+	Low	142.9 ± 7.1 ^{Ca}	19.75 ± 0.74 ^{Ab}	26.46 ± 1.98 ^{Ab}	0.75 ± 0.01 ^{Ca}	10.27 ± 0.41 ^{Ca}
+	Control	178.6 ± 6.3 ^{Aa}	17.29 ± 1.08 ^{Ba}	21.13 ± 1.10 ^{Cb}	0.82 ± 0.07 ^{Aa}	12.10 ± 0.24 ^{Aa}
+	High	165.0 ± 6.2 ^{Ba}	18.41 ± 0.57 ^{Bb}	23.71 ± 1.21 ^{Bb}	0.78 ± 0.01 ^{Ba}	11.40 ± 0.45 ^{Ba}
Interaction effects						
EBR × Zn supply (F-test)		**	*	**	**	*
Abaxial face						
–	Low	130.0 ± 5.6 ^{Cb}	20.73 ± 0.61 ^{Aa}	28.38 ± 1.35 ^{Aa}	0.72 ± 0.01 ^{Cb}	12.90 ± 0.34 ^{Ba}
–	Control	181.4 ± 7.9 ^{Ab}	17.66 ± 0.73 ^{Ba}	22.94 ± 0.94 ^{Ba}	0.78 ± 0.01 ^{Ab}	13.58 ± 0.29 ^{Ab}
–	High	142.9 ± 1.2 ^{Bb}	20.75 ± 0.80 ^{Aa}	27.71 ± 1.93 ^{Aa}	0.75 ± 0.01 ^{Bb}	11.34 ± 0.47 ^{Cb}
+	Low	150.0 ± 5.6 ^{Ca}	19.30 ± 0.79 ^{Ab}	25.10 ± 1.39 ^{Ab}	0.77 ± 0.01 ^{Aa}	12.08 ± 0.45 ^{Bb}
+	Control	197.1 ± 1.8 ^{Aa}	17.08 ± 0.82 ^{Ba}	20.61 ± 0.86 ^{Bb}	0.83 ± 0.01 ^{Aa}	14.27 ± 0.32 ^{Aa}
+	High	171.4 ± 8.2 ^{Ba}	18.67 ± 0.70 ^{Ab}	23.52 ± 2.06 ^{Ab}	0.80 ± 0.01 ^{Aa}	13.77 ± 0.31 ^{Aa}
Interaction effects						
EBR × Zn supply (F-test)		**	*	*	*	*

EBR = 24-epibrassinolideo; Zn = zinc; SD = stomatal density; PDS = polar diameter of the stomata; EDS = equatorial diameter of the stomata; SF = stomatal functionality; SI = stomatal index. Columns with different uppercase letters between Zn supplies (low, control and high Zn supply under equal EBR level) and lowercase letters between EBR level (with and without EBR under equal Zn supply) indicate significant differences from the Scott-Knott test ($P < 0.05$). Interaction effects linked to EBR × Zn supply are described by **, * and ns, corresponding to significant at 1% probability level, significant at 5% probability level and not significant, in this order. Means ± SD, n = 5.

Table 5

Photosynthetic pigments, chlorophyll fluorescence and gas exchange in tomato plants sprayed with EBR and exposed to different Zn supplies.

