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LEIDY ALVES DOS SANTOS

BRASSINOSTEROIDS SYSTEMATICALLY TRIGGER TOLERANCE TO MAGNESIUM STRESS IN SOYBEAN PLANTS

BELÉM-PA
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Thesis submitted to Universidade Federal Rural da Amazônia, as part of the requirements for obtaining the *Doctor Scientiae* degree in Agronomy.
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To God, author and provider of life; to my farmers grandparents Amilton and José (in memorian) for the inspiration of life and to my parents, José and Angela, fundamental in my human and professional growth.

I DEDICATED

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"Grandes são as obras do SENHOR,
Dignas de estudo para quem as ama".

Salmos 111.2

"Great are the works of the Lord; they are
pondered by all who delight in them".

Psalm 111.2

RESUMO

O objetivo geral deste estudo foi avaliar se a aplicação foliar de 24-Epibrassinolide (EBR) pode mitigar o estresse oxidativo em plantas de soja submetidas a alta e baixa adição de Mg e avaliar suas possíveis repercussões nos comportamentos anatômicos, nutricionais, bioquímicos, fisiológicos e na biomassa. Para isso, o experimento seguiu um delineamento fatorial completamente casualizado com duas concentrações de 24-Epibrassinolide (0 e 100 nM EBR, descrito como - EBR e + EBR, respectivamente) e três suprimentos de Mg (0,0225, 2,25 e 225 mM de $MgCl_2$, descrito como baixo, controle e alto suprimento de Mg). De modo geral, suprimentos baixos e altos de Mg promoveram efeitos deletérios no metabolismo antioxidante, pigmentos fotossintéticos e mudanças negativas nos parâmetros anatômicos avaliados. No entanto, nos tratamentos com baixo e alto Mg + EBR foram observados aumentos na espessura da epiderme da raiz, endoderme, córtex, cilindro vascular e metaxilema. De igual modo o EBR promoveu incrementos na densidade de estômatos, espessura da epiderme foliar, espessura do parênquima em paliçada e esponjoso, confirmando a ação do EBR sobre a divisão celular e diferenciação dos tecidos. Plantas expostas a baixo e alto Mg e pulverizadas com EBR apresentaram melhorias no acumulo de Mg e conteúdo de macronutrientes (K, Ca, S) e micronutrientes (Mn, Cu e Bo) sugerindo que este esteróide melhorou a absorção, transporte e acúmulo de nutrientes nos tecidos avaliados. O EBR promoveu aumentos das atividades das enzimas antioxidantes em plantas sob estresse de Mg, revelando o efeito benéfico de mitigar os danos oxidativo aos fotossistemas e membranas dos cloroplastos. Aliado a isto, o EBR mitigou os impactos negativos induzidos pela baixa e alta concentração de Mg na taxa de fotossíntese liquida e carboxilação instantânea associados aos incrementos obtidos na taxa de transporte de elétrons e densidade estomática. Concluímos que o EBR reduziu o estresse oxidativo ocasionado pela baixa e alta adição de Mg com repercussões positivas em enzimas antioxidantes, pigmentos fotossintéticos e biomassa da raiz e folha.

Palavras-chave: Anatomia de plantas, Características fotossintéticas, 24-epibrassinolideo, Estresse oxidativo.

ABSTRACT

The general objective of this study was to evaluate whether the foliar application of 24-epibrassinolide (EBR) can mitigate oxidative stress in soybean plants submitted to high and low addition of Mg and to evaluate its possible repercussions on anatomical, nutritional, biochemical, physiological and morphological behaviors. For this, 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 Mg supplies (0.0225, 2.25 and 225 mM Mg, described as low, control and high Mg supply). In general, low and high Mg supplies promoted deleterious effects on antioxidant metabolism, photosynthetic pigments and negative changes in the anatomical parameters evaluated. However, in treatments with low and high Mg + EBR, increases in the thickness of the epidermis of the root, endoderm, cortex, vascular cylinder and metaxylem were observed. Likewise, EBR promoted increases in stomata density, leaf epidermis thickness, palisade and spongy parenchyma thickness, confirming the action of EBR on cell division and tissue differentiation. Plants exposed to low and high Mg and sprayed with EBR showed improvements in the accumulation of Mg and content of macronutrients (K, Ca, S) and micronutrients (Mn, Cu and Bo) suggesting that this steroid improved the absorption, transport and accumulation of nutrients in the evaluated tissues. The EBR promoted increases in the activities of antioxidant enzymes in plants under Mg stress, revealing the beneficial effect of mitigating oxidative damage to chloroplast photosystems and membranes. Allied to this, the EBR mitigated the negative impacts induced by the low and high concentration of Mg in the liquid photosynthesis rate and instant carboxylation associated with the increments obtained in the electron transport rate and stomatal density. We conclude that EBR reduced oxidative stress caused by low and high addition of Mg with positive repercussions on antioxidant enzymes, photosynthetic pigments and root and leaf biomass.

Keywords: Plant anatomy, Photosynthetic characteristics, 24-epibrassinolideo, Oxidative stress.

SUMMARY

CONTEXTUALIZATION

Soybean [*Glycine max* (L.) Merr.], it is an annual plant, herbaceous, erect, autogamous, has a carbon fixation mechanism of type C3, and belongs to Fabaceae and Papilionoideae. Cultivated soybeans were domesticated from wild *Glycine soja* approximately 5.000 to 6.000 years ago in China, their geographical distribution is limited to central and northern East Asia, which includes China, Korea, Japan and the Far East of Russia (WANG; LI; LIU, 2012).

According to Da Silva et al. (2017), soybean has three types of leaves during its development, cotyledonary, unifoliolate, trifoliolate and sessile leaves. The cotyledon leaves have an elliptical oval shape and remain until their reserves are depleted, to the point that they turn yellow, wither and fall. The unifoliolates are primary leaves with opposite disposition, narrow base and auricular, truncated or lanceolate shape. The trifoliate, are alternately arranged, with long petioles and composed of three large leaflets, usually oval. The flowers are axillary or terminal, papillary, white or violet, depending on the variety. The fruits are oblong and hanging pods, pubescent, with a number of grains varying from one to five per pod.

Its cycle, which corresponds to the number of days from emergence to maturation, can take 75 for the earliest cultivars and 200 days for the later ones (BOREM, 1999). The stem has hairiness, is erect, often branched, with a height between 30 and 200 cm and can present indeterminate, semi-determined or determined growth (MÜLLER, 1981). The root system is pivoting, with a main root and profuse lateral branches with the ability to establish symbiosis with bacteria fixing atmospheric nitrogen (CARDOSO et al. 2006).

The soybean cycle is divided into stages of development according to the methodology proposed by Fehr and Caviness (1977), and distinguishes the stages of soybean development between vegetative stage, represented by the letter V and reproductive stage by the letter R. The letters V and R follow with numbers to determine the specific stage the plant is in. Exceptions are the emergency and opening stages of the cotyledons as the letters VE and VC respectively represent them.

For the vegetative stage count, the number of nodes that start from the unifoliate leaves immediately above the cotyledon nodes (V1, V2, V3, ..., Vn) is taken into account. The reproductive stages describe the period of flowering until the maturation of the grain, ranging from R1 to R8 (EMBRAPA, 2020).

Soybean is an important oilseed crop, its seeds are used as a source of protein in both human and animal food, and it is used as a raw material for biofuels. It is responsible for about 56% of the total oilseed production, 25% of the global edible oil and about two thirds of the protein concentrate for animal feed. According to FAO (2020) global soybean production is estimated at 337.9 million tons for the 2019/2020 harvest, with the main world producers of soybean in order of importance, the United States of America, Brazil, Argentina, China and India. In Brazil, the first report on the emergence of soybeans through its cultivation is from 1882, in the state of Bahia (BLACK, 2000). Then, Japanese immigrants took it to São Paulo, and in 1914 soybeans were introduced in the state of Rio Grande do Sul, which was, finally, the

place where the varieties brought from the United States, best adapted to the edaphoclimatic conditions, mainly in relation to the photoperiod (BONETTI, 1981).

According to CONAB (2020), the harvest estimate for Brazil's 2018/19 crop is 120.9 million tons, a historic record, representing an increase of 5.1% in relation to the previous crop, with the states of Mato Grosso, Paraná, Goiás, São Paulo, Tocantins, Maranhão, Rondônia and Distrito Federal presented the highest productivity. Brazilian soy consumption is expected to be 47 million tons, which corresponds to 37,75% of total production, exports for the 2019/2020 harvest are expected to total 76 million tons, which represents 61% of Brazilian production (CONAB, 2020).

The State of Pará is the second largest producer of soybeans in the northern region. In the 2018/19 harvest, the state of Pará produced 1.859 million tons of soybeans, 8,8% higher than the previous harvest (CONAB, 2020). Currently, Pará has twelve soy producing microregions, Paragominas being the main one, responsible for 65% of the production in the state and also being the main export microregion, with a turnover of US$ 458 million in 2017 (SIDRA / IBGE, 2019).

Soy is in full agricultural expansion in Brazil, especially in the North and Northeast regions (ODERICH, 2020), however the cultivations occur in soils of the oxisol and argisol type, predominant in these regions, these soils are characterized by high weathering, leaching, aluminum contents and low cation exchange capacity (CTC), soils with low Mg^{2+} content available to plants (LUMBRERAS et al., 2015).

Magnesium (Mg) is an essential element in plants, it is a constituent of the chlorophyll molecule and acts in phosphorylation, aggregation of ribosome subunits, translocation of photoassimilates and in the activation of multiple enzymes, such as glutathione synthetase, phosphoenolpyruvate (PEP) carboxylase and Rubisco (ribulose 1,5 bisphosphate oxidase/ carboxylase). Therefore, crop growth and productivity are highly affected by Mg deficiency in areas of intensive agricultural production (ALTARUGIO et al., 2017).

Several factors can cause Mg deficiency in plants, such as a low concentration of Mg in the soil-forming rocks, excessive acidity, high levels of aluminum or manganese, salinity, low availability of water in the soil and low transpiration of the plants (GERENDÁS; FÜHRS, 2013). The imbalance in the relationship between calcium (Ca), potassium (K) and magnesium is also pointed out as one of the main causes of Mg deficiency (MEIRELES; SILVA, 2013). Mg is mainly supplied by liming, which cannot always supply sufficient amounts of this element to plants, as many limestones have low Mg levels, in addition to low reactivity and solubility (ALTARUGIO et al., 2017).

According to EMBRAPA (2020), liming, despite being a mandatory practice in the implantation of crops, is not a common practice in the State of Pará, the main factors being the difficulties of operationalizing its application in large areas due to the excessive rainfall in the region, price of the product in the market burdened by the cost of transportation and the lack of professionals in the field of soil fertility to raise awareness of the importance of applying soil correctives.

The absorbed Mg is transported in the plant in the form of Mg^{2+}, and accumulates in the young tissues of the plants due to its high mobility (WATERS, 2011). Magnesium deficiency in soy is characterized by having, in the initial stage of the deficiency, short plants and pale green in appearance, and if it continues, the visual symptoms progress to pale yellow internerval chlorosis in the old leaves, as the symptoms progress, pale yellow chlorosis turns into pale brown necrosis in the internerval regions (APARECIDA VIECELLI, 2017). The mechanism of Mg^{2+} absorption by the roots occurs mainly by mass flow and root interception, so it is highly dependent on the availability of water in the soil and transpiration of plants (EMBRAPA 2020).

The main targets of magnesium deficiency are photosynthesis and sugar transport from the source organs to the drains (ELKHOUNI; ZORRIG; SMAOUI, 2016). Mg is required for the synthesis and structural stability of chlorophyll (ALBUS et al., 2012), and in the photochemical phase of photosynthesis, the synthesis of ATP in chloroplasts is increased by the extreme supply of Mg, as this process has an absolute requirement for Mg as a binding component between ADP and the ATPase enzyme (KIRKBY, 2011). In the chemical phase of photosynthesis, the modulation of the Rubisco enzyme requires Mg to increase the enzyme's affinity for its substrate (CO_2) and the rate of new enzyme synthesis (CAKMAK; YAZICI, 2010).

Magnesium-deficient plants have lower root growth and lower root/shoot ratio, this is because the loading of carbohydrates in the phloem is an active process, dependent on the consumption of ATP, which in turn also requires Mg (BARROSO, 2013). The accumulation of non-structural sugars in the leaves and the change in the carbohydrate partition observed under Mg deficiency are attributed to an inhibition of phloemic loading (HAWKESFORD et al. 2012).

Stress due to high concentrations of magnesium are not common, but serpentine soils and semi-arid regions can have a high Mg: Ca ratio and thus affect the growth and development of crops, as high concentrations of Mg^{2+} restricts water absorption by the roots, causing high osmolarity, ionic toxicity, and as a consequence oxidative stress, decreased nutrient availability, especially Ca^{2+}, K^+ and Mn^{2+} and reductions in the growth rate (GERENDÁS; FÜHRS, 2013; NIU et al. 2018; PARIHAR; SINGH; SINGH; 2015).

According to EMBRAPA (2020), the references of Mg^{2+} contents for the purpose of interpreting the chemical analysis of the soil, for soybean crops are: low <0.5 $cmol_c/dm^3$ medium 0,5-0,9 $cmol_c/dm^3$ and high > 0,9 $cmol_c/dm^3$ for soils with CTC <5 $cmol_c/dm^3$. And for soils with CTC $\geq$ 5 $cmol_c/dm^3$ the reference levels are, low <0,9 $cmol_c/dm^3$; medium 0,9-2,5 $cmol_c/dm^3$ and high> 2,5 $cmol_c/dm^3$. And the Mg^{2+} reference values for interpreting the results of the analysis of leaves, without petiole, of soybeans are: low <2,8g/ kg; enough 2,8 to 5,0 g/ kg and high> 5,0 g/ kg.

A possible solution to the damage caused by low and high addition of Mg^{2+} in plants may be the use of 24-epibrassinolide (EBR), since the ability of this steroid to modulate positive responses to situations of abiotic stresses such as stimulation of the antioxidant system (FARIDUDDIN et al., 2015), increased levels of chlorophyll (FERREIRA et al., 2018),

efficiency of photosystem II (YUSUF; FARIDUDDIN; AHMAD, 2011), and growth rate (DA FONSECA ; DA SILVA; LOBATO, 2020).

Brassinoids (BRs) are polyhydroxylated plant hormones, active in very low concentrations, indicated by their pleiotropic involvement in various physiological processes and defense strategies during stress in plants (BAJGUZ, 2010; COSTA et al., 2018; KANWAR et al., 2017).

Brassinosteroids were found in several species of plants (including algae), distributed in different organs, being pollens, immature seeds, roots and flowers, which had the highest steroid content (TANG; HAN; CHAI, 2016). The first works were carried out by Mitchell and colleagues in 1970, and identified a steroid substance in Brassica's pollen and called it 'brassinas' (MITCHELL et al. 1970). Following years, it was extracted chemically by Grove et al. (1979) of rapeseed plant pollen (Brassica napus L.) which was called 'brassinolide'.

Currently, about 70 types of natural analogues of brassinolide have been isolated from tissues of various plant species (KUTSCHERA; WANG, 2012), with emphasis on brassinolide, 24-epibrassinolide and 28-homobrassinolide, which have been shown to be the most biologically active, and widely used in physiological studies of cultivated plants (BAGHEL et al. 2019).

The biosynthesis of BRs in plants occurs from campesterol, sinosterol and cholesterol. In general, the route starts with sterol campesterol, which is derived from cycloartenol. Campesterol is converted to castasterone through two routes called early route and late oxidation route at C-6, the two routes converge to castasterone which is then converted to brassinolide, the most active BR (BAGHEL et al., 2019).

The proposed model to explain brassinosteroid signaling in plants includes the direct binding of a BR molecule in the extracellular domain of the plasma membrane to the kinase receptor rich in leucine repeats called BRASSINOSTEROID INSENSITIVE 1 (BRI1) (CLOUSE; LANGFORD; MCMORRIS, 1996) . Research with *A. thaliana* has shown that BRs are perceived by receptor kinases that transduce the signal from the cell surface to the nucleus by an intracellular cascade of protein-protein interactions, involving kinases, phosphatases, 14-3-3 proteins and nuclear transcription factors, in addition, BR signaling is regulated by the plant's endocytic machinery because the increased endosomal location of the BR receptor increases signaling (CODREANU; RUSSINOVA, 2011).

Studies indicate that BRs give tolerance to plants to abiotic stresses, stimulating the activity of antioxidant enzymes and promoting the maintenance of the photosynthetic rate (AHANGER et al., 2018; RAMAKRISHNA; RAO, 2015; WANG; LI; ZHANG, 2012). Research by DOS SANTOS et al. (2020) revealed that 24-epibrassinolide mitigated oxidative stress induced by low and high Zn concentration in *Glycine max*. Likewise, RODRIGUES et al. (2020) found that the exogenous application of EBR (100 nM) promoted the maintenance of photosynthetic pigments (*Chl a*, *Chl b*, *total Chl* and *Car*) and less accumulation of EROs (O_2^- and H_2O_2) in soybean plants (*Glycine max*) with deficiency and toxicity of Mn.

The general hypothesis of the work considers the deleterious effects promoted by the inadequate supply of Mg^{2+} (deficiency or toxicity) on antioxidant metabolism, gas exchange,

photosynthetic pigments and possible repercussions on the anatomy and growth of *Glycine max* plants. On the other hand, EBR represents a biodegradable substance that efficiently modulates tolerance in plants exposed to deficiency or toxicity.

In this sense, EBR can be a possible solution to mitigate the damage caused by deficiencies and excess of Mg in plants because this steroid has a set of actions linked to increases in nutrient content (YUAN et al. 2015), elimination of reactive species oxygen (DA SILVA CUNHA et al. 2020) and biomass stimulation (PEREIRA et al. 2018). Therefore, the general objective of this study was to evaluate whether the foliar application of EBR can mitigate oxidative stress in soybean plants submitted to high and low addition of Mg and to evaluate its possible repercussions on anatomical, nutritional, biochemical, physiological and morphological behaviors.

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Foliar-applied 24-epibrassinolide systemically triggers tolerance to magnesium stress in soybean plants: plausible responses focused on root and leaf structures

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ABSTRACT

The adverse effects caused by inadequate magnesium (Mg) supplies (deficiency or toxicity) cause disorders in root and leaf structures, compromising the uptake and transport of nutrients and gas exchange. However, 24-epibrassinolide (EBR) is a biodegradable and ecologically viable steroid that exhibits benefits on cell division and vascular differentiation. This study aimed to evaluate whether exogenous EBR application can attenuate the deleterious effects linked to inadequate Mg supplies on the anatomical structures of roots and leaves in soybean plants. 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 Mg supplies (0.0225, 2.25 and 225 mM Mg, described as low, control and high supplies of Mg). In general, low and high Mg supplies promoted negative changes in the anatomical variables evaluated. In root tissue, pretreatment with 100 nM EBR stimulated the root structures, confirming the systemic action of EBR and benefits related to the differentiation of these structures. Treatment with low Mg + EBR promoted increases in epidermis thickness from the abaxial leaf side (ETAb) and spongy parenchyma thickness (SPT) in 20% and 13%, respectively. High Mg and EBR were increased in ETAb (31%) and SPT (15%) compared with equal treatment without steroids, improving protection and CO2 fixation. Our results indicate that the foliar application of EBR mitigated the deleterious effects linked to inadequate Mg supply in the anatomical structures of the root and leaf, confirming the systemic action of this steroid.

Introduction

Soybean (*Glycine max* L.) is an oleaginous crop with significant economic potential as a major nutritional resource for humans and animals worldwide. Its grains contain approximately 40% proteins, 20% oil, and 30% carbohydrates, with good proportions of vitamins, fibers and minerals (Bakhshandeh and Gholamhossieni 2018). Grains may be used in industrialized products aiming at human or animal consumption (Nishinari et al. 2014; Sanjukta and Rai 2016). The area cultivated with soybean plants covers approximately 123 million ha, with an annual production of 352.6 million tons. The United States, Brazil and Argentina have the largest shares in the global production (FAO 2019).

Magnesium (Mg) is the third most abundant cation in plant tissues and plays key roles in plant metabolism (Billard et al. 2016). Mg is required for the synthesis and stabilization of the chlorophyll molecule (Zhang et al. 2018), for stacking of the granum in the chloroplast (Ceppi et al. 2012) and for several catalyzing enzymes, such as for instance phosphoenolpyruvate carboxylase, glutathione synthase, phosphate kinases,

RNA polymerases and ATPases (Verbruggen and Hermans 2013). According to Cakmak and Yazici (2010), Mg is a macronutrient that is poorly studied and often neglected despite its crucial roles in plant metabolism.

Mg stress (deficiency or toxicity) delays plant development, significantly affecting the production potential of crops (Khaitov 2018; Niu et al. 2018). Mg deficiency in plants often occurs due to low levels of exchangeable Mg in acidic soils (Wang et al. 2020) or in areas fertilized only with nitrogen, phosphorus and potassium, interfering with Mg availability due to the antagonistic relationship between these elements (Farhat et al. 2016). High Mg^{2+} contents can occur in serpentine soils, areas contaminated by heavy metals and semiarid regions under water stress conditions, presenting low water availability for the assimilation of essential nutrients (Conn et al. 2011).

In plants, Mg deficiency compromises light absorption and photosynthetic carbon dioxide (CO_2) fixation because this element is a limiting factor for chlorophyll biosynthesis (Neuhaus et al. 2013). Another problem is related to electron accumulation that is not used during light-dependent reactions in chloroplasts,

resulting in overproduction of reactive oxygen species, photooxidation, membrane damage and leaf senescence (Tanoi and Kobayashi 2015). Indirectly, a low Mg supply also impairs the nutrient transport and transpiration process (Huang et al. 2019), whereas high Mg affects the stability of the cell wall, membrane permeability, and uptake and translocation of essential nutrients (Guo et al. 2014) due to osmotic stress and ionic toxicity (Nawaz et al. 2017).

The root is an organ with essential functions, which contributes to nutrient uptake and water influx. It plays a fundamental role in abiotic stress tolerance due to morphological modulations that include the length and diameter of the root (York et al. 2013). Anatomically, the epidermis, cortex and vascular cylinder are structures with interesting properties that can serve to improve plant protection in stressed environments, facilitating the transport of water and mineral ions (Cui 2015; Barberon et al. 2016). In other words, the stimulation of these tissues can improve the tolerance to abiotic stresses and maximize the symplastic transport of nutrients (Lobato et al. 2020).

Leaf anatomy reveals important structures for plant growth and yield because these structures are related to physiological processes, such as photosynthesis and water use efficiency (Terashima et al. 2011). The leaf epidermis is a tissue with functions that are linked to the protection of internal structures and is composed of cells specialized in controlling water loss and the regulation of gas exchange (Zhang et al. 2011; Glover et al. 2016). Palisade and spongy parenchyma tissues have cells responsible for the equilibrium in light distribution and CO_2 diffusion for photosynthesis (Tholen et al. 2012). In this context, the stomatal density, development of the mesophyll surface area, and geometry of the palisade parenchyma often improve CO_2 availability, photon absorption and temperature control (Hao et al. 2019; Retta et al. 2020).

Exogenous 24-epibrassinolide (EBR) application may represent a possibility to mitigate the damage caused by Mg stress in higher plants. This molecule, which is extracted from plant tissue, is a biodegradable, ecologically viable polyhydroxylated steroid that is involved in a wide range of activities (Hou et al. 2018; Jan et al. 2018), including elongation and cell division (Zhiponova et al. 2013), vascular differentiation (Lee et al. 2019; Fan et al.

2020) and root development (Wei and Li 2016). Additionally, brassinosteroids (BRs) are recognized to positively modulate plant responses to environmental stresses (Ahammed et al. 2020; Trevisan et al. 2020; Kour et al. 2021), such as drought, salinity, heavy metals and herbicides, with benefits in biomass production (Oliveira et al. 2019) and ionic homeostasis in plant tissues (Yuan et al. 2015).

The hypothesis of this research considered the adverse effects caused by the inadequate supply of Mg (deficiency or toxicity) on anatomical responses. However, EBR is a plant growth regulator with promising effects on plant metabolism, more specifically stimulating root development (Nazir et al. 2021), xylem differentiation (Zheng et al. 2020) and leaf anatomy (Fonseca et al. 2020). Therefore, this study aimed to evaluate whether exogenous EBR application can attenuate the deleterious effects linked to inadequate Mg supplies on the anatomical structures of roots and leaves in soybean plants.

Materials and methods

Location and growth conditions

This experiment was performed on the Campus of Paragominas of the Universidade Federal Rural da Amazônia, Paragominas, Brazil (2°55′ S, 47°34′ W). The study was conducted in a greenhouse with controlled temperature and humidity. The minimum, maximum, and median temperatures were 23.1, 28.7 and 26.3 °C, respectively. The relative humidity during the experimental period varied between 60% and 80%.

Containers, seeds and Mg supplies

Soybean plants were cultivated under semi-hydroponic conditions, and the pots had one hole in the bottom covered with mesh to maintain the substrate and aerate the roots. Solution absorption was performed by capillary action, with these pots placed into other containers (0.15 m in height and 0.15 m in diameter) containing 500 mL of distilled water for eight day. Seeds of *Glycine max* (L.) Merr. var. M8644RR Monsoy™ were germinated and grown in 1.2-L pots (0.15 m in height and 0.10 m in diameter) filled with a mixed substrate of sand

Table 1. Root anatomy in soybean plants sprayed with EBR and exposed to different Mg supplies.

EBR	Mg supply	RET (µm)	RDT (µm)	RCD (µm)	VCD (µm)	RMD (µm)
−	Low	14.7 ± 1.0 Ab	19.8 ± 1.0 Bb	66.9 ± 0.8 Bb	67.8 ± 2.1 Bb	43.9 ± 1.5 Bb
−	Control	15.0 ± 0.7 Ab	23.0 ± 0.5 Ab	106.4 ± 6.2 Ab	112.7 ± 4.3 Aa	51.5 ± 0.9 Ab
−	High	11.1 ± 0.6 Bb	16.7 ± 0.7 Cb	58.3 ± 2.8 Cb	47.4 ± 2.6 Cb	23.0 ± 0.7 Cb
+	Low	22.2 ± 0.7 Ba	22.0 ± 0.2 Ba	94.0 ± 1.3 Ba	94.3 ± 3.4 Ba	52.8 ± 0.8 Ba
+	Control	24.0 ± 0.9 Aa	24.1 ± 0.5 Aa	115.9 ± 1.9 Aa	113.5 ± 2.0 Aa	58.4 ± 1.4 Aa
+	High	18.1 ± 0.5 Ca	19.0 ± 0.3 Ca	87.9 ± 0.6 Ca	82.6 ± 2.0 Ca	44.2 ± 0.8 Ca

RET = Root epidermis thickness; RDT = Root endodermis thickness; RCD = Root cortex diameter; VCD = Vascular cylinder diameter; RMD = Root metaxylem diameter. Columns with different uppercase letters between Mg supplies (low, control and high Mg supply under equal EBR level) and lowercase letters between EBR level (with and without EBR under equal Mg supply) indicate significant differences from the Scott-Knott test (P < 0.05). Means ± SD, n = 5.
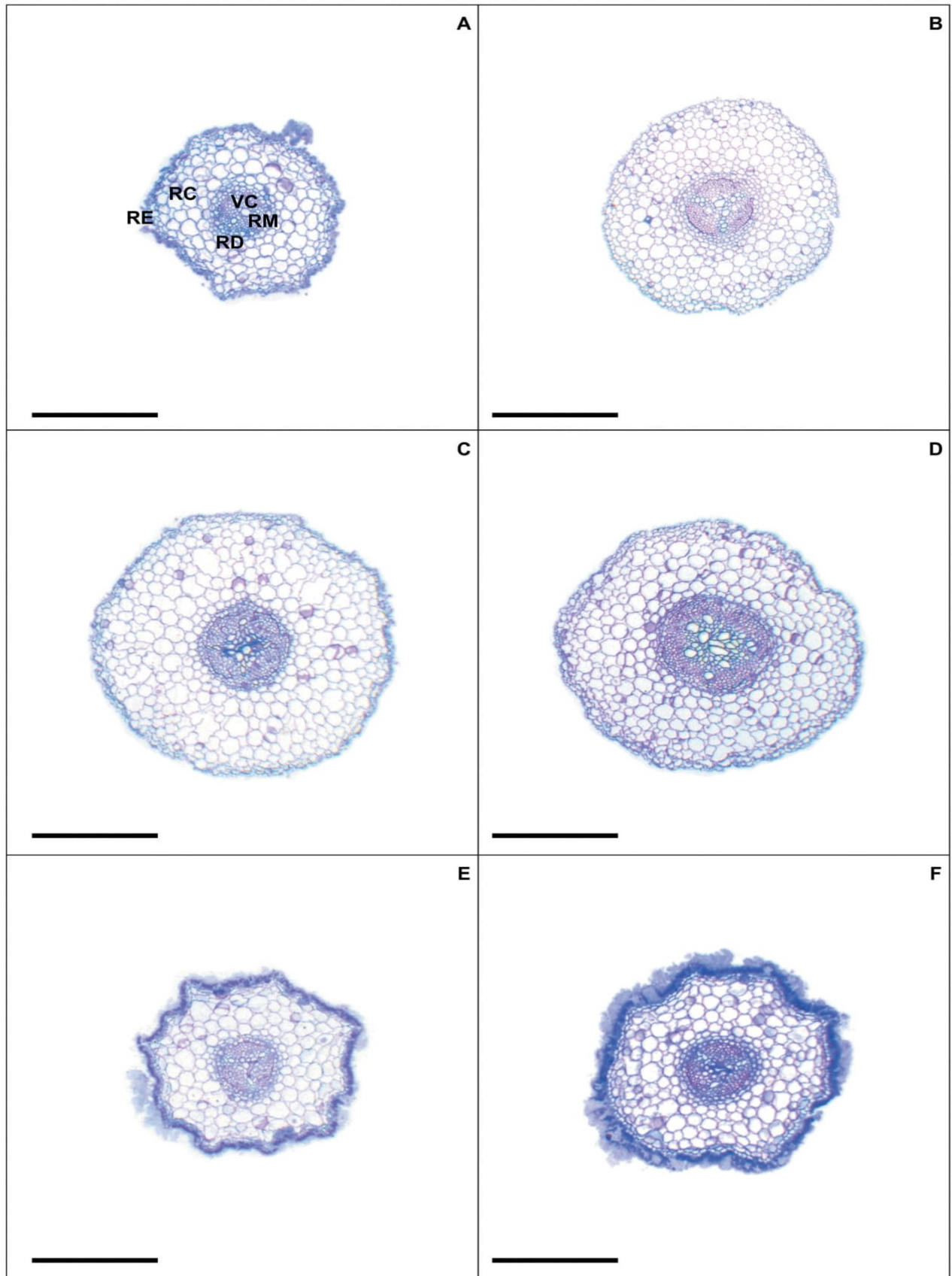
Figure 1. Root cross sections in soybean plants sprayed with EBR and exposed to different Mg supplies. Low Mg without EBR (A), low Mg with EBR (B), control Mg without EBR (C), control Mg with EBR (D), high Mg without EBR (E) and high Mg with EBR (F). RE = Root epidermis; RC = Root cortex; RD = Root endodermis; VC = Vascular cylinder; RM = Root metaxylem. Bars: 200 μm.