EBR	Zn supply	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
–	Low	6.93 ± 0.47 ^{Bb}	1.15 ± 0.11 ^{Bb}	8.08 ± 0.42 ^{Bb}	0.51 ± 0.04 ^{Bb}	6.06 ± 0.50 ^{Aa}	16.03 ± 1.27 ^{Aa}
–	Control	7.99 ± 0.45 ^{Ab}	1.43 ± 0.06 ^{Ab}	9.51 ± 0.26 ^{Ab}	0.60 ± 0.04 ^{Ab}	5.61 ± 0.39 ^{Aa}	15.84 ± 0.75 ^{Aa}
–	High	7.00 ± 0.45 ^{Bb}	1.17 ± 0.07 ^{Bb}	8.18 ± 0.05 ^{Bb}	0.58 ± 0.04 ^{Ab}	5.98 ± 0.20 ^{Aa}	13.84 ± 0.82 ^{Bb}
+	Low	10.67 ± 0.63 ^{Ba}	1.80 ± 0.11 ^{Ba}	12.47 ± 0.59 ^{Ba}	0.76 ± 0.03 ^{Ca}	5.94 ± 0.32 ^{Aa}	16.52 ± 1.55 ^{Aa}
+	Control	11.78 ± 0.40 ^{Aa}	2.31 ± 0.20 ^{Aa}	14.03 ± 0.88 ^{Aa}	1.02 ± 0.07 ^{Aa}	5.13 ± 0.42 ^{Ba}	13.76 ± 0.92 ^{Bb}
+	High	11.53 ± 0.12 ^{Aa}	2.23 ± 0.20 ^{Aa}	13.76 ± 0.28 ^{Aa}	0.83 ± 0.03 ^{Ba}	5.20 ± 0.41 ^{Bb}	16.58 ± 1.26 ^{Aa}
Interaction effects							
EBR × Zn supply (F-test)							
EBR	Zn supply	Φ _{PSII}	q _p	NPQ	ETR (μmol m ⁻² s ⁻¹)	EXC (μmol m ⁻² s ⁻¹)	ETR/P _N
–	Low	0.21 ± 0.01 ^{Ba}	0.26 ± 0.01 ^{Cb}	0.61 ± 0.04 ^{Aa}	30.6 ± 0.5 ^{Bb}	0.73 ± 0.01 ^{Aa}	3.00 ± 0.13 ^{Aa}
–	Control	0.25 ± 0.01 ^{Ab}	0.34 ± 0.02 ^{Ab}	0.24 ± 0.01 ^{Ca}	36.4 ± 1.9 ^{Ab}	0.69 ± 0.01 ^{Ba}	2.68 ± 0.12 ^{Ba}
–	High	0.22 ± 0.01 ^{Bb}	0.29 ± 0.01 ^{Bb}	0.42 ± 0.02 ^{Ba}	32.5 ± 1.0 ^{Cb}	0.72 ± 0.01 ^{Aa}	3.13 ± 0.17 ^{Aa}
+	Low	0.21 ± 0.01 ^{Ca}	0.31 ± 0.01 ^{Ab}	0.41 ± 0.01 ^{Ab}	31.5 ± 0.3 ^{Ba}	0.70 ± 0.01 ^{Ab}	2.78 ± 0.09 ^{Ab}
+	Control	0.28 ± 0.01 ^{Aa}	0.40 ± 0.03 ^{Aa}	0.25 ± 0.01 ^{Ca}	41.9 ± 1.2 ^{Aa}	0.65 ± 0.01 ^{Bb}	2.77 ± 0.10 ^{Aa}
+	High	0.25 ± 0.01 ^{Ba}	0.32 ± 0.01 ^{Ba}	0.31 ± 0.01 ^{Bb}	37.3 ± 1.8 ^{Ca}	0.69 ± 0.01 ^{Cb}	2.87 ± 0.08 ^{Ab}
Interaction effects							
EBR × Zn supply (F-test)							
EBR	Zn supply	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)
–	Low	10.21 ± 0.35 ^{Bb}	2.99 ± 0.16 ^{Ab}	0.29 ± 0.01 ^{Ab}	276.8 ± 12.1 ^{Aa}	3.42 ± 0.18 ^{Ca}	0.037 ± 0.001 ^{Bb}
–	Control	13.62 ± 0.50 ^{Ab}	2.97 ± 0.10 ^{Ab}	0.30 ± 0.02 ^{Ab}	267.2 ± 4.9 ^{Aa}	4.60 ± 0.39 ^{Aa}	0.051 ± 0.002 ^{Ab}
–	High	10.37 ± 0.41 ^{Bb}	2.74 ± 0.12 ^{Bb}	0.29 ± 0.02 ^{Ab}	277.4 ± 21.5 ^{Aa}	3.81 ± 0.20 ^{Bb}	0.038 ± 0.003 ^{Bb}
+	Low	11.31 ± 0.45 ^{Ca}	3.10 ± 0.17 ^{Aa}	0.34 ± 0.03 ^{Ba}	273.0 ± 5.9 ^{Aa}	3.65 ± 0.24 ^{Ca}	0.041 ± 0.002 ^{Ca}
+	Control	15.12 ± 0.55 ^{Aa}	3.25 ± 0.14 ^{Aa}	0.39 ± 0.01 ^{Aa}	263.4 ± 2.5 ^{Aa}	4.66 ± 0.24 ^{Aa}	0.057 ± 0.003 ^{Aa}
+	High	13.01 ± 0.41 ^{Ba}	3.08 ± 0.18 ^{Aa}	0.34 ± 0.02 ^{Ba}	273.0 ± 11.1 ^{Aa}	4.23 ± 0.18 ^{Ba}	0.048 ± 0.003 ^{Ba}
Interaction effects							
EBR × Zn supply (F-test)							
		*	*	*	ns	*	*

EBR = 24-epibrassinolide; Zn = zinc; 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 CO₂ concentration; WUE = water-use efficiency; P_N/C_i = carboxylation instantaneous efficiency. Columns with different uppercase letters between Zn supplies (low, control and high Zn supply under equal EBR level) and lowercase letters between EBR level (with and without EBR under equal Zn supply) indicate significant differences from the Scott-Knott test (P < 0.05). Interaction effects linked to EBR × Zn supply are described by **, * and ns, corresponding to significant at 1% probability level, significant at 5% probability level and not significant, in this order. Means ± SD, n = 5.

EBR presented reductions of 4%, 7%, and 11% under low, control, and high treatments, respectively, compared to the same treatment without EBR. Concerning F_m, F_v, and F_v/F_m, with the application of EBR, there were increases of 3%, 7% and 5% (low Zn); 2%, 4%, and 2% (control treatment); and 3%, 7%, and 4% (high Zn), respectively, when compared to the equal treatment without EBR. The low and high Zn supplies negatively affected Φ_{PSII}, q_p and ETR (Table 5). Plants sprayed with EBR and exposed to the low treatment had increased q_p and ETR of 19% and 3%, respectively, while NPQ, EXC, and ETR/P_N decreased by 33%, 4% and 7%, respectively. In the high Zn + EBR treatment, increases of 14%, 10%, and 15% were observed for Φ_{PSII}, q_p and ETR, as well as reductions of 26%, 4% and 8% in NPQ, EXC and ETR/P_N, when compared to treatment equal without EBR. Regarding gas exchange, plants exposed to low and high Zn supplies suffered decreases (Table 5). However, EBR spray promoted increases in P_N, E, g_s, WUE and P_N/C_i of 11%, 4%, 17%, 7% and 11% (low Zn); 11%, 9%, 30%, 1% and 12% (control); 25%, 12%, 17%, 11% and 26% (high Zn), respectively, when compared to the same treatment without EBR. Concerning C_i, reductions of 1%, 1% and 2% were detected

3.6. EBR maximized antioxidant enzymes, mitigating oxidative damages in plants exposed to Zn inadequate

Plants submitted to low and high levels of Zn had increases in the activities of antioxidant enzymes (Fig. 3), being found interaction effects (F < 0.05) linked to EBR × Zn supply. For SOD, EBR induced increments of 10%, 31%, and 19% in low, control, and high Zn treatments in this order. In tomatoes treated with EBR, the activity of CAT was increased by 46%, 40% and 41% for low, control, and high Zn, respectively, compared to the same treatment without EBR.

Regarding APX, the application of EBR caused increases of 58%, 33%, and 79% in the treatments low, control and high Zn, respectively. In POX, plants sprayed with EBR presented increases of 132%, 203%, and 149% under low, control and high Zn treatments, respectively, when compared with the same treatment without EBR. Regarding stress indicators (Fig. 4) also was detected interaction effects (F < 0.05) linked to EBR × Zn supply, low and high supplements of Zn promoted the overproduction of oxidant compounds. However, plants sprayed with EBR mitigated these effects. For O₂⁻, we detected reductions of 49%, 7%, and 30% in the treatments with low, control and high Zn, respectively, compared to the same treatment without EBR. Concerning H₂O₂, plants sprayed with EBR presented decreases of 24%, 23%, and 25% under low, control, and high Zn supplies, respectively. Regarding MDA, EBR spray promoted reductions of 10%, 9%, and 9% in low, control, and high Zn treatments, respectively. Concerning EL, there were decreases of 12%, 7%, and 14% in plants exposed to low, control, and high Zn, respectively, compared to equal treatment without EBR.

3.7. EBR clearly triggered tolerance to Zn stress

The stress caused by Zn's low and high supplies promoted reductions in the variables related to growth (Fig. 5), with interaction effects (F < 0.05) linked to EBR × Zn supply. However, the EBR spray attenuated these negative interferences. In LDM, plants sprayed with EBR and exposed to low treatment, control, and high Zn presented increases of 26%, 6% and 29%, respectively. RDM increased by 13%, 14%, and 22% under low treatment, control, and high Zn, respectively, compared to the same treatment without EBR. Concerning SDM, plants with EBR had increased with low, control, and high Zn supplies

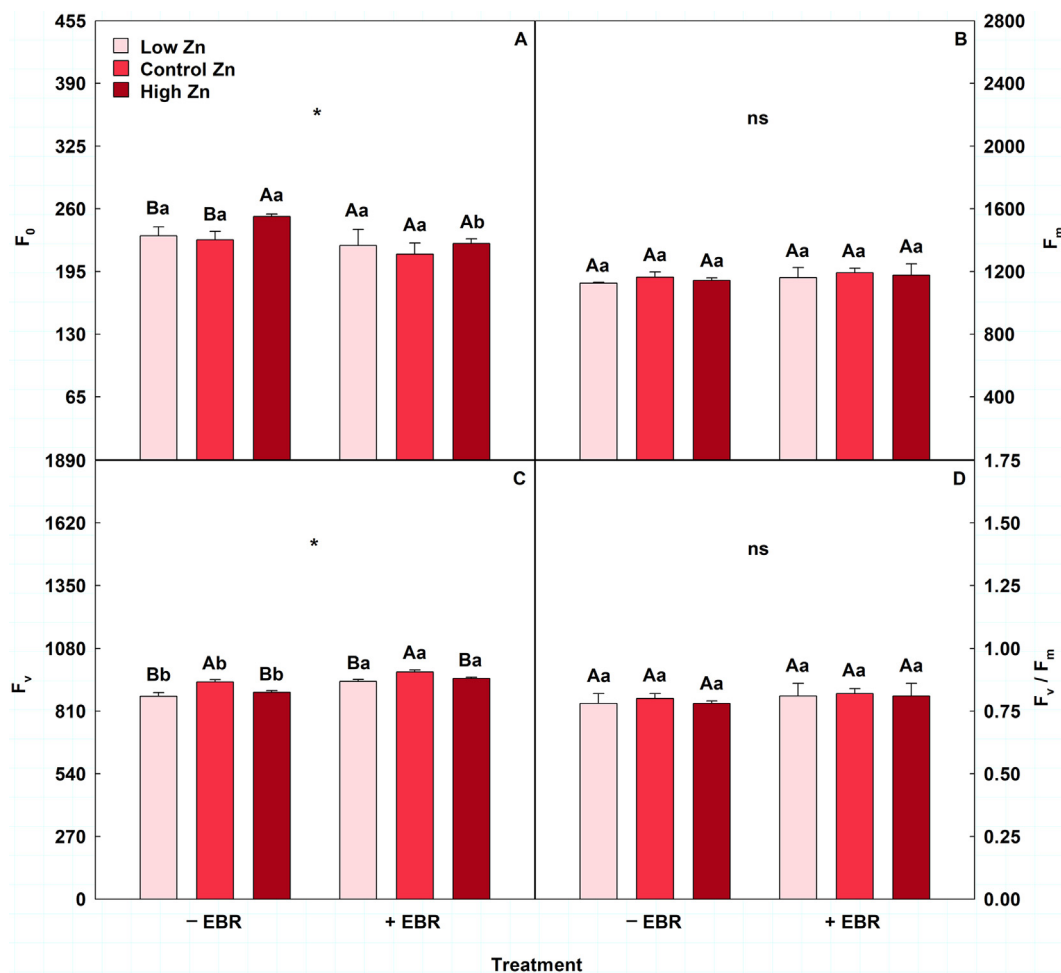


Fig. 2. Minimal fluorescence yield of the dark-adapted state (F_0 ; A), maximal fluorescence yield of the dark-adapted state (F_m ; B), variable fluorescence (F_v ; C) and maximal quantum yield of PSII photochemistry (F_v/F_m ; D) in tomato plants sprayed with EBR and exposed to different Zn supplies. Columns with different uppercase letters between Zn supplies (low, control and high Zn supply under equal EBR level) and lowercase letters between EBR level (with and without EBR under equal Zn supply) indicate significant differences from the Scott-Knott test ($P < 0.05$). Interaction effects linked to EBR \times Zn supply (F -test) are described by **, * and ns, corresponding to significant at 1% probability level, significant at 5% probability level and not significant, in this order. Means \pm SD, $n = 5$.