Table 2. Stomatal characteristics in soybean plants sprayed with EBR and exposed to different Mg supplies.

EBR	Mg supply	SD (stomata per mm^2)	PDS (µm)	EDS (µm)	SF	SI (%)
Adaxial face						
–	Low	169 ± 8 Bb	12.6 ± 0.3 Ba	20.7 ± 0.5 Aa	0.61 ± 0.03 Aa	5.7 ± 0.3 Ab
–	Control	221 ± 11 Aa	12.0 ± 0.3 Ba	19.8 ± 0.3 Ba	0.61 ± 0.04 Aa	5.8 ± 0.4 Ab
–	High	160 ± 13 Ba	13.2 ± 0.2 Aa	21.3 ± 0.4 Aa	0.62 ± 0.05 Aa	5.4 ± 0.2 Ab
+	Low	209 ± 6 Aa	11.4 ± 0.2 Bb	19.8 ± 0.4 Aa	0.58 ± 0.02 Aa	7.0 ± 0.4 Aa
+	Control	231 ± 24 Aa	11.1 ± 0.3 Bb	18.9 ± 0.3 Bb	0.59 ± 0.03 Aa	7.5 ± 0.6 Aa
+	High	178 ± 6 Ba	12.0 ± 0.2 Ab	20.3 ± 0.4 Ab	0.59 ± 0.03 Aa	6.1 ± 0.3 Ba
Abaxial face						
–	Low	284 ± 17 Bb	11.5 ± 0.4 Aa	19.5 ± 0.7 Aa	0.59 ± 0.03 Aa	8.8 ± 0.4 Ab
–	Control	323 ± 17 Ab	11.0 ± 0.5 Aa	19.2 ± 0.6 Aa	0.58 ± 0.02 Aa	9.2 ± 0.6 Ab
–	High	252 ± 15 Ba	12.0 ± 0.5 Aa	20.5 ± 0.9 Aa	0.59 ± 0.02 Aa	7.8 ± 0.2 Bb
+	Low	368 ± 28 Aa	10.7 ± 0.4 Aa	17.7 ± 0.3 Bb	0.60 ± 0.04 Aa	10.2 ± 0.6 Ba
+	Control	525 ± 70 Aa	9.6 ± 0.3 Bb	16.3 ± 0.2 Cb	0.61 ± 0.04 Aa	11.9 ± 0.5 Aa
+	High	294 ± 9 Ca	11.2 ± 0.6 Aa	18.9 ± 0.5 Ab	0.59 ± 0.03 Aa	9.1 ± 0.4 Ba

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

and vermiculite at a ratio of 3:1. A nutritive solution was used for nutrients, with the ionic strength (treatment) beginning at 50% (8th day) and later modified to 100% after two days (10th day). The plants received the following macro- and micronutrients contained in the nutrient solution: 8.75 mM KNO_3, 7.50 mM $Ca(NO_3)_2 \cdot 4H_2O$, 3.25 mM $NH_4H_2PO_4$, 0.75 mM K_2SO_4, 62.50 µM KCl, 31.25 µM H_3BO_3, 2.50 µM $MnSO_4 \cdot H_2O$, 2.50 µM $ZnSO_4 \cdot 7H_2O$, 0.63 µM $CuSO_4 \cdot 5H_2O$, 0.63 µM $NaMoO_4 \cdot 5H_2O$, and 250.00 µM $NaEDTAFe \cdot 3H_2O$, with Mg concentrations adjusted to each treatment. For Mg treatments, $MgCl_2$ was used at concentrations of 0.0225 mM (low), 2.25 mM (control) and 225 mM (high) applied over 10 days (days 25–35 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 35 of the experiment, root and leaf tissues were harvested for anatomical analyses.

Preparation and application of 24-epibrassinolide (EBR)

Twelve-day-old plants were sprayed with 24-epibrassinolide (EBR) or Milli-Q water (containing a proportion of ethanol that was equal to that used to prepare the EBR solution) at 5-day intervals until day 35. EBR (0 and 100 nM, Sigma-Aldrich, USA) solutions were prepared by dissolving the solute in ethanol followed by dilution with Milli-Q water [ethanol:water (v/v) = 1:10,000] (Ahammed et al. 2013).

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 Mg supplies

(0.0225, 2.25 and 225 mM Mg, described as low, control and high supply of Mg). With five replicates for each of six treatments, a total of 30 experimental units were used in the experiment, with one plant in each unit.

Measurements of anatomical parameters

Samples were collected from the middle region of the leaf blades of fully expanded leaves of 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 Leica™ (Leica, Nussloch, Germany). Transverse sections with a thickness of 5 µm were obtained with a rotating microtome (model Leica RM 2245, Leica Biosystems) and stained with toluidine blue (O'Brien et al. 1964). For stomatal characterization, the epidermal impression method was used according to Segatto et al. (2004). The slides were observed and photomicrographed under an optical microscope (Motic BA 310; Motic Group Co. LTD.) coupled to a digital camera (Model Motic 2500; Motic Group Co., LTD.). The images were analysed with 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 adaxial leaf side (ETAd), the epidermis thickness from the abaxial leaf side (ETAb), the palisade parenchyma thickness (PPT), the spongy parenchyma thickness (SPT) and the PPT/SPT ratio. For both leaf faces, the stomatal density (SD) was calculated as the number of stomata per unit area and the stomatal functionality (SF) was calculated as the PDS/EDS ratio, as described by Castro et al. (2009). The stomatal index (SI) was calculated as the percentage of stomata in relation to total epidermal cells, by area. In
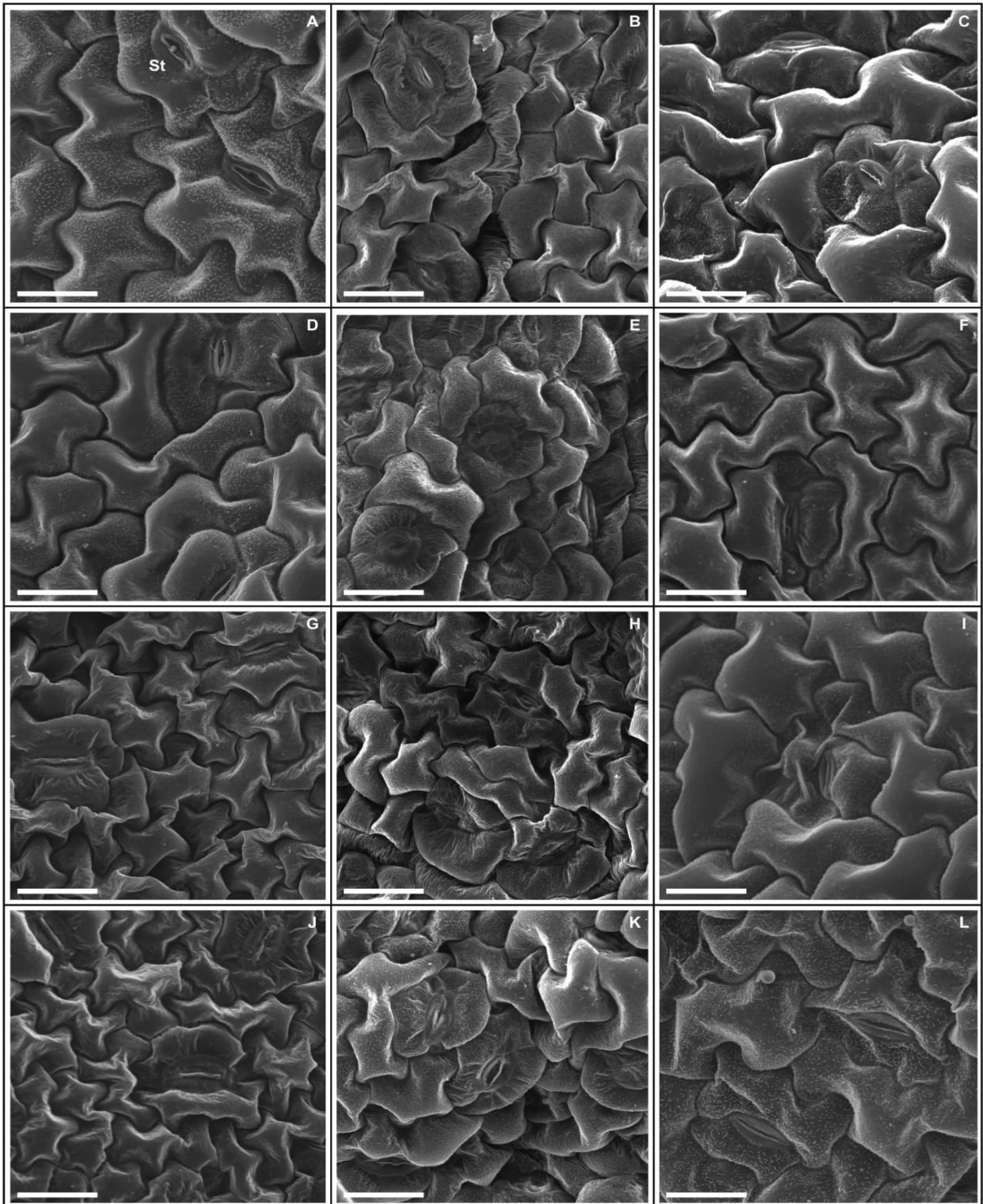
Figure 2. Adaxial (A-F) and abaxial leaf surface (G-L) in scanning electron microscopy showing stomata in soybean plants sprayed with EBR and exposed to different Mg supplies. Low Mg without EBR (A and G), low Mg with EBR (D and J), control Mg without EBR (B and H), control Mg with EBR (E and K), high Mg without EBR (C and I) and high Mg with EBR (F and I). Legend: St = Stomata. Bars: 20 μm.

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.

Scanning electron microscopy

The middle region of the leaf blade previously fixed in 70% fixation solution was dehydrated in an ethyl

Table 3. Leaf anatomy in soybean plants sprayed with EBR and exposed to different Mg supplies.

EBR	Mg supply	ETAd (µm)	ETAb (µm)	PPT (µm)	SPT (µm)	Ratio PPT/SPT
_	Low	18.8 ± 0.5 Bb	18.7 ± 0.7 Bb	68.1 ± 1.3 Bb	59.9 ± 1.3 Bb	1.13 ± 0.02 Aa
_	Control	20.6 ± 0.4 Ab	20.7 ± 0.6 Ab	73.6 ± 1.5 Ab	67.3 ± 0.6 Ab	1.09 ± 0.01 Ba
_	High	16.0 ± 0.7 Cb	13.1 ± 0.6 Cb	61.9 ± 1.1 Cb	52.4 ± 0.7 Cb	1.18 ± 0.02 Aa
+	Low	21.7 ± 0.8 Ba	22.4 ± 0.5 Ba	72.7 ± 0.9 Ba	67.6 ± 1.0 Ba	1.08 ± 0.02 Ab
+	Control	23.5 ± 0.4 Aa	23.6 ± 0.5 Aa	77.2 ± 1.5 Aa	76.3 ± 1.4 Aa	1.01 ± 0.03 Bb
+	High	18.6 ± 0.3 Ca	17.1 ± 0.4 Ca	66.5 ± 1.9 Ca	60.2 ± 1.5 Ca	1.10 ± 0.03 Ab

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

series, processed in a critical point CO_2 dryer and coated with gold (one layer approximately 20 nm thick) under a current of 25 mA. Micrographs were obtained by scanning electron microscopy (model LEO 1450 VP; Zeiss).

Data analysis

The data were subjected to an analysis of variance, and significant differences between the means were determined using the Scott-Knott test at a probability level of 5% (Steel et al. 2006). Standard deviations were calculated for each treatment. For all statistical procedures the Assistat software was used.

Results

EBR systemically induced root protection against Mg^{2+} stress

Low and high Mg supplies reduced the root structures that were measured (Table 1 and Figure 1). However, EBR spray in plants submitted to low Mg promoted increases in RET, RDT, RCD, VCD and RMD of 51%, 11%, 41%, 39% and 20%, respectively, compared with the same treatment without EBR, while the high Mg + EBR treatment resulted in increases of 63%, 14%, 51%, 74% and 92%, respectively. In the root cross-section (Figure 1), EBR treatment in plants submitted to different Mg supplies resulted in more developed structures, with higher amounts of cortical cell layers and more evident endoderm, when compared with plants without EBR treatment. In parallel, the vascular cylinder of plants sprayed with EBR exhibited elements of vessels with a larger diameter, demonstrating the late maturation of vascular cells in relation to plants without EBR.

Steroids attenuated the negative effects linked to low and high Mg supplies on stomatal characteristics

The low and high Mg supplies caused negative effects on stomatal characteristics; however, EBR mitigated these deleterious impacts (Table 2 and Figure 2). On

the adaxial side, plants exposed to low Mg + EBR had increases of 24% and 23% in SD and SI, respectively, and decreases in PDS (10%) and EDS (4%). High Mg + EBR resulted in increments of 11% and 13% in SD and SI, respectively, and decreases in PDS (9%) and EDS (5%), respectively, compared with the same treatment without EBR. For the abaxial side, the low Mg and 100 nM EBR spray resulted in increases of 30%, 2% and 16% in the variables SD, SF and SI, respectively, and reductions in PDS (7%) and EDS (9%), when compared with similar treatment without EBR. Under high Mg + EBR, increases in SD (17%) and SI (17%) were observed, and reductions in PDS (7%) and EDS (8%) occurred.