(of 40%, 8%, and 42%, respectively). In TDM, increases were observed for the low, control and high Zn treatments (of 24%, 9%, and 29%, respectively) compared to the same treatment without EBR.

4. Discussion

In general, this research revealed that plants exposed to low and high Zn suffered intense disturbances in metabolism, being detected in anatomical, biochemical, nutritional, physiological, and morphological aspects, but exogenous EBR application relieved the stress caused by Zn in tomato plants, synergistically stimulating leaf structures, photosynthesis, and growth.

EBR application promoted increases in Zn contents in the root, stem, and leaves of tomato plants under low and controlled Zn supplies. These results are due to the steroids enhancing the absorption and transport of this metallic nutrient. In other words, during the Zn absorption, plants use several strategies, including the association of the Zn^{2+} ion with organic molecules, inorganic acids, and specialized organelles (Sbartai et al., 2012). And there is likely a beneficial interaction between steroids and metallic ions. On the other hand, EBR spray also alleviated the toxic effects of the Zn excess because this natural molecule favors the plant defense against ionic toxicity. This steroid stimulates the synthesis and accumulation of glutathione and phytochelatins, compounds that are part of the detoxification

mechanism of metals, including Zn (Adediran et al., 2016; Arora et al., 2010). Phytochelatin synthesis promotes the Zn accumulation in leaves of plants grown in soil with adequate Zn supply, benefiting the plant performance (Kühnlénz et al., 2016). Talarek-Karwel et al. (2019), investigating the EBR roles in *Acutodesmus obliquus* under lead (Pb) stress, reported that the application of 1 μ M EBR resulted in increased synthesis and activation of phytochelatins. Santos et al. (2020) studying the EBR effects on root anatomy and antioxidant system of soybean plants subjected to Zn stress, described that 100 nM EBR induced increases in leaf, stem and root Zn contents in plants under low Zn supplementation (0.2 μ M Zn).

Plants exposed to Zn stress (low and high Zn) suffered reductions in root structures. However, EBR spray positively modulated these results, corroborated by increased RET, RDT, RCD, VCD, and RDM. Increases in RET, RDT, and RCD suggest that EBR contributed to root protection of plants exposed to high and low Zn supplies. The thickness of the epidermis and endodermis and the diameter of the cortex are important indicators, as they act on the protection and selectivity of the root, acting as a barrier and protection against biotic and abiotic stresses (Maia et al., 2018). Concerning increases verified in VCD and RMD, these results suggest that EBR mitigated stress caused by Zn, maximizing the diameter of the vascular structures and maintaining absorption and conductivity of the water and mineral (Meyer et al., 2011). Vassilev et al. (2011), cultivating *Phaseolus vulgaris* plants under

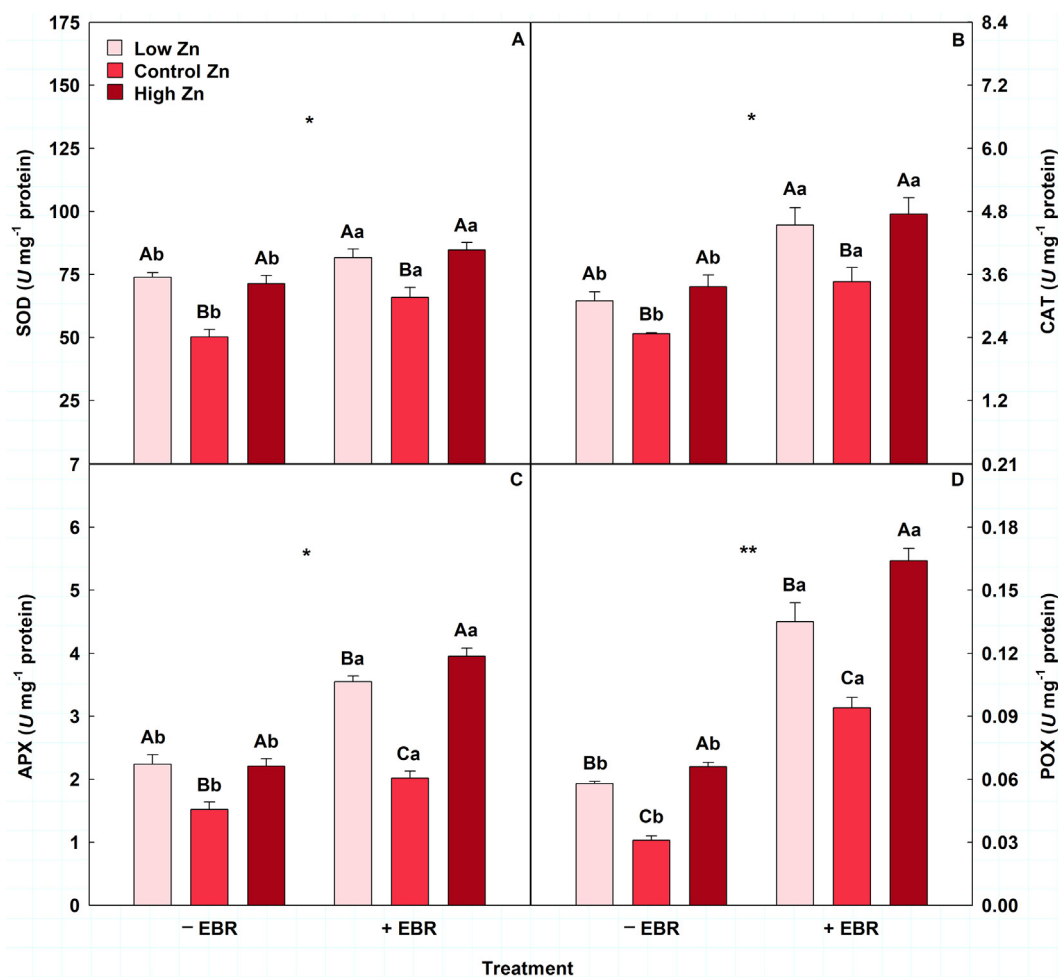


Fig. 3. Activities of superoxide dismutase (SOD; A), catalase (CAT; B), ascorbate peroxidase (APX; C) and peroxidase (POX; D) in tomato plants sprayed with EBR and exposed to different Zn supplies. Columns with different uppercase letters between Zn supplies (low, control and high Zn supply under equal EBR level) and lowercase letters between EBR level (with and without EBR under equal Zn supply) indicate significant differences from the Scott-Knott test ($P < 0.05$). Interaction effects linked to EBR \times Zn supply (F -test) are described by **, * and ns, corresponding to significant at 1% probability level, significant at 5% probability level and not significant, in this order. Means \pm SD, $n = 5$.

different Zn concentrations (1, 200, 300, 400, and 500 μ M) for 10 days, detected a decrease of RMD in plants submitted to 500 μ M Zn.

Pre-treatment with EBR in plants under low and high Zn supplementation promoted positive effects on macronutrient contents (Mg, K, Ca) and micronutrients (Mn, Fe, Cu). These results demonstrate the ability of this steroid to modulate ion uptake in plant cells, probably through cation diffusion facilitators (CDF), being responsible for transferring divalent cations out of the cytoplasm and allocating extracellular spaces (Gustin et al., 2011; Khrupach, 2000). Zn excess in plants often affects normal ionic homeostasis, impacting the uptake, transport, and regulation of essential ions and consequently decreasing nutrient absorption (Cherif et al., 2012). An increase in Ca content indicates improvements in membrane integrity, benefiting root elongation (Cristancho et al., 2014; Ribeiro et al., 2013). Furthermore, Mn is a component of enzymes involved in essential processes in plant metabolism, including antioxidant metabolism, respiration and photosynthesis, more specifically during the decomposition of the water molecule in PSII (Aravind and Prasad, 2004; Bityutskii et al., 2014). Wang et al. (2009) investigated Zn toxicity in *Brassica napus* seedlings (0.07–1.12 mM) for 7 days; they found reductions in Fe, Mg, and Mn contents in the root tissue, as well as a decrease in Cu in leaves.

Plants treated with EBR and under low and high Zn supplies had positive effects on SD, PDS, EDS, SF, and SI, revealing the beneficial actions of this steroid on leaf anatomical variables. The increases in SD, SF, and SI are intrinsically related to the increase in g_s and E ,

indicating that EBR improved stomatal performance and attenuated stomatal disturbances caused by high and low Zn (Kasim, 2006; Misra et al., 2019). The reductions in PDS and EDS suggest that EBR maximized water utilization during gas exchange, proved by the increase in WUE, resulting in higher SF, often characterized by more elliptical stomata (Sha Valli Khan et al., 2003). Sagardoy et al. (2010), analyzing the effects of Zn toxicity on photosynthesis and respiration in *Beta vulgaris* plants hydroponically grown with 1.2, 100, and 300 μ M Zn detected a decrease in SD and pore size.

In both inadequate Zn supplies (low and high), pre-treatment with EBR induced increases in photosynthetic pigments (Chl *a*, Chl *b*, total Chl, and Car). These results indicate that EBR may directly or indirectly stimulate biosynthesis of these pigments or inhibit chlorophyllase activity, attenuating damage caused to chloroplasts due to oxidative stress and pigment degradation caused by the toxicity or deficiency of Zn (Hayat et al., 2011; Sharma et al., 2012). He et al. (2015) evaluated the EBR effects in *Solanum melongena* seedlings exposed to two Zn concentrations (0 and 10 mM) for 8 days. They reported increased Chl *a*, Chl *b* and total Chl levels after EBR spray. Zhao et al. (2013) found that exogenous application of EBR in *Solanum nigrum* seedlings reduced the negative impacts of Cd toxicity on plants, increasing Chl *a*, Chl *b* and Car levels, corroborating the results obtained in this study.