Pretreatment with EBR affected the leaf epidermis in plants exposed to inadequate Mg supplies

The low and high Mg caused alterations, in general reduction in leaf structure (Table 3 and Figure 3). Despite these changes, the treatment with low Mg + EBR promoted increments in ETAd (15%), ETAb (20%), PPT (7%) and SPT (13%) and a reduction in PPT/SPT (4%), compared with the same treatment without EBR. At high Mg and EBR, increases in ETAd (16%), ETAb (31%), PPT (7%) and SPT (15%) were obtained, and a decrease in the PPT/SPT ratio (7%) was observed. For the leaf cross-section (Figure 3), plants sprayed with EBR had an altered arrangement of the mesophyll, mainly in the treatment under low Mg and + EBR, presenting a lower degree of compaction of the palisade and spongy tissues. EBR application induced the development of the vascular system, with higher extensions of the bundle-sheath cells along the leaf cross-section in relation to plants without EBR. In the frontal view, plants treated with EBR had an apparent expansion of epidermal cells, mainly on abaxial surfaces.

Discussion

EBR attenuated the negative effects related to low and high concentrations of Mg^{2+} on the root anatomy, inducing increases in root structures (RET, RDT, RCD, VCD and RMD) (Table 1, Figure 1). The
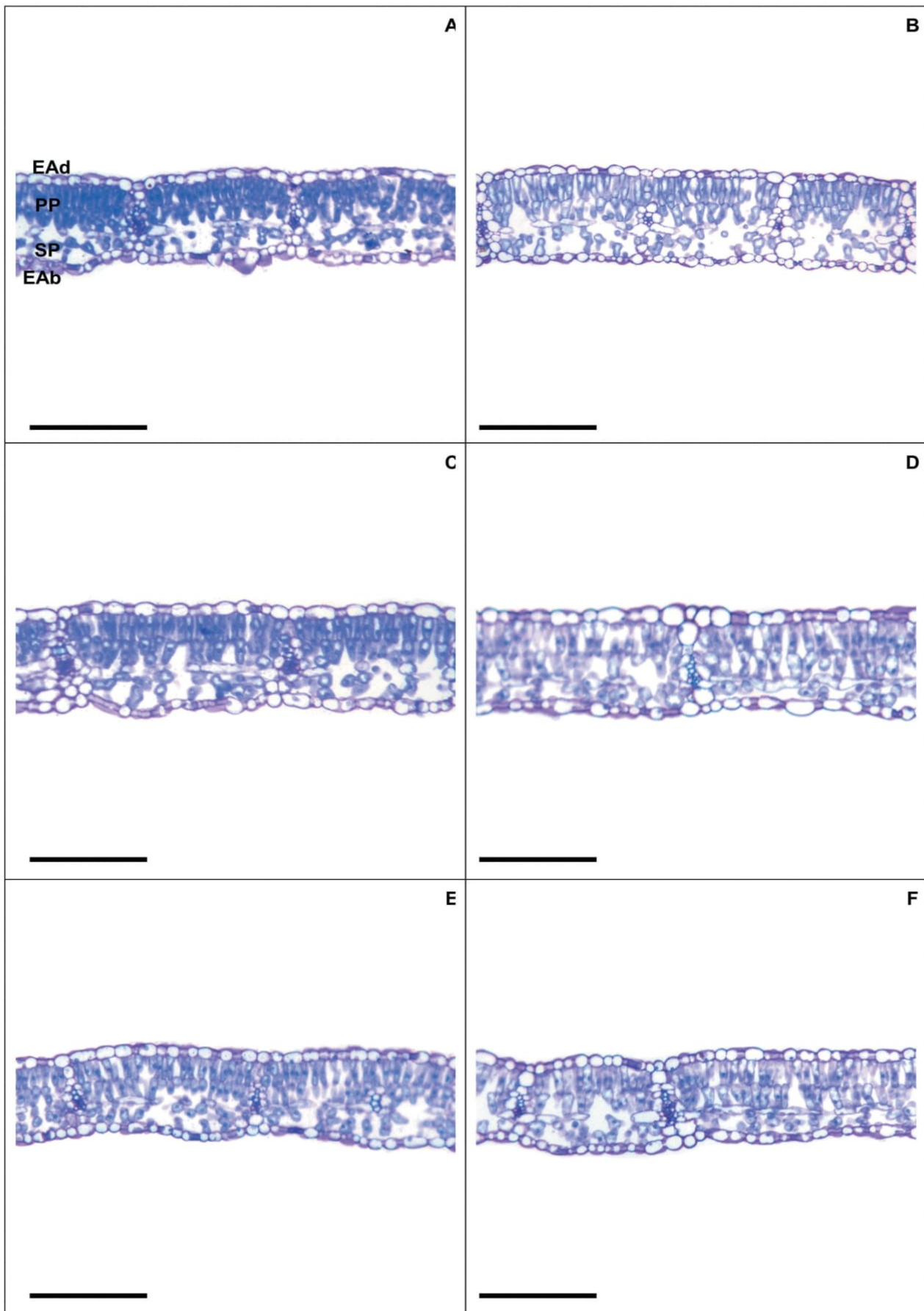
Figure 3. Leaf cross sections in soybean plants sprayed with EBR and exposed to different Mg supplies. Low Mg without EBR (A), low Mg with EBR (B), control Mg without EBR (C), control Mg with EBR (D), high Mg without EBR (E) and high Mg with EBR (F). EAd = Adaxial epidermis; EAb = Abaxial epidermis; PP = Palisade parenchyma; SP = Spongy parenchyma. Bars: 200 μm.

increments induced in RET, RCD and VCD confirm that the EBR stimulates cell multiplication and differentiation of the root tissues, improving the hydraulic conductivity and protection of this organ (Hacham et al. 2011). The epidermis, endoderm and cortex are specialized tissues for protection and selectivity in the roots, and increases detected in these tissues may contribute as a barrier and protection of the root against abiotic stresses (Javelle et al. 2011; Doblas et al. 2017). EBR improves the absorption of water and nutrients because VCD and RMD are fully vascularized and the diameter of the xylem vessels is directly related to the maintenance of water conductivity in the xylem, in which larger vessels of the xylem are useful to improve water extraction from deeper layers of the soil (Singh et al. 2013). Studies conducted by Maia et al. (2018) and Lee et al. (2019) confirm the role of EBR linked to xylem differentiation and the secondary growth of *Solanum lycopersicum* L., increasing VCD and RMD. Santos et al. (2020) found that the application of 100 nM EBR to *Glycine max* plants subjected to different Zn stresses (0.2, 20 and 2000 μM) promoted increases in RET (16%), RDT (3%), RCD (14%), VCD (33%) and RMD (74%). These authors suggested that the higher densities of VCD and RMD must facilitate transport of water and nutrients by the symplastic pathway.

EBR attenuated the negative effects linked to low and high Mg supplies on SD, SI, PDS and EDS (Table 2, Figure 2). This steroid stimulates the production of stomata by regulating specific proteins of stomatal development, such as BIN2 kinase, MAP kinase and SPCH (Serna 2014), and increments in SD suggest a higher concentration of CO_2 that is absorbed and available for the photosynthetic process. Tanaka et al. (2013) studied *Arabidopsis thaliana* (L.) Heynh. lines with overexpression or silencing of the *STOMAGEN* gene, an upregulator of stomatal density, and used them to produce plants with DS two to three times higher than in wild-type plants, demonstrating that increases in SD positively modulated gas exchange, mainly P_N, E and g_s. Stomata are epidermal pores that are essential for gas exchange between plants and the environment. Stomatal regulation and density guarantee the capture of CO_2 to maximize photosynthetic activity and minimize water loss through transpiration (Lawson and Blatt 2014). Reductions observed in PDS and EDS reveal the beneficial influence of the EBR on stomatal shape, inducing an elliptical format in which these structures continuously adjust to environmental and intracellular conditions (Drake et al. 2013; Raven 2014; Martins et al. 2018). Wang et al. (2015) evaluated *Vitis vinifera* L. seedlings under water stress and observed increases in stomatal density after exogenous EBR application (0.05, 0.10 and 0.20 mg L^{-1}). Oliveira et al. (2019) also found increases in SD and SI in *Eucalyptus urophylla* S.T.

Blake plants subjected to salt stress (250 mM NaCl) and treated with EBR (50 nM), related to beneficial roles triggered by this plant steroid, stimulating the stomata production. Sun et al. (2014) evaluated the plasticity of stomatal size and density in *Solanum tuberosum* L. leaves under three irrigation regimes and different phosphorus concentrations, detecting a reduction in SD in treatments under irrigation deficit and 0 mg P kg^{-1} of soil, concluding that indicate that stomatal morphology is plastic to changes of soil moisture status. Adamski et al. (2012) researched the impacts of Fe on the anatomy of *Ipomoea batatas* (L.) Lam. and found a decrease in SD and increase in PDS and EDS on the abaxial leaf side at high Fe concentrations (4.5 and 9.0 mmol L^{-1} Fe). These effects were accompanied by the inefficient detoxification of reactive oxygen species, delaying the stomatal development.

Our results shows that plants pretreated with EBR and submitted to low and high concentrations of Mg present alterations in leaf anatomy (increase in ETAd, ETAb, PPT and SPT) (Table 3, Figure 3). These beneficial effects on ETAd and ETAb may be associated with EBR's ability to enhance growth (Fàbregas and Caño-Delgado 2014; Latha and Vardhini 2016), cell division and elongation (González-García et al. 2011), improving the stability and integrity of membranes (Karlidag et al. 2011; Khan et al. 2015). Increase in ETAd and ETAb also suggest a positive effect of EBR on the protection and adaptation of the mesophyll and epidermis by maximizing the efficiency of water use. The epidermis is indeed a covering tissue involved in avoiding excessive water losses during the transpiration process (Polizel et al. 2011). An increase in the thickness of the palisade parenchyma (PPT) can maximize the fixation and absorption of CO_2 because this tissue contains a large amount of chlorophyll and is responsible for the penetration of light into deeper mesophilic layers (Tholen et al. 2012; Asmar et al. 2013). In relation to the spongy parenchyma, the increases in interconnected intercellular spaces clearly facilitate the accumulation and later diffusion of CO_2 to the carbon fixation sites (Ennajeh et al. 2010; Ho et al. 2016). Zhiponova et al. (2013) evaluated the roles of EBR in cell division, expansion and differentiation in the leaves of two *Arabidopsis thaliana* genotypes (wild and EBR-deficient mutant) sprayed with 100 nM EBR and found significant increases in epidermal cell size, mesophyll cell size, and leaf blade area (60%) in the EBR-deficient mutant compared with the same mutant without EBR. Oliveira et al. (2019) found that pretreatment with 50 nM EBR in young *Eucalyptus urophylla* plants subjected to saline stress (250 mM NaCl) generated increases in ETAd, ETAb, PPT and SPT of 7%, 22%, 14%

and 25%, respectively, compared with the equal treatment without EBR. These authors detected the ability of EBR to stimulate the processes of cell division and elongation due to modulation of Ca^{2+} levels in the leaf, with beneficial repercussions on the stability and integrity of membranes.

Conclusion

This research clearly demonstrates that inadequate Mg supplies (low and high) promotes negative changes in root and leaf anatomy. In root tissue, pretreatment with 100 nM EBR stimulated RET, RDT, RCD, VCD and RMD, confirming the systemic action of EBR and benefits related to the differentiation of these structures and protection of the root system against simulated stress, in addition to suggesting benefits in the transport of water and nutrients by the xylem. Concomitantly, this study reveals that steroids attenuate the negative effects associated with Mg stress on leaf structures, improving the protection and adaptation of this organ (ETAd and ETAb) and facilitating the diffusion and fixation of CO_2 by the mesophyll (PPT + SPT). Therefore, our results indicate that the foliar application of EBR mitigated the deleterious effects linked to inadequate Mg supply in the anatomical structures of the root and leaf in soybean plants, confirming the systemic action of this steroid.

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Disclosure statement

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Data availability statement

Data are available upon request to the corresponding author.

Author contributions

AKSL was the advisor of this project, planned all phases of the research and critically revised the manuscript. LAS and BRSS conducted the experiment, performed anatomical determinations and wrote and edited the manuscript. All authors read and approved the final version of the manuscript.

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14 **Title page**

15
16 **24-epibrassinolide simultaneously delays chlorophyll degradation and stimulates the photosynthetic**

17 **machinery in magnesium stressed soybean plants**

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34 **Author contribution statement**
35 AKSL was the advisor of this project, planning all phases of the research and critically revised the manuscript.

36 LAS conducted the experiment and performed physiological, biochemical and morphological determinations, as

37 well as wrote and edited the manuscript. BLB performed nutritional determinations. All authors read and approved

38 final version of manuscript.

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40 **Data availability statement**
41 Data are available upon request to the corresponding author.

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

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56   **Abbreviations**

APX	Ascorbate peroxidase
BRs	Brassinosteroids
B	Boron
Ca	Calcium
CAR	Carotenoids
CAT	Catalase
Chl a	Chlorophyll a
Chl b	Chlorophyll b
C_i	Intercellular CO_2 concentration
CO_2	Carbon dioxide
Cu	Copper
E	Transpiration rate
EBR	24-epibrassinolide
EL	Electrolyte leakage
ETR	Electron transport rate
ETR/P_N	Ratio between the apparent electron transport rate and net photosynthetic rate
EXC	Relative energy excess at the PSII level
F_0	Minimal fluorescence yield of the dark-adapted state
F_m	Maximal fluorescence yield of the dark-adapted state
F_v	Variable fluorescence
F_v/F_m	Maximal quantum yield of PSII photochemistry
g_s	Stomatal conductance
H_2O_2	Hydrogen peroxide
K	Potassium
LDM	Leaf dry matter
MDA	Malondialdehyde
Mg	Magnesium
Mn	Manganese
NPQ	Nonphotochemical quenching
O_2^-	Superoxide
P_N	Net photosynthetic rate
P_N/C_i	Instantaneous carboxylation efficiency
POX	Peroxidase
PSII	Photosystem II
q_P	Photochemical quenching
RDM	Root dry matter
ROS	Reactive oxygen species
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SDM	Stem dry matter

SOD	Superoxide dismutase
S	Sulfur
TDM	Total dry matter
Total Chl	Total chlorophyll
WUE	Water-use efficiency
Φ_{PSII}	Effective quantum yield of PSII photochemistry

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90 **24-epibrassinolide simultaneously delays chlorophyll degradation and stimulates the photosynthetic**

91 **machinery in magnesium stressed soybean plants**

92

93 **Abstract**

94 Adverse effects caused by inadequate magnesium (Mg) supply (deficiency or excess) often cause oxidative stress

95 into chloroplast, declining in photosynthetic activity. On the other hand, 24-Epibrassinolide (EBR) is a plant

96 growth regulator natural, biodegradable and ecologically viable, with multiple roles in plant metabolism. This

97 research aims to answer if the foliar application of EBR (1) can delay the chlorophyll degradation and/or (2) to

98 mitigate the oxidative stress on photosynthetic machinery in magnesium stressed soybean plants. The experiment

99 followed a completely randomized factorial design with two concentrations of 24-Epibrassinolide (0 and 100 nM

100 EBR, described as - EBR and + EBR, respectively) and three Mg supplies (0.0225, 2.25 and 225 mM Mg,

101 described as low, control and high supply of Mg). Inadequate Mg supplies (deficiency and excess) negatively

102 interfered on photosynthetic pigments, chlorophyll fluorescence and gas exchange. However, exogenous EBR

103 improved the antioxidant enzyme activities (superoxide dismutase, catalase, ascorbate peroxidase and peroxidase),

104 protecting against oxidative stress and delaying the chlorophyll degradation in plants grown with low and high Mg

105 supplies. Concomitantly, plants sprayed with this steroid had increases in Mg contents, improving the

106 photochemical efficiency and gas exchange, because the Mg exercises essential role during light capture process.