The organic molecule tested mitigated the negative effects of both Zn supplies (low and high) on F_0 , F_m , F_v , and F_v/F_m . These results

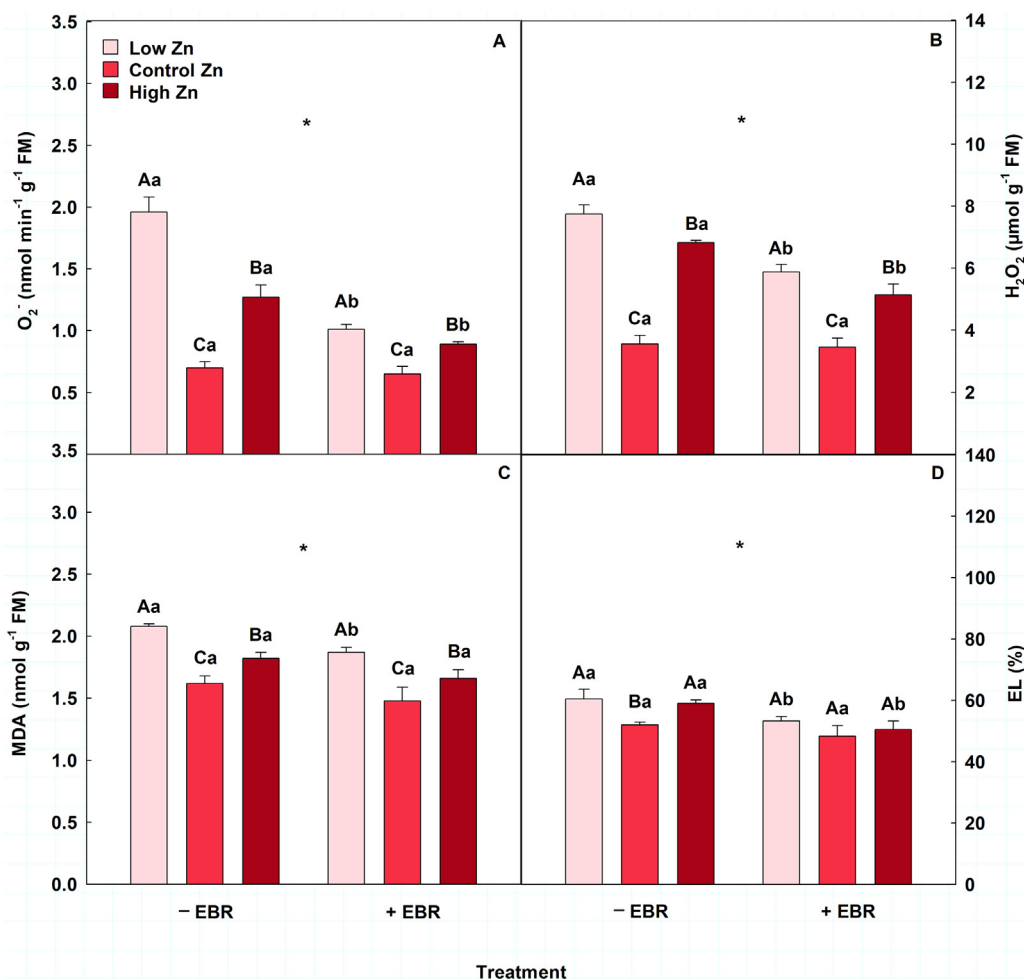


Fig. 4. Superoxide (O_2^- ; A), hydrogen peroxide (H_2O_2 ; B), malondialdehyde (MDA; C) and electrolyte leakage (EL; D) in tomato plants sprayed with EBR and exposed to different Zn supplies. Columns with different uppercase letters between Zn supplies (low, control and high Zn supply under equal EBR level) and lowercase letters between EBR level (with and without EBR under equal Zn supply) indicate significant differences from the Scott-Knott test ($P < 0.05$). Interaction effects linked to EBR \times Zn supply (F -test) are described by **, * and ns, corresponding to significant at 1% probability level, significant at 5% probability level and not significant, in this order. Means \pm SD, $n = 5$.

suggest that the EBR potentiated the activity in photosynthetic apparatus due to the better organization of the chloroplast ultrastructure. The decline in F_v/F_m values caused by both high and low Zn supply is related to photoinhibition (Calatayud and Barreno, 2004; Maxwell and Johnson, 2000; Murchie and Lawson, 2013), revealed by the decrease in the proportion of open reaction centers (F_v), besides reductions in chlorophyll levels, verified in this study. Concerning F_0 , this decrease indicates that EBR increased the electron flow from the light-harvesting complex to the reaction centers of the PSII (Baker and Rosenqvist, 2004). Mir et al. (2015), evaluating the effect of EBR on gas exchange and antioxidant system of *Vigna radiata* plants subjected to salt stress and Zn toxicity for 30 days, observed a decrease in F_v/F_m values. However, treatment with EBR mitigated the harmful effects of NaCl and Zn stress. Andrejić et al. (2018), studying the effects of different Zn concentrations (control, 250, 500, and 1000 mg Zn kg⁻¹ soil) on the pigments and photosynthetic parameters in *Miscanthus \times giganteus* plants, observed significant reductions in F_v and F_v/F_m and increase in F_0 in all treatments with excess Zn, compared to the control treatment.

Exogenous EBR application positively impacted Φ_{PSII} , q_p , and ETR in plants submitted to low Zn and high supplies, being these effects related to increases in F_0 , F_v , and reduction in EXC described in this study. The maximization in Φ_{PSII} after treatment with EBR suggests that this steroid improved the absorption of photon energy (F_0 and F_v) and decreased the super-excitation generated in PSII (EXC),

reducing the structural damages caused by excess electrons in the membranes of the thylakoids into chloroplasts (Ogwenko et al., 2008). Increases in ETR and q_p indicate that EBR increased the flow of electrons previously captured by plastoquinone Buonasera et al. (2011). Similar to our results, Sagardoy et al. (2009), investigating the physiological behavior and nutritional status in *Beta vulgaris* plants exposed to high concentrations of Zn (50, 100, and 300 μ M), described decreases in Φ_{PSII} . On the other hand, this steroid promoted reductions in NPQ, EXC, and ETR/ P_N in plants exposed to low and high Zn supplies. These decreases suggest that the EBR promoted better use of electrons for photochemical activity and reduced alternative drains of electrons (Oliveira et al., 2019). The reduction in EXC is intrinsically related to the decline in NPQ, demonstrating that EBR reduced the photochemical damages in PSII, while the reduction in ETR/ P_N indicates that less photochemical energy was directed to other drains, such as photorespiration. In other words, more energy will be available for CO_2 assimilation reactions (Ribeiro et al., 2009; Silva et al., 2010). Lima and Lobato (2017), evaluating the photosystem II, gas exchange and antioxidant enzyme in *Vigna unguiculata* plants pre-treated with EBR (100 nM), found increases of 30% in NPQ, corroborated by our research.

Steroid spray in plants exposed to low and high Zn supplies resulted in increased P_N , E , g_s , WUE, and P_N/C_i values related to beneficial effects on SD, SI, and SF, verified in this study. Zn toxicity promotes stomatal closure and decreases g_s , limiting CO_2 assimilation in

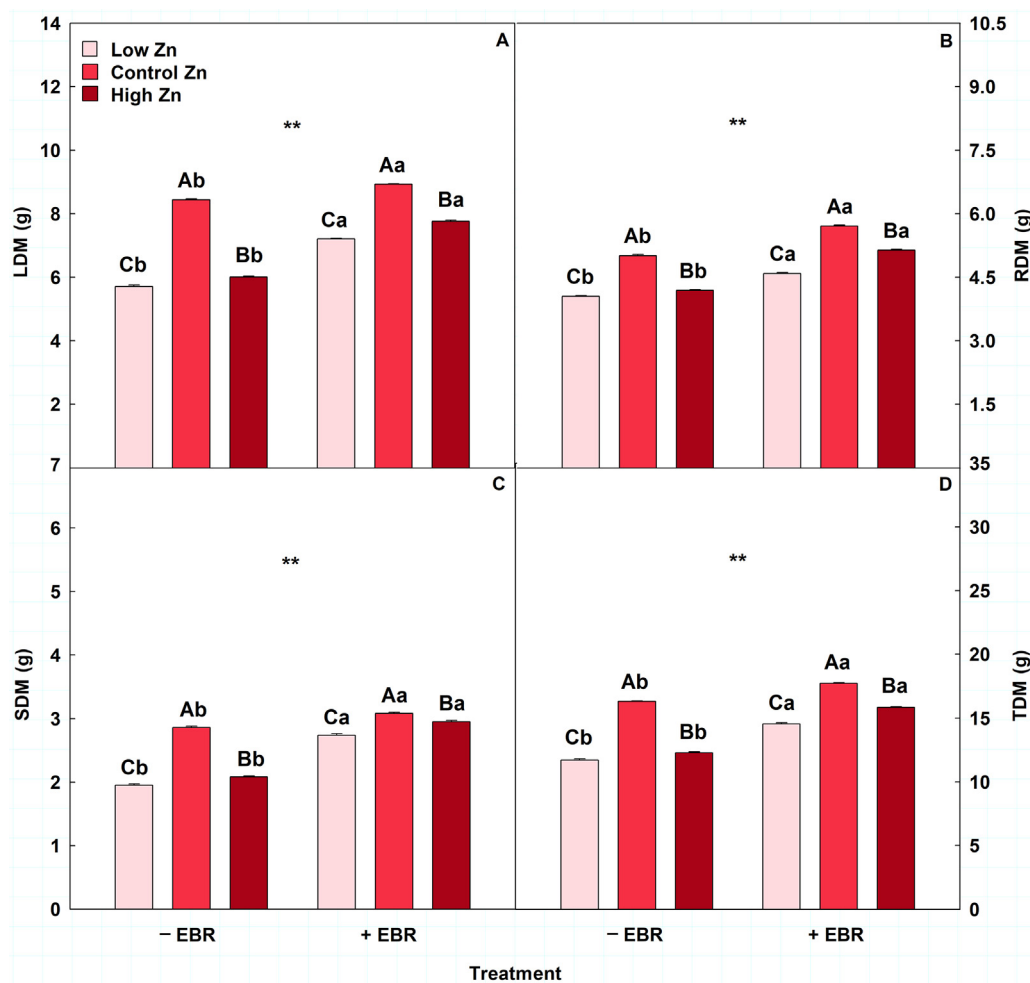


Fig. 5. Leaf dry matter (LDM; A), root dry matter (RDM; B), stem dry matter (SDM; C) and total dry matter (TDM; D) in tomato plants sprayed with EBR and exposed to different Zn supplies. Columns with different uppercase letters between Zn supplies (low, control and high Zn supply under equal EBR level) and lowercase letters between EBR level (with and without EBR under equal Zn supply) indicate significant differences from the Scott-Knott test ($P < 0.05$). Interaction effects linked to EBR \times Zn supply (F -test) are described by **, * and ns, corresponding to significant at 1% probability level, significant at 5% probability level and not significant, in this order. Means \pm SD, $n = 5$.

the photosynthetic process (Paula et al., 2015). However, EBR stimulates the activity of the RuBisCO enzyme, which is the main contributor to the fixation and reduction of CO_2 during photosynthesis (Galmés et al., 2013; Parry et al., 2013; Xia et al., 2006) and improves the content and uptake of water (Ali et al., 2008; Farooq et al., 2009). The EBR spray also mitigated the negative effects of the low and high Zn on C_i , which can be explained by the increases in P_N and P_N/C_i observed in this study. These results corroborate an EBR action on CO_2 assimilation, reducing C_i due to activity linked to the RuBisCO enzyme (Evans, 2013). Pavlíková et al. (2014), evaluating nitrogen metabolism and gas exchange in transformed and non-transformed *Nicotiana tabacum* plants under Zn stress, detected a reduction in P_N , E and g_s . Mir et al. (2015) found an increase in WUE after EBR treatment in *Vigna radiata* plants exposed to Zn stress by 30 days. Sagar-doy et al. (2009), studying the effects of EBR on *Beta vulgaris* plants hydroponically cultivated and subjected to Zn toxicity, found a reduction of 24% in C_i .