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108 **Keywords** Brassinosteroids ● Chloroplastic pigment ● Essential macronutrient ● *Glycine max* ● Photosystem II

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Introduction

Soybean (*Glycine max* (L.) Merr.) is one of the most important leguminous in the world due to high levels of oils (18 to 20%) and proteins (35 to 40%) contained in its seeds, being a fundamental crop for human nutrition, animal feed and energy source in biofuels (Teixeira et al. 2020). Global soybean production reached 364 million tons in 2018/2019 harvest, with the United States, Brazil and Argentina as the main producers (Marocco and Milo 2019).

Magnesium (Mg) is one of the essential macronutrients for plants and plays key functions in several physiological processes, including photosynthesis. Mg is the central element of the chlorophyll molecule and activation of the enzyme ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCo) (Assunção et al. 2020; Gransee and Führs 2013). In parallel, this ion has multiple metabolic functions, including cofactor in several enzymes and an essential component in proteins, for example, in the synthesis of ATP (Verbruggen and Hermans 2013; Wang et al. 2020), a important step during respiration and synthesis of organic compounds (Shameer et al. 2019).

Plants exposed to low magnesium supply often presents decreases in carbon dioxide (CO_2) assimilation (Yang et al. 2012), reductions in leaf pigments and chlorophyll fluorescence (Zhou et al. 2011), excessive accumulation of carbohydrates in the leaves (Mengutay and Ceylan 2013), significant reductions in the electron transport rate (Tang et al. 2012), increase in the production of reactive oxygen species (ROS) and photooxidative damages (Chao et al. 2012). On the other hand, high cytoplasmic concentrations of Mg^{2+} block the K^+ channels in internal membranes of the chloroplasts, acidifying the stroma by preventing the removal of H^+ ions, which inactivate enzymes linked to carbon fixation, potentiating the production of free radicals and generating damages in cellular structures (Venkatesan and Jayaganesh 2010). Nutritionally, Mg excess interferes negatively on the absorption and transport of other essential elements, mainly Ca, K and Mn (Conn et al. 2011; Hermans et al. 2013).

Brassinosteroids (BRs) are plant growth regulators, with multiple roles in plant metabolism (Ahammed et al. 2020). Exogenous application of EBR can mitigate damages to plants exposed to abiotic stresses, because this molecule exhibit beneficial effects on antioxidant enzymes (Amraee et al. 2020), increments in chlorophyll levels (Tadaiesky et al. 2020), increases in photosystem II efficiency (Kolomeichuk et al. 2020), stimulation on gas exchange (Wei and Li 2016) and higher biomass (Ribeiro et al. 2019).

Our hypothesis is that the adverse effects caused by inadequate Mg supply (deficiency or excess) cause an increase in oxidative stress and a decline in photosynthetic activity. On the other hand, EBR is a natural and biodegradable molecule that exhibits improvements in the performance of photosystem II, gas exchange and activity of antioxidant enzymes (Cai et al. 2020; Cunha et al. 2020; Pereira et al. 2020) with positive repercussions on the nutrient contents (Santos et al. 2020) and biomass of plants under conditions of environmental stress (Fonseca et al. 2020). Therefore, this research aimed to answer if the foliar application of EBR (1) can delay the chlorophyll degradation and/or (2) to mitigate the oxidative stress on photosynthetic machinery in magnesium stressed soybean plants, evaluating the responses associated with nutrient concentrations, gas exchange, chlorophyll fluorescence and morphological parameters.

168 **2. Materials and Methods**

169 Location and growth conditions

170 The experiment was performed at the Campus of Paragominas of the Universidade Federal Rural da Amazônia,

171 Paragominas, Brazil (2°55' S, 47°34' W). The study was conducted in a greenhouse with the temperature and

172 humidity controlled. The minimum, maximum, and median temperatures were 23.1, 28.7 and 26.3 °C,

173 respectively. The relative humidity during the experimental period varied between 60% and 80%.

174

175 Plants, containers and acclimation

176 Seeds of *Glycine max* var. M8644RR Monsoy™ were germinated and grown in 1.2-L pots filled with a mixed

177 substrate of sand and vermiculite at a ratio of 3:1. The plants were cultivated under semi-hydroponic conditions

178 containing 500 mL of distilled water for four days. A nutritive solution described by Pereira et al. (2019) was used

179 to plant nutrition, with ionic strength beginning at 50% (8th day) and later modified to 100% after two days (10th

180 day). After this period, the nutritive solution remained at total ionic strength.

181

182 Experimental design

183 The experiment followed a completely randomized factorial design with two concentrations of 24-Epibrassinolide

184 (0 and 100 nM EBR, described as - EBR and + EBR, respectively) and three Mg supplies (0.0225, 2.25 and 225

185 mM Mg, described as low, control and high supply of Mg). With five replicates for each of six treatments, a total

186 of 30 experimental units were used in the experiment, with one plant in each unit.

187

188 24-epibrassinolide (EBR) preparation and application

189 Twelve-day-old plants were sprayed with 24-epibrassinolide (EBR) or Milli-Q water (containing a proportion of

190 ethanol that was equal to that used to prepare the EBR solution) at 5-day intervals until day 35. The 0 and 100 nM

191 EBR (Sigma-Aldrich, USA) solutions were prepared by dissolving the solute in ethanol followed by dilution with

192 Milli-Q water [ethanol:water (v/v) = 1:10,000] (Ahammed et al. 2013).

193

194 Plant conduction and Mg supplies

195 Plants received the following macro- and micronutrients contained in the nutrient solution in agreement with

196 Pereira et al. (2019). For Mg treatments, $MgCl_2$ was used at concentrations of 0.0225 mM (low) and 2.25 mM

197 (control) and 225 mM (high) applied over 10 days (days 25–35 after the start of the experiment). During the study,

198 the nutrient solutions were changed at 07:00 h at 3-day intervals, with the pH adjusted to 5.5 using HCl or NaOH.

199 On day 35 of the experiment, physiological and morphological parameters were measured for all plants, and leaf

200 tissues were harvested for biochemical and nutritional analyses.

201 Determining of Mg and nutrients

202 Milled samples (100 mg) of root, stem and leaf tissues were pre-digested using conical tubes (50 mL) with 2 ml of

203 sub boiled HNO_3. Subsequently, 8 ml of a solution containing 4 ml of H_2O_2 (30% v/v) and 4 ml of ultra-pure water

204 were added, and transferred to a Teflon digestion vessel in agreement with Paniz et al. (2018). The determination

205   of Mg, K, Ca, S, Mn, Cu and B were performed using an inductively coupled plasma mass spectrometer (model

206   ICP-MS 7900; Agilent).

207

208   Measurement of chlorophyll fluorescence and gas exchange

209   Chlorophyll fluorescence was measured in fully expanded leaves under light using a modulated chlorophyll

210   fluorometer (model OS5p; Opti-Sciences). Preliminary tests determined the location of the leaf, the part of the leaf

211   and the time required to obtain the greatest F_v/F_m ratio; therefore, the acropetal third of the leaves, which was the

212   middle third of the plant and was adapted to the dark for 30 min, was used in the evaluation. The intensity and

213   duration of the saturation light pulse were 7.500 $\mu mol\ m^{-2}\ s^{-1}$ and 0.7 s, respectively. Gas exchange was evaluated

214   in all plants and measured in the expanded leaves in the middle region of the plant using an infrared gas analyser

215   (model LCPro+; ADC BioScientific) in a chamber under constant CO_2, photosynthetically active radiation, air-

216   flow rate and temperature conditions at 360 $\mu mol\ mol^{-1}$ CO_2, 800 $\mu mol\ photons\ m^{-2}\ s^{-1}$, 300 $\mu mol\ s^{-1}$ and 28 °C,

217   respectively, between 10:00 and 12:00 h.

218

219   Determination of the antioxidant enzymes, superoxide and soluble proteins

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

221   tissues according to the method of Badawi et al. (2004). The total soluble proteins were quantified using the

222   methodology described by Bradford (1976). The SOD assay was measured at 560 nm (Giannopolitis and Ries

223   1977), and the SOD activity was expressed in mg^{-1} protein. The CAT assay was detected at 240 nm (Havir and

224   McHale 1987), and the CAT activity was expressed in $\mu mol\ H_2O_2\ mg^{-1}$ protein min^{-1}. The APX assay was

225   measured at 290 nm (Nakano and Asada 1981), and the APX activity was expressed in $\mu mol\ AsA\ mg^{-1}$ protein

226   min^{-1}. The POX assay was detected at 470 nm (Cakmak and Marschner 1992), and the activity was expressed in

227    $\mu mol\ tetraguaiacol\ mg^{-1}$ protein min^{-1}. O_2^- was measured at 530 nm (Elstner and Heupel 1976).

228

229   Quantification of hydrogen peroxide, malondialdehyde and electrolyte leakage

230   Stress indicators (H_2O_2 and MDA) were extracted using the methodology described by Wu et al. (2006). H_2O_2 was

231   measured using the procedures described by Velikova et al. (2000). MDA was determined by the method of

232   Cakmak and Horst (1991) using an extinction coefficient of 155 $mM^{-1}\ cm^{-1}$. EL was measured according to Gong

233   et al. (1998) and calculated by the formula EL (%) = (EC1/EC2) × 100.

234

235   Determination of photosynthetic pigments and biomass

236   Chlorophyll and carotenoid determinations were performed using a spectrophotometer (model UV-M51; Bel

237   Photonics) according to the methodology of Lichtenthaler and Buschmann (2001). The biomass of roots and

238   shoots was measured based on constant dry weights (g) after drying in a forced-air ventilation oven at 65 °C.

239

240 Data analysis

241 The data were subjected to an analysis of variance, and significant differences between the means were determined

242 using the Scott-Knott test at a probability level of 5% (Steel et al. 2006). Standard deviations were calculated for

243 each treatment.

244

245 **3. Results**

246 EBR maximizes Mg and nutrient contents in plants grown with low Mg supply

247 The low and high Mg supplies occasioned interferences on contents of this element in the tissues of the root, stem

248 and leaf (Table 1). Plants sprayed with EBR and exposed to low Mg presented increases in Mg contents of 14%

249 (root), 68% (stem) and 100% (leaf), when compared to equal treatment without EBR. Interestingly, plants with

250 high Mg + EBR had decreases in root, stem and leaf tissues of 18%, 12% and 7%, in this order, compared with

251 equal treatment in the absence of EBR. To nutrient contents, plants exposed to low and high Mg had reductions in

252 tissues evaluated (Table 2). However, plants submitted to low Mg supply and sprayed with EBR increased the K,

253 Ca, S, Mn, Cu and Bo contents in 3%, 30%, 4%, 19%, 45% and 34% (root); 3%, 13%, 1%, 5%, 6% and 9%

254 (stem); 20%, 4%, 3%, 2%, 3% and 20% (leaf), respectively, compared to the same treatment without EBR. High

255 Mg + EBR treatment promoted also increases in K, Ca, S, Mn, Cu and Bo contents of 25%, 31%, 15%, 9%, 19%

256 and 43% in roots, in this order, 14%, 16 %, 8%, 5%, 5% and 10% in stems and 3%, 4%, 3%, 11%, 10% and 24%

257 in leaves, compared to the same treatment in the absence of EBR.

258

259 Steroid delays chlorophyll degradation and stimulates the photosynthetic machinery in plants exposed to
260 magnesium stress

261 Low and high supplies of Mg resulted in reductions in Chl a, Chl b, Total Chl and Car values (Table 5) and

262 increases in Chl a/Chl b and Total Chl/Car ratios. Under low Mg with EBR, the variables Chl b, Total Chl and Car

263 had increases of 32%, 7% and 33%, respectively. To high Mg + EBR the increments were of 1%, 31%, 9% and

264 53% for Chl a, Chl b, Total Chl and Car, in this order, when compared to the same treatment without EBR. For

265 Chl a/Chl b, under low and high Mg both with EBR, decreases of 24% and 19% were detected, in this order. In the

266 Total Chl/Car ratio were verified reductions in low and high Mg combined with EBR of 20% and 29%,

267 respectively. To chlorophyll fluorescence, plants with low and high Mg supplies expressed decreases (P<0.05) in

268 F_m, F_v and F_v/F_m values (Fig. 1), in relation to control treatment, with the exception of F_0, which did not show

269 significant changes in the treatment with low Mg. In F_m, the EBR application induced increments of 8% and 44%

270 in low and high supplies, respectively, when related to the same treatment without EBR. In F_v, plants under low

271 and high Mg supplementation and sprayed with EBR had increases of 10% and 128%, in the same order. In F_v/F_m,

272 the low Mg supply with EBR increased by 2%, while the high Mg + EBR treatment showed an increase of 61%,

273 compared to the equal treatments without EBR. In relation to photosystem II, plants exposed to low and high Mg

274 suffered negative interferences (Table 3). However, plants exposed to low Mg and treated with 100 nM EBR had

275 increases of 9% and 9% for Φ_{PSII} and ETR, respectively, and decreases in the values of EXC (3%) and ETR/P_N

276 (6%), compared to the same treatment without EBR. In relation to high Mg with EBR, there were increases of

277 43%, 27%, 50% for Φ_{PSII}, q_P and ETR, respectively and decreases in the values of NPQ (19%) and EXC (6%),

278 compared to the same treatment in the absence of EBR. To gas exchange, low and high Mg provoked deleterious

279   effects (Table 3). However, the EBR application in plants with low Mg resulted in increases for P_N, WUE and

280   P_N/C_i of 16%, 23% and 120%, respectively, and a decrease of 47% for C_i, when compared to the same treatment

281   without EBR. While the high Mg + EBR treatment, the values of P_N, E, g_s, WUE and P_N/C_i were increased by

282   33%, 7%, 9%, 22% and 25%, respectively, and in C_i a reduction of 7%.

283

284   Antioxidant enzymes control the oxidative stress generated by low and high Mg supplies

285   Plants exposed to low and high Mg had increases ($P<0.05$) in SOD, CAT, APX and POX (Fig. 2). The application

286   of 100 nM EBR in plants under low Mg caused increases of 18%, 22%, 55%, and 39%, respectively, compared to

287   the low Mg + 0 nM EBR, while high Mg with EBR treatment occurred significant increases in SOD (37%), CAT

288   (34%), APX (48%) and POX (49%) activities, compared to the same treatment in the absence of EBR. To stress

289   indicators (Fig. 3), plants exposed to low and high Mg supplies presented increases. However, plants with low Mg

290   supply and 100 nM EBR suffered significant reductions in O_2^- (21%), H_2O_2 (10%) and MDA (28%), compared to

291   the low Mg and 0 nM EBR. In relation to high Mg supply combined with EBR, occurred significant decreases in

292   O_2^- (17%), H_2O_2 (18%), MDA (42%) and EL (10%), compared to the same treatment in the absence of EBR.

293

294   EBR reduced the deleterious effects provoked by the Mg stress on biomass

295   The low and high Mg supplies promoted reductions in morphological parameters, if compared to control treatment

296   (Fig. 4). In low Mg + EBR treatment, increases of 1%, 13% and 3% were detected for LDM, RDM and TDM,

297   respectively, compared to the low Mg + 0 nM EBR treatment. High Mg + EBR treatment had increments in LDM,

298   RDM and TDM of 2%, 1% and 2%, in this order.

299

300   **4. Discussion**

301   Plants exposed to low Mg and sprayed with EBR presented increases in Mg contents, suggesting that this steroid

302   improved the absorption, transport and accumulation of Mg in tissues evaluated. EBR probably improved Mg

303   transport due to increased activity of the H^+-ATPase enzyme (Song et al. 2016a), responsible by the active

304   transport of cations and protons at the cellular level through membrane, forming an essential electrochemical

305   gradient of protons to maintain ionic balance in higher plants (Falhof et al. 2016). Our results also suggest that

306   EBR maximized the Mg absorption, activating the expression of genes that encode high-affinity transport proteins

307   for Mg^{2+} (Gransee and Führs 2013), including the proteins of the *AtMRS2/AtMGT* family, in which they are known

308   as the main membrane transporters in the root and shoot tissues (Bose et al. 2013). *MGT1*, *MGT3*, *MGT4*, *MGT5*

309   and *MGT6* proteins have been identified in *Arabidopsis thaliana* (Yan et al. 2018), *Brassica napus* (Sun et al.

310   2019), *Oryza* (Chen and Ma 2013) and *Cucurbita moschata* (Huang et al. 2016), which comprise the main

311   transporters expressed in the membranes of the root cells. Therefore, those responsible for capturing and

312   distributing Mg^{2+} under low Mg concentrations. Concomitantly, EBR increased the xylem loading efficiency,

313   through increments in RMD and RDT, favoring the simplistic transport to the vascular system and improving the

314   upward transport of Mg in the plants (Huang et al. 2019). On the other hand, exogenous EBR reduced the toxic

315   effects of Mg, reducing the Mg content in tissues exposed to high Mg supply, related to the regulation of Mg^{2+}/H^+

316   transporters for the vacuole, identified as responsible for the osmotic adjustment in conditions of Mg excess (Conn

317   et al. 2011; Waters 2011), combined with the protective roles of this steroid, maintaining the membrane integrity

318 (Yue et al. 2019). Yuan et al. (2015) investigating the EBR effects (0.1 μM) on uptake and ion distribution in

319 *Cucumis sativus* plants stressed with Ca (NO$_3$)$_2$ (80 mM) obtained decreases and increases in Ca and Mg contents,

320 respectively. Karlidag et al. (2011) verified that *Fragaria × ananassa* plants exposed to salinity (35 mM NaCl)

321 suffered reductions in Mg contents in roots and leaves, however EBR treatments (0.5 and 1.0 μM) promoted

322 significant increases in the contents of this macronutrient.

323   Plants submitted to low and high Mg concentrations and sprayed with 100 nM EBR had increases in

324 contents of macronutrient (K, Ca and S) and micronutrients (Mn, Cu, and Bo). These results are probably related

325 to the increments promoted by the EBR on root structures, because this steroid regulates the root meristem size,

326 development of root bundles, formation of root hairs and lateral root initiation, providing increases in root contact

327 surface exposed to the substrate and potentiating the uptake and transport of nutrients by the xylem from the root

328 to shoot (Fàbregas et al. 2010; Noh et al. 2015; Wei and Li 2016). Additionally, our study revealed that EBR

329 mitigated the negative impacts of the Mg concentrations (low and high) on ionic homeostasis of these elements,

330 probably regulating transporters linked to K$^+$ (SKOR, AKT / KAT and HAK / KUP), Ca^{2+} (Ca^{2+} channel, Ca^{2+}/H$^+$

331 and Ca^{2+}-ATPase), SO$_4^{2-}$ (*GmSULTR1*; 2b and H+/ SO$_4^{2-}$), Mn^{2+} (*OsNRAMP3*), Cu^{2+} (*COPT1*, *Cu/ZnSOD* and

332 *FeSOD*) and H$_3$BO$_3$ (*BOR1*), and optimizing the transport process and assimilation of ions linked to the

333 photosynthetic process and constitution of antioxidant enzymes (Ahmad and Maathuis 2014; Ding et al. 2016;

334 Gaspar 2011; Herrera-Rodríguez et al. 2010; Siddiqui et al. 2018; Wang et al. 2016a; Yang et al. 2013), in which

335 future studies are essential to elucidate these informations.

336   K plays important roles connected to osmotic regulation of plants and stomatal mechanism, directly

337 influencing on gas exchange (Jákli et al. 2017). Ca has a structural function in membranes and cell wall (Hepler

338 and Winship 2010), reactions involving O$_2$ in Photosystem II (Yachandra and Yano 2011), and stomatal regulation

339 (Dodd et al. 2010). S is absorbed by the plant as sulfate, and subsequently reduced and incorporated into amino

340 acids, such as cysteine (Cys), in which it is the precursor of several compounds containing reduced S, such as

341 glutathione (GSH), an antioxidant enzyme that participates in the removal of H$_2$O$_2$ in excess. In parallel, Cys are

342 fundamental in the control of redox status in chloroplasts (Anjum et al. 2011). Mn-containing molecules are part

343 of the catalytic center of the water decomposition complex in PSII and contribute to antioxidant metabolism,

344 acting as a cofactor for the enzymes Mn-CAT and Mn-SOD (McAlpin et al. 2012; Shen 2015; Signorella et al.

345 2018; Srivastava and Dubey 2011). Cu is a cofactor for plastocyanin (PC), an electron transporter in the lumen of

346 thylacoids, and antioxidant enzymes, including Cu/Zn-SOD (Mohammadi et al. 2020; Ravet et al. 2011).

347 Straltsova et al. (2015) testing three BRs analogs (24-epibrassinolide, 28- homobrassionolide or 24-

348 epicastasterone) induced gradual increases in free cytosolic Ca^{2+} in *Arabidopsis thaliana* root cells. Research

349 conducted by Yan et al. (2015) indicated that BRs (10 nM) induced significant increase in cytosolic Ca^{2+}

350 concentration in protoplasts from *Zea mays* mesophyll, as well as these authors provided that Ca^{2+} stimulated the

351 BRs-induced antioxidant defense, more specifically SOD and APX enzymes. Ekinci et al. (2012) evaluating the

352 EBR application (3 μM) in *Lactuca sativa* seedlings exposed to salt stress (100 mM NaCl) found increases in K, S,

353 Mn, Cu and B contents, similar to results obtained in this study.

354   EBR delayed the deleterious effects on photosynthetic pigments (Chl *a*, Chl *b*, Chl Total and Car) in

355 plants exposed to Mg^{2+} stress, being these results explained by the reductions in oxidative damages generated by

356 O$_2^-$ and H$_2$O$_2$, detected in this research. Subsequently, higher efficiency of the PSII was verified, being confirmed

357 by the increments in Φ$_{PSII}$ and ETR, suggesting that the EBR improved the structural organization of the thylakoid

membrane, maximizing the performance of the pigments and the energy distribution in the PSII (Dobrikova et al. 2014). Plants under Mg deficiency and excess suffered reductions of photosynthetic pigments. Magnesium is a structural element, occupying the central position of the porphyrin ring of the chlorophyll molecules, it also acts as an activator or regulator of several kinases, such as ATPases and ribulose-1,5-bisphosphate carboxylase / oxygenase (RuBisCO), in which Mg^{2+} stress (high and low) can affect several physiological processes associated with chlorophyll biosynthesis, plant growth and development (Cakmak 2013; Zhou et al. 2011). Mg^{2+} is also important for stacking of the chloroplast granum, negatively compromising the photosynthetic performance of plants under low Mg^{2+} supply (Ceppi et al. 2012). Meireles and Brandão (2014) found reductions in Chl a, Chl b and Car levels and increases on H_2O_2 concentrations in two *Coffea arabica* cultivars exposed to Mg deficiency. Thussagunpanit et al. (2015) verified that the exogenous application of EBR (1 nM) alleviated losses related to Total Chl in *Oryza sativa* plants under heat stress (40/30 °C temperature day/night for seven days). Efimova et al. (2014) demonstrated that the EBR treatment (10^{-10} M) mitigated the inhibitory effects of salt stress (175 mM NaCl for seven days) in *Brassica napus* promoting increases in Chl a and Chl b levels, besides reduction in MDA, suggesting maintenance of membrane integrity.

Exogenous EBR spray attenuated the negative impacts caused by low and high Mg supplies on F_m, F_v and F_v/F_m. These results indicate the beneficial effects of this steroid on the components of the thylacoid membrane (Farhat et al. 2015), increasing the proportion of oxidized quinone (Q_A) (Liu et al. 2017) and reducing the damages on photosynthetic apparatus (Talaat 2020), clearly demonstrating a reduction in photoinhibition and improvements in the photochemical efficiency of PSII reaction centers (Guo et al. 2016). Studies confirm that plants grown under Mg stress have negative impacts on the photochemical efficiency of PSII, reductions in photosynthetic pigments, disorganization of thylakoid membranes and inhibition of photosynthesis (Farhat et al. 2015; Huang et al. 2019, 2016). Lima et al. (2018), evaluating the effects induced by the EBR application (100 nM) in young *Eucalyptus urophylla* plants exposed to Fe deficiency detected that this substance induced significant increases in F_m (48%) and F_v (78%), reducing the negative effects on chlorophyll fluorescence. Hussain et al. (2019) found that EBR increased the photochemical efficiency of *Brassica juncea* under Mn toxicity (150 mg kg^{-1}), promoting an increase in F_v/F_m (25%), compared to the same treatment without EBR.

Beneficial effects promoted by EBR in F_m, F_v and F_v/F_m in soybean plants submitted to low and high Mg supplies contributed to increases in Φ_{PSII}, q_P and ETR values, revealing that EBR improves the efficiency of photosystem II and the electron transference during photochemical reactions. This steroid maximized the fluorescence dissipation, facilitating the electrons flow into chloroplasts (Jiang et al. 2012), with a probable positive impact on the generation of ATP and NADPH, in which they are used in the next stage of CO_2 fixation, more specifically in Calvin cycle (Kumari et al. 2017). EBR spray also caused reductions in NPQ and EXC in plants exposed to low and high Mg supplementations, evidencing that EBR promoted less dissipation of excitation energy in the form of heat and consequently improves the quantum fluorescence yield (Nath et al. 2013).This fact is very interesting, because the EBR alleviated energy losses (heat), caused by a high incidence of light energy and that often lead to photooxidation and damages to photosystems (Hu et al. 2013; Kangasjärvi et al. 2012; Nishiyama and Murata 2014; Siddiqui et al. 2018). Decrease in ETR/P_N value occurred in the treatment with EBR and low Mg reveals that in situations of deficiency of this element, this steroid provides better use of electrons in photochemical activity and decreases the use of alternative electron drains, such as photorespiration and Mehler reactions (Fang et al. 2011; Krumova et al. 2013; Pereira et al. 2019). Similar results were confirmed by Zhang et

398 al. (2014) describing increases in ΦPSII and qP, but decreases in NPQ, after EBR treatments in *Cucumis melo*

399 cultivars submitted to high temperatures. Wu et al. (2014) suggested that EBR may improve the protection

400 mechanism of plants under cold stress by obtaining a reduction in NPQ in *Solanum melongena* seedlings treated

401 with EBR (0.05 and 0.1 μM). Rodrigues et al. (2020) evaluating the effects of EBR spray (100 nM) in *Glycine*

402 *max* plants exposed to low (0.25 μM) and high (2500 μM) Mn supplies obtained increases in Φ_{PSII} (4%), q_P (10%),

403 ETR (4%) and EXC (3%). Yuan et al. (2012) investigating the benefits of the EBR application on the

404 photosynthetic characteristics of *Cucumis sativus* under toxicity of 80 mM Ca $(NO_3)_2$ reported significant increases

405 in Φ_{PSII} and q_P of plants sprayed with steroid (0.1 μM).

406   EBR attenuated the negative effects caused by low and high Mg supplementations in gas exchange. In

407 this context, plants exposed to low Mg and sprayed with EBR (100 nM) had increases in P_N and P_N/C_i and WUE,

408 confirming the improvements promoted by EBR on photosynthetic machinery and CO_2 uptake, evidenced by the

409 increase in P_N and reduction of C_i, suggesting higher activity of the RuBisCO enzyme, main enzyme involved

410 during CO_2 fixation in Calvin cycle (Pociecha et al. 2016). On the other hand, plants under Mg deficiency suffered

411 a decline in P_N, E, g_s, and WUE, being these results related to impaired CO_2 fixation, inducing electron

412 accumulation unused and increased energy absorbed in chloroplasts, resulting in generation reactive oxygen

413 species (ROS), which cause photooxidation and damages to the chlorophyll and chloroplast membranes

414 (Kobayashi et al. 2013; Tang et al. 2012; Tränkner et al. 2016). Li et al. (2017) investigating the deleterious effects

415 provoked by the Mg deficiency (0 mM MgSO4 for 16 weeks) on gas exchange and RuBisCO activity in *Citrus*

416 *sinensis* seedlings described that plants under Mg deficiency suffered significant decreases in g_s, RuBisCO and

417 CO_2 assimilation in lower leaves. Li et al. (2016) studying the roles triggered by the EBR endogenous on

418 photosynthesis regulation and photosynthetic performance using three *Solanum lycopersicum* genotypes (dwarf

419 mutant deficient to EBR biosynthesis, dwarf line efficient to EBR biosynthesis and wild plants), revealing that

420 high endogenous levels of EBR induced increases in P_N, E, g_s, and P_N/C_i, accompanied by higher capacity *in vivo*

421 for carboxylation and regeneration of the RuBisCO enzyme, compared to mutant deficient to EBR and wild

422 genotypes. Santos et al. (2018) investigating the effects connected to exogenous EBR spray (100 nM) in *Glycine*

423 *max* plants exposed to Cd stress (500 μM) verified improvements in gas exchange, more specifically increases in

424 P_N, E, g_s, WUE and P_N/C_i.

425   Gas exchange were maximized in plants under excess of Mg + EBR (100 nM), with increases in P_N, E, g_s,

426 WUE, P_N/C_i and decrease in C_i, in which these results can be explained by the positive impacts on stomatal

427 performance, confirmed by the increase in the values of g_s, SD and SI, previously detected in this study.

428 Additionally, increase in g_s facilitate CO_2 absorption, with probable increase in RuBisCO activity (increase in de

429 P_N/C_i and decrease in C_i). Plants under high Mg and sprayed with EBR presented increase in WUE value, being

430 this fact attributed to the increases in P_N and E. In other words, WUE is the result of the relationship between P_N

431 and E, being associated with the stomatal mechanism the regulation of gas exchange and water use, therefore

432 improving photosynthetic efficiency (Bertolli et al. 2012; Kim et al. 2012). Hayat et al. (2012) investigating the

433 effect of foliar spraying of two EBR analogs (28-homobrassinolide and 24-epibrassinolide at 10^{-8} M) in

434 *Lycopersicon esculentum* plants exposed to Cd toxicity verified benefits in photosynthetic attributes, more

435 specifically increases in P_N, g_s, E and WUE.