Under low and high Zn supplies, EBR-sprayed plants had benefits on leaf anatomy, resulting in increases in ETAd, ETAb, PPT, and SPT. The increases in ETAd and ETAb evidenced that EBR caused the thickening of the epidermal cell walls on both sides, which this response aims to prevent water loss through the transpiration process, corroborating the increases found in WUE (Vollenweider et al., 2006). Increases in PPT and SPT suggest that EBR improved the transport and distribution of CO_2 in these tissues (Khalil et al., 2006), as

confirmed by the increase in P_N and reduction in C_i shown in this study. Maruthi Sridhar et al. (2005) detected reductions of palisade and epidermal cells in *Brassica juncea* exposed to Zn and Cd for 15 and 16 days, respectively.

Plants sprayed with EBR and exposed to low and high Zn supplies presented increased SOD, CAT, APX, and POX activities. Zn toxicity induces oxidative stress through the overproduction of ROS, such as O_2^- and H_2O_2 , causing antioxidant imbalance, lipid peroxidation, protein oxidation, and membrane damage (Alonso-Blázquez et al., 2015). However, the results reveal the beneficial actions of EBR on the antioxidant system, proved by the reduction of oxidative damages occasioned on photosynthetic apparatus and membranes, being corroborated by the reductions in NPQ, EXC and increases in ETR, as well as decreases in MDA and EL, found in this study after treatment with EBR. Ramakrishna and Rao (2012), investigating the EBR effects on ROS levels and antioxidant system in *Raphanus sativus* seedlings submitted to stress by Zn, found significant increases in antioxidant enzymes (SOD, CAT and POX). He et al. (2015) described that *Solanum melongena* plants sprayed with EBR and exposed to two concentrations of Zn (0 and 10 mM) reduced the toxicity of this element, increasing the activities of SOD, CAT, APX and POX, confirming the results obtained in this study.

EBR application mitigated the harmful effects of low and high Zn supplies on O_2^- , H_2O_2 , MDA, and EL. Zn toxicity or deficiency causes oxidative stress, which is characterized by the overproduction of

reactive oxygen species (ROS), besides to impact negatively the antioxidant defense system (Vázquez et al., 2013). However, our results suggest that the EBR maximized the activities of antioxidant enzymes intrinsically related to ROS elimination, reducing oxidative damages, proved by the decreases of O_2^- and H_2O_2 , due to an increase in SOD activity. This enzyme catalyzes the dismutation from O_2^- to H_2O_2 , subsequently converted to H_2O by the enzymes CAT APX and POX, mitigating the production of oxidant compounds (Bhaduri and Fulekar, 2012; Li et al., 2013). Similar to our study, Arora et al. (2010), investigating the EBR interference on the antioxidant system in *Brassica juncea* plants with 30-day-old under Zn stress, found reductions in MDA, EL, O_2^- and H_2O_2 .

EBR attenuated the deleterious effects of low and high Zn supplies on biomass, with increases observed in all variables (LDM, RDM, SDM, and TDM). These increases reported are closely related to benefits observed on photosynthetic pigments, gas exchange, root anatomy, and chlorophyll fluorescence. Zn deficiency caused growth retardation and increased ROS due to photo-oxidative damages, with consequent reduction of P_N and ETR (Bae et al., 2011; Zhao and Wu, 2017). On the other hand, EBR stimulates chlorophyll synthesis or reduces its degradation (Hayat et al., 2011), besides maximizing cell elongation and/or cell division (Ashraf et al., 2010; Jin et al., 2014). Ramakrishna and Rao (2014), studying the EBR role of EBR on the growth and development of *Raphanus sativus* plants subjected to high Zn for 30 days, verified attenuation of the adverse effects related to Zn toxicity on biomass after exogenous application of this steroid. Wu et al. (2016), investigating the application of 0.1 μ M EBR on growth regulation, oxidative damages, and metabolism of *Solanum melongena* seedlings exposed to 10 mM Zn, observed increases of 78% in SDM and 13% in RDM, compared to treatment without EBR. Both results corroborate the results found in this study.

5. Conclusion

This research has shown that the application of EBR relieved the stress caused by Zn in tomato plants, synergistically stimulating leaf structures, photosynthesis, and growth. EBR benefited leaf structure by promoting increases in the epidermis thickness from the abaxial leaf side, the palisade parenchyma thickness, and the spongy parenchyma thickness. EBR also improved the photosynthesis rate; this fact is related to increasing stomatal density and effective quantum yield of photosystem II photochemistry. Parallely, EBR attenuated the harmful effects of low and high Zn supplies on biomass. Therefore, our results show that EBR application could help improve tomato plants' tolerance to zinc stress.

Author contribution statement

AKSL was the advisors of this project, planning all phases of this research. EJFL and ATR conducted the experiment in the greenhouse and performed physiological, biochemical and morphological determinations, while YCP measured anatomical parameters, as well as CNL and BLB performed nutritional determinations and critically revised the manuscript. HSB and AB critically revised the manuscript. All authors read and approved the final version of manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sajb.2023.06.034.

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