436   EBR promoted increases in the activities of the SOD, APX and POX enzymes in plants submitted to low

437 and high Mg, revealing the beneficial effects promoted by the EBR, mitigating oxidative damages to photosystems

and membranes. These enzymes act in the antioxidant system, detoxifying membranes and reducing the impact generated by reactive oxygen species (ROS), such as H_2O_2 and O_2^- (Ramakrishna and Rao 2015). BRs are involved in the regulation of the antioxidant metabolism through the expression of antioxidant genes in *Glycine max* plants, such as *Fe-SOD*, *Cu/ Zn-SOD*, *CAT1*, *APX1*, *APX2* and *POX10* (Hamurcu et al. 2013; Hossain et al. 2012; Kausar et al. 2012; Wang et al. 2016b; Wu et al. 2013). Activities of these enzymes improve the tolerance to oxidative stress (Ding et al. 2012; Hayat et al. 2012). Several abiotic stresses lead to ROS overproduction in plants, in which these compounds are highly reactive, and toxic, causing damages mainly to components of PSII, proteins, lipids, carbohydrates and DNA, resulting in oxidative stress (Gill and Tuteja 2010). The EBR promoted increases in the activities of SOD (43%), POX (17%) and CAT (29%) in a study with *Arachis hypogea* plants under Fe deficiency (Song et al. 2016b) and *Arabidopsis thaliana* under B toxicity (0.8 or 1.6 mM) (Surgun et al. 2016).

Mg stress (low and high) caused increases in the contents of O_2^- and H_2O_2, MDA and EL, but the exogenous EBR application (100 nM) promoted reductions in levels of these stress indicators. Under situations of low Mg, the activation state linked to RuBisCO enzyme and photochemical activity are reduced, limiting the CO_2 assimilation and the absorbed light energy for the transport of photosynthetic electrons, resulting in excess photons and subsequent overproduction of ROS (Yang et al. 2012). In other hand, high Mg generates high osmolarity, restricting the H_2O molecules in the solution, resulting in ionic toxicity and consequent nutritional imbalances, mainly of Ca^{2+} and Mn^{2+} (Conn et al. 2011; Zhang et al. 2018). Several impacts are described in literature, including decreases in g_s, negative interferences on electron transport into chloroplast, declining ETR and leading to ROS accumulation, damages to lipid membranes and other essential macromolecules, such as proteins and nucleic acids (Bose et al. 2014; Niu et al. 2018; Ozgur et al. 2013; Shabala et al. 2016).

Reductions in O_2^-, H_2O_2, MDA and EL under low or high Mg supplies and sprayed with EBR indicate its function as a secondary messenger, inducing increases in the activities of antioxidant enzymes (SOD, CAT, APX and POX), and improving redox homeostasis ROS, thus contributing to membrane stability, integrity and permeability (Vardhini and Anjum 2015). Ding et al. (2012) evaluating the benefits of different concentrations of EBR (0, 0.025, 0.05, 0.1 and 0.2 mg dm^{-3} EBR) in *Solanum melongena* plants exposed to saline stress (90 mM NaCl) obtained reductions in O^{2-} and H_2O_2, EL and MDA under concentration of 0.05 mg dm^{-3} EBR. Surgun et al. (2016) also obtained mitigation of oxidative stress generated by the B toxicity (1.60 mM H_3BO_3) after treatment with 1 μM EBR on *Arabidopsis thaliana*.

EBR reduced the deleterious effects on plant biomass (LDM, RDM, SDM and TDM) caused by low and high Mg supplies, being explained by the benefits on nutrient contents and antioxidant system, combined with increments on photosynthetic pigments and gas exchange, positively modulating the biomass (Dalio et al. 2011). Huang et al. (2019) investigating the effects connected to Mg deficiency on *Citrus sinensis* found significant reductions in RDM (27%), SDM (25%) and TDM (26%), related to decreases in RDT and RDM, impacting the assimilation and transport of the nutrients, such as P, B, Cu and Fe, and decreases in physiological variables (P_N, g_s, E, Chl total and F_v/F_m). Lima and Lobato (2017) working with *Vigna unguiculata* plants under water deficit reported decreases in RDM, however the EBR spray (100 nM) promoted increases in LDM (11%), SDM (7%), RDM (10%) and TDM (10%), results directly related to the improvements promoted by EBR on gas exchange, presenting increases in P_N (96%), E (24%), g_s (33%), WUE (49%) and P_N/C_i (141%).

5. Conclusion

This research revealed that inadequate supplies of Mg (deficiency and excess) negatively interfered on photosynthetic pigments, chlorophyll fluorescence and gas exchange, being intrinsically related to inadequate Mg contents and intense oxidative stress. However, the foliar application of EBR delayed the chlorophyll degradation occasioned by the oxidative stress in plants under low and high Mg supplies, improving the activity of enzymes linked to the antioxidant mechanism (superoxide dismutase, catalase, ascorbate peroxidase and peroxidase), reducing the concentrations of superoxide and hydrogen peroxide into chloroplast. Concomitantly, plants sprayed with this steroid had increases in the contents of macronutrients and micronutrients, including Mg, which benefited the photochemical efficiency of photosystem II and gas exchange, because the Mg is the central element of the chlorophyll molecule, exercising central role during light capture. Therefore, these results indicate that the exogenous application of EBR increases the tolerance to deficiency/excess of Mg in soybean plants.

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7. References

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Figures
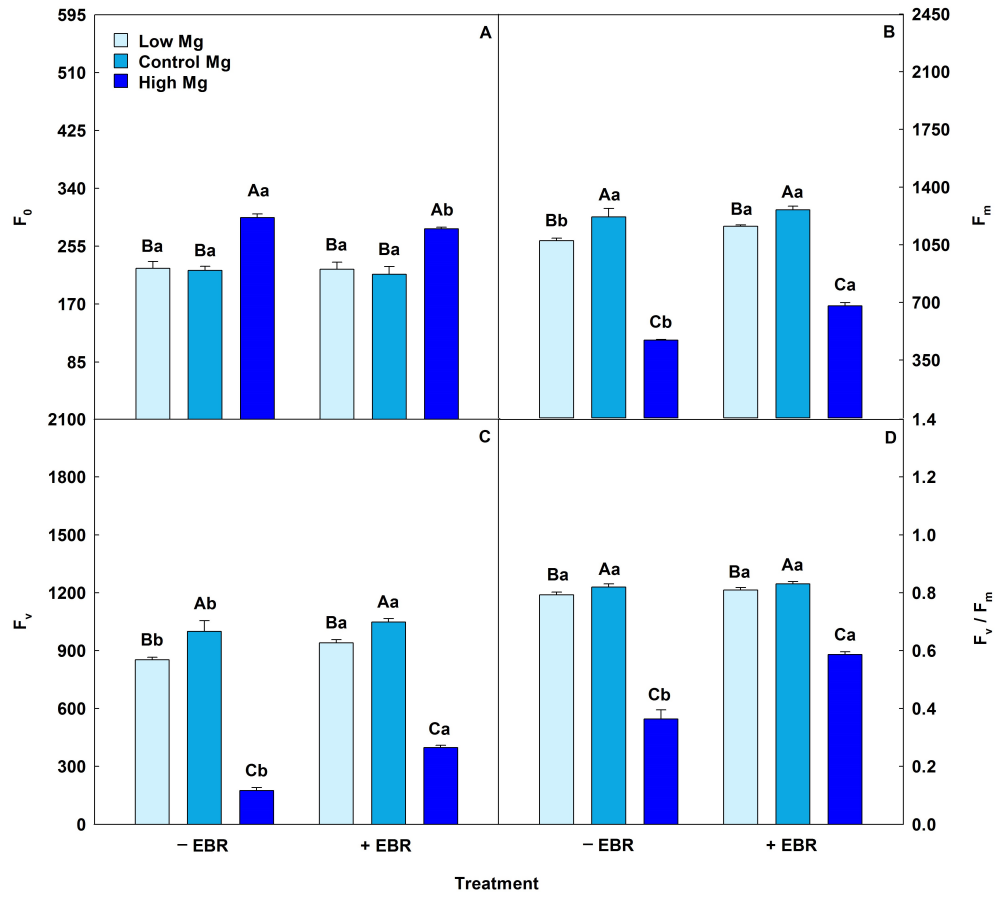
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860 Fig. 1. Minimal fluorescence yield of the dark-adapted state (F_0), maximal fluorescence yield of the dark-adapted

861 state (F_m), variable fluorescence (F_v) and maximal quantum yield of PSII photochemistry (F_v/F_m) in soybean plants

862 sprayed with EBR and exposed to different Mg supplies. Columns with different letters indicate significant

863 differences from the Scott-Knott test ($P<0.05$). Columns corresponding to means from five repetitions and

864 standard deviations.

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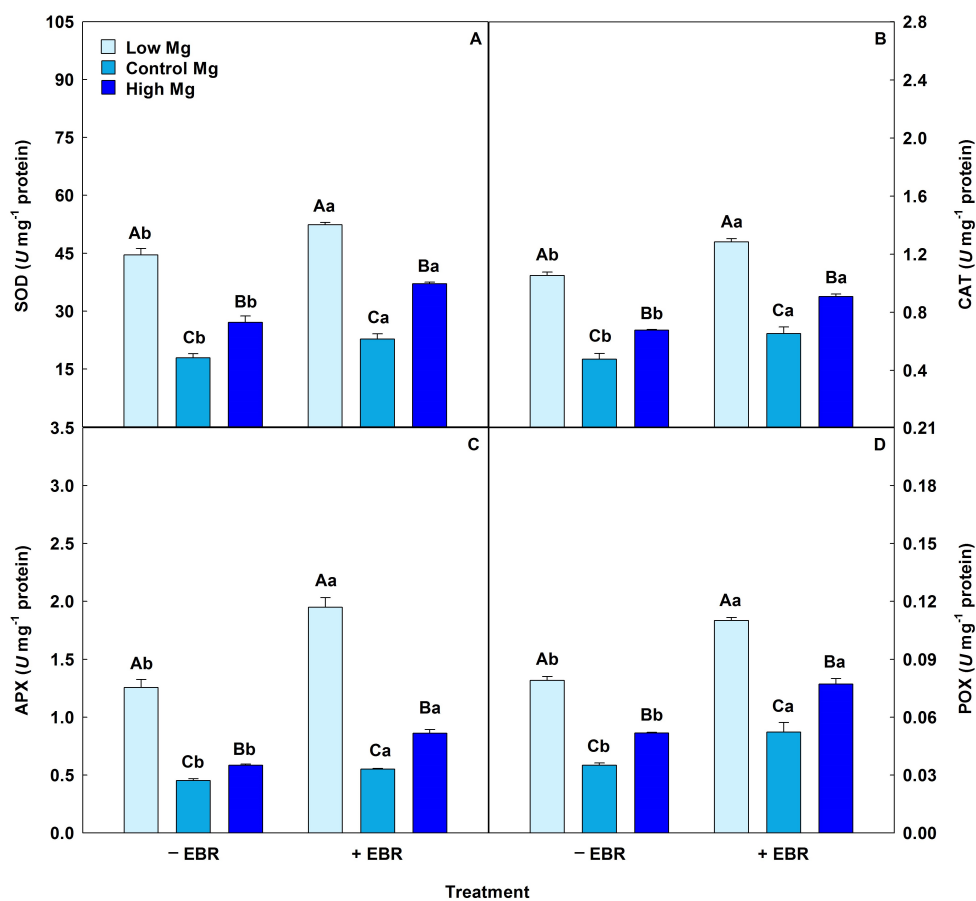
Fig. 2. Activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POX) in soybean plants sprayed with EBR and exposed to different Mg supplies. Columns with different letters indicate significant differences from the Scott-Knott test ($P<0.05$). Columns corresponding to means from five repetitions and standard deviations.
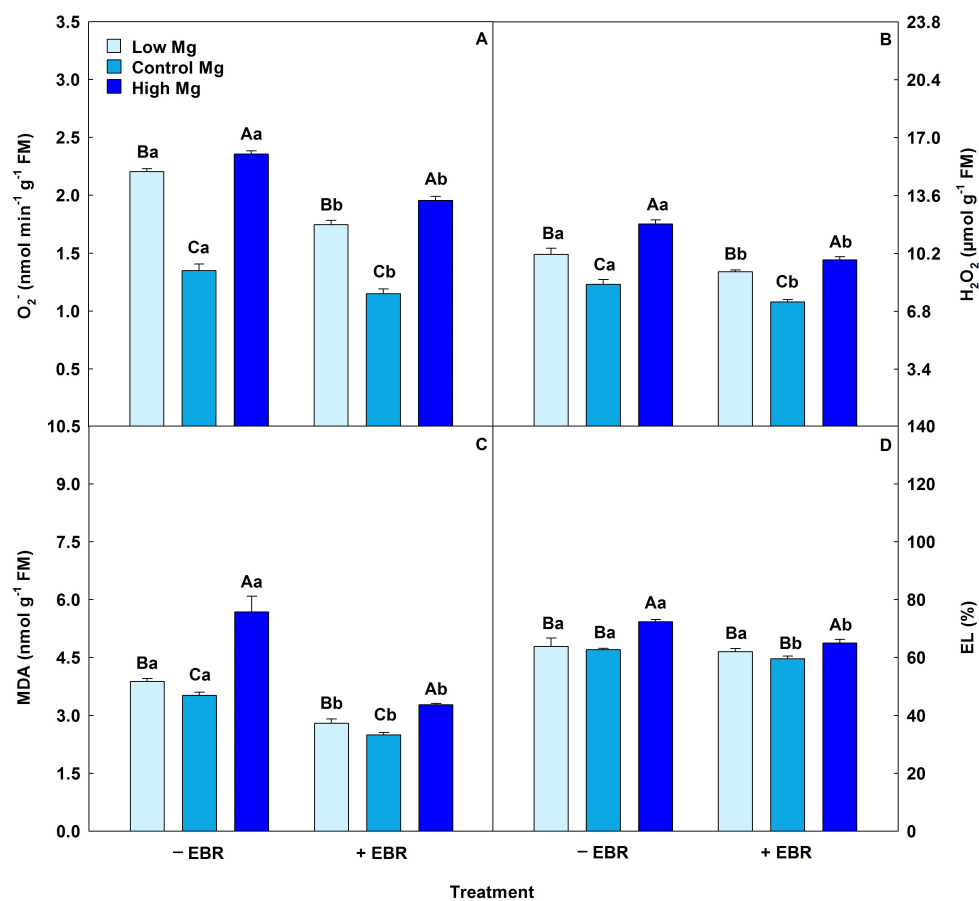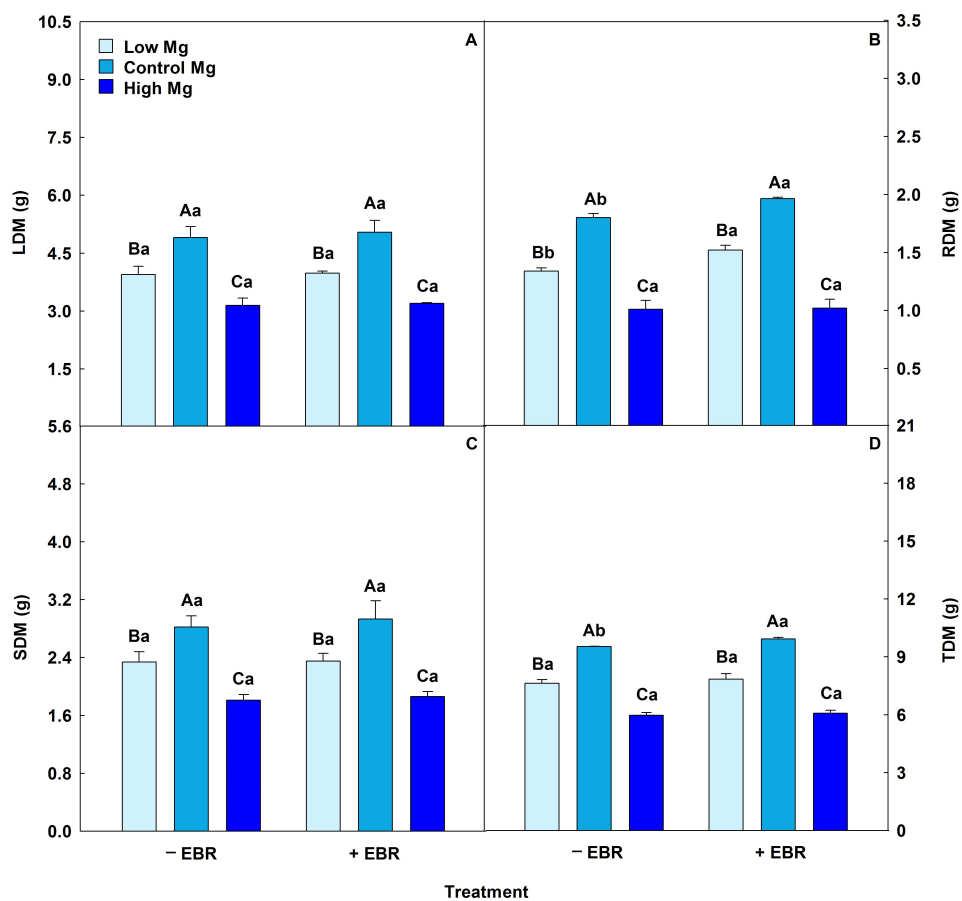
Fig. 3. Superoxide (O_2^-), hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and electrolyte leakage (EL) in soybean plants sprayed with EBR and exposed to different Mg supplies. Columns with different letters indicate significant differences from the Scott-Knott test ($P<0.05$). Columns corresponding to means from five repetitions and standard deviations.

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891 Fig. 4. Leaf dry matter (LDM), root dry matter (RDM), stem dry matter (SDM) and total dry matter (TDM) in
892 soybean plants sprayed with EBR and exposed to different Mg supplies. Columns with different letters indicate
893 significant differences from the Scott-Knott test ($P<0.05$). Columns corresponding to means from five repetitions
894 and standard deviations.

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911 **Tables**

912 Table 1. Mg contents in soybean plants sprayed with EBR and exposed to different Mg supplies.

EBR	Mg supply	Mg in root (mg g DM^{-1})	Mg in stem (mg g DM^{-1})	Mg in leaf (mg g DM^{-1})
_	Low	$9.58 \pm 0.52Cb$	$1.46 \pm 0.04Bb$	$1.08 \pm 0.01Cb$
_	Control	$13.71 \pm 0.10Ba$	$1.70 \pm 0.03Bb$	$3.53 \pm 0.08Ba$
_	High	$29.03 \pm 0.19Aa$	$27.80 \pm 0.41Aa$	$29.79 \pm 0.35Aa$
+	Low	$10.89 \pm 0.40Ca$	$2.46 \pm 0.03Ba$	$2.16 \pm 0.02Ca$
+	Control	$14.29 \pm 0.47Ba$	$2.60 \pm 0.03Ba$	$3.60 \pm 0.05Ba$
+	High	$23.94 \pm 0.69Ab$	$24.59 \pm 0.28Ab$	$27.73 \pm 0.07Ab$

913 Mg = Magnesium. Columns with different uppercase letters between Mg supplies (low, control and high Mg supply under equal EBR level) and lowercase letters between

914 EBR level (with and without EBR under equal Mg supply) indicate significant differences from the Scott-Knott test ($P<0.05$). Means $\pm$ SD, n = 5.

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929 Table 2. Nutrient contents in soybean plants sprayed with EBR and exposed to different Mg supplies.

EBR	Mg supply	K (mg g DM^{-1})	Ca (mg g DM^{-1})	S (mg g DM^{-1})	Mn (µg g DM^{-1})	Cu (µg g DM^{-1})	Bo (µg g DM^{-1})
Contents in root							
_	Low	39.37 ± 0.69Bb	14.93 ± 0.70Bb	2.50 ± 0.09Ba	507.17 ± 21.19Cb	9.05 ± 0.33Cb	28.73 ± 0.80Cb
_	Control	41.90 ± 0.48Aa	23.83 ± 0.50Aa	2.69 ± 0.09Aa	585.73 ± 6.69Ab	15.68 ± 0.64Ab	39.76 ± 0.66Ab
_	High	22.15 ± 0.25Cb	6.20 ± 0.20Cb	2.25 ± 0.03Cb	556.42 ± 17.65Bb	11.89 ± 0.40Bb	30.59 ± 0.79Bb
+	Low	40.52 ± 0.53Ba	19.39 ± 0.51Ba	2.61 ± 0.09Aa	605.00 ± 8.31Ba	13.15 ± 0.74Ca	38.45 ± 0.89Ca
+	Control	42.47 ± 0.43Aa	24.40 ± 0.04Aa	2.71 ± 0.07Aa	627.61 ± 8.48Aa	19.59 ± 0.33Aa	51.30 ± 0.93Aa
+	High	27.65 ± 0.93Ca	8.15 ± 0.13Ca	2.59 ± 0.12Aa	606.13 ± 2.18Ba	14.16 ± 0.22Ba	43.89 ± 0.57Ba
Contents in stem							
_	Low	50.91 ± 0.73Bb	13.83 ± 0.48bB	1.48 ± 0.02Ba	8.90 ± 0.37Ca	1.62 ± 0.07Bb	31.60 ± 0.19Bb
_	Control	53.01 ± 0.46Ab	22.31 ± 0.28Ab	1.58 ± 0.03Ab	15.71 ± 0.31Aa	1.76 ± 0.01Ab	34.67 ± 0.41Ab
_	High	37.85 ± 0.64Cb	6.59 ± 0.21Cb	1.31 ± 0.05Cb	10.73 ± 0.26Bb	1.72 ± 0.02Ab	24.50 ± 0.80Cb
+	Low	52.38 ± 0.61Ba	15.64 ± 0.34Ba	1.50 ± 0.07Ba	9.33 ± 0.07Ca	1.72 ± 0.09Ca	34.52 ± 0.95Ba
+	Control	55.89 ± 0.72Aa	23.14 ± 0.28Aa	1.64 ± 0.01Aa	16.19 ± 0.12Aa	1.91 ± 0.03Aa	35.74 ± 0.65Aa
+	High	43.19 ± 0.48Ca	7.64 ± 0.16Ca	1.42 ± 0.04Ca	11.24 ± 0.64Ba	1.80 ± 0.02Ba	26.97 ± 0.18Ca
Contents in leaf							
_	Low	28.21 ± 0.31Bb	13.24 ± 0.03Bb	2.84 ± 0.08Aa	56.47 ± 0.31Cb	1.48 ± 0.04Cb	44.04 ± 0.73Bb
_	Control	30.12 ± 0.21Ab	21.19 ± 0.19Ab	2.89 ± 0.06Aa	65.23 ± 0.25Ab	1.73 ± 0.00Ab	51.18 ± 0.29Ab
_	High	25.89 ± 0.29Cb	11.47 ± 0.27Cb	2.43 ± 0.06Ba	58.98 ± 0.23Bb	1.65 ± 0.04Bb	36.52 ± 0.33Cb
+	Low	33.88 ± 0.13Aa	13.71 ± 0.24Ba	2.92 ± 0.10Aa	57.60 ± 0.27Ca	1.52 ± 0.01Ca	52.72 ± 0.63Ba
+	Control	34.00 ± 0.46Aa	21.96 ± 0.17Aa	2.99 ± 0.06Aa	76.06 ± 0.84Aa	2.15 ± 0.03Aa	58.08 ± 0.41Aa
+	High	26.66 ± 0.25Ba	11.90 ± 0.14Ca	2.50 ± 0.04Ba	65.21 ± 0.61Ba	1.82 ± 0.02Ba	45.26 ± 0.64Ca

930 Mg = Magnesium; K = Potassium; Ca = Calcium; S = Sulfur; Mn = Manganese; Cu = Copper; Bo = Boron. Columns with different uppercase letters between Mg supplies

931 (low, control and high Mg supply under equal EBR level) and lowercase letters between EBR level (with and without EBR under equal Mg supply) indicate significant

932 differences from the Scott-Knott test ($P<0.05$). Means ± SD, n = 5.

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934   Table 3. Chlorophyll fluorescence in soybean plants sprayed with EBR and exposed to different Mg supplies.

EBR	Mg supply	Φ_{PSII}	q_P	NPQ	ETR (μmol m^{-2} s^{-1})	EXC (μmol m^{-2} s^{-1})	ETR/P_N
_	Low	0.23 ± 0.01Ab	0.33 ± 0.01Aa	0.62 ± 0.03Ba	34.9 ± 0.9Ab	0.70 ± 0.01Ba	2.17 ± 0.10Ba
_	Control	0.24 ± 0.01Ab	0.33 ± 0.01Ab	0.59 ± 0.05Ba	36.1 ± 1.8Ab	0.70 ± 0.01Ba	1.99 ± 0.13Ba
_	High	0.07 ± 0.01Bb	0.15 ± 0.01Bb	0.83 ± 0.05Aa	10.3 ± 0.5Bb	0.81 ± 0.02Aa	6.63 ± 0.50Ab
+	Low	0.25 ± 0.01Ba	0.33 ± 0.02Ba	0.62 ± 0.04Aa	38.1 ± 2.1Ba	0.68 ± 0.02Ba	2.05 ± 0.11Ba
+	Control	0.31 ± 0.01Aa	0.45 ± 0.03Aa	0.48 ± 0.03Bb	46.4 ± 2.8Aa	0.62 ± 0.02Cb	2.36 ± 0.18Ba
+	High	0.10 ± 0.01Ca	0.19 ± 0.01Ca	0.67 ± 0.03Ab	15.5 ± 1.1Ca	0.77 ± 0.03Ab	7.63 ± 0.56Aa

935   Φ_{PSII} = Effective quantum yield of PSII photochemistry; q_P = Photochemical quenching coefficient; NPQ = Nonphotochemical quenching; ETR = Electron transport rate;

936   EXC = Relative energy excess at the PSII level; ETR/P_N = Ratio between the electron transport rate and net photosynthetic rate. Columns with different uppercase letters

937   between Mg supplies (low, control and high Mg supply under equal EBR level) and lowercase letters between EBR level (with and without EBR under equal Mg supply)

938   indicate significant differences from the Scott-Knott test ($P<0.05$). Means ± SD, n = 5.

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952 Table 4. Gas exchange in soybean plants sprayed with EBR and exposed to different Mg supplies.

EBR	Mg 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	16.0 ± 0.6Bb	2.28 ± 0.11Ba	0.21 ± 0.01Ba	241 ± 6Ba	7.06 ± 0.21Ab	0.066 ± 0.003Bb
_	Control	18.1 ± 0.6Ab	2.47 ± 0.20Aa	0.24 ± 0.01Aa	224 ± 6Ca	7.38 ± 0.56Ab	0.081 ± 0.004Ab
_	High	1.5 ± 0.1Ca	1.70 ± 0.13Ca	0.11 ± 0.01Ca	378 ± 8Aa	0.92 ± 0.08Ba	0.004 ± 0.000Ca
+	Low	18.5 ± 0.7Ba	2.14 ± 0.10Aa	0.12 ± 0.01Bb	128 ± 6Bb	8.70 ± 0.41Aa	0.145 ± 0.011Ba
+	Control	19.7 ± 0.7Aa	2.19 ± 0.05Ab	0.14 ± 0.01Ab	100 ± 6Cb	9.01 ± 0.33Aa	0.197 ± 0.013Aa
+	High	2.0 ± 0.1Ca	1.82 ± 0.16Ba	0.12 ± 0.01Ba	353 ± 16Ab	1.12 ± 0.09Ba	0.005 ± 0.000Ca

953 P_N = Net photosynthetic rate; E = Transpiration rate; g_s = Stomatal conductance; C_i = Intercellular CO_2 concentration; WUE = Water-use efficiency; P_N/C_i = Carboxylation

954 instantaneous efficiency. Columns with different uppercase letters between Mg supplies (low, control and high Mg supply under equal EBR level) and lowercase letters

955 between EBR level (with and without EBR under equal Mg supply) indicate significant differences from the Scott-Knott test ($P<0.05$). Means ± SD, n = 5.

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970 Table 5. Photosynthetic pigments in soybean plants sprayed with EBR and exposed to different Mg supplies.

EBR	Mg 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	6.37 ± 0.37Ba	1.93 ± 0.14Cb	8.30 ± 0.45Ba	0.27 ± 0.01Cb	3.30 ± 0.20Aa	31.18 ± 1.65Aa
_	Control	8.46 ± 0.36Ab	3.26 ± 0.13Ab	11.72 ± 0.25Ab	0.49 ± 0.02Ab	2.60 ± 0.20Ba	24.24 ± 1.70Ba
_	High	8.35 ± 0.26Aa	2.79 ± 0.06Bb	11.14 ± 0.24Ab	0.38 ± 0.03Bb	3.00 ± 0.14Aa	29.57 ± 2.49Aa
+	Low	6.38 ± 0.87Ca	2.54 ± 0.17Ca	8.92 ± 0.26Ca	0.36 ± 0.04Ca	2.51 ± 0.30Ab	24.81 ± 1.98Ab
+	Control	9.88 ± 0.18Aa	5.00 ± 0.13Aa	14.88 ± 0.31Aa	0.74 ± 0.06Aa	1.98 ± 0.02Bb	20.30 ± 1.93Bb
+	High	8.45 ± 0.21Ba	3.65 ± 0.79Ba	12.10 ± 0.83Ba	0.58 ± 0.02Ba	2.42 ± 0.61Ab	20.96 ± 1.96Bb

971 Chl a = Chlorophyll a; Chl b = Chlorophyll b; Total chl = Total chlorophyll; Car = Carotenoids. Columns with different uppercase letters between Mg supplies (low,

972 control and high Mg supply under equal EBR level) and lowercase letters between EBR level (with and without EBR under equal Mg supply) indicate significant

973 differences from the Scott-Knott test ($P<0.05$). Means ± SD, n = 5.

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