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

LORENE BIANCA ARAÚJO TADAIESKY

IRON TOLERANCE MODULATED BY BRASSINOSTEROIDS IS TRIGGERED ENHANCING AERENCHYMA AREA AND METAL HOMEOSTASIS, COUPLED TO BENEFITS ON ROS SCAVENGING AND CO_2 ASSIMILATION IN RICE PLANTS

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

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LIST OF ABBREVIATIONS

APX	Ascorbate peroxidase
BCD	Bulliform cell diameter
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
EDS	Equatorial diameter of the stomata
EL	Electrolyte leakage
ETAb	Epidermis thickness from abaxial leaf side
ETAd	Epidermis thickness from adaxial leaf side
ETR	Electron transport rate
ETR/P_N	Ratio between the apparent electron transport rate and net photosynthetic rate
EXC	Relative energy excess at the PSII level
F_0	Minimal fluorescence yield of the dark-adapted state
Fe	Iron
F_m	Maximal fluorescence yield of the dark-adapted state
F_v	Variable fluorescence
F_v/F_m	Maximal quantum yield of PSII photochemistry
g_s	Stomatal conductance
H_2O_2	Hydrogen peroxide
K	Potassium
LAA	Leaf aerenchyma area
MDA	Malondialdehyde
Mg	Magnesium

Mn	Manganese
MT	Mesophyll thickness
NPQ	Nonphotochemical quenching
O_2^-	Superoxide
PDS	Polar diameter of the stomata
P_N	Net photosynthetic rate
P_N/C_i	Instantaneous carboxylation efficiency
POX	Peroxidase
PSII	Photosystem II
q_P	Photochemical quenching
RAA	Root aerenchyma area
RCD	Root cortex diameter
RDM	Root dry matter
RMD	Root metaxylem diameter
RDT	Root endodermis thickness
RET	Root epidermis thickness
ROS	Reactive oxygen species
RUBISCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RXT	Root exodermisthickness
SD	Stomatal density
SDM	Shootdrymatter
SF	Stomatal functionality
SOD	Superoxide dismutase
TD	Trichome density
TDM	Total dry matter
Total Chl	Total Chlorophyll
VCD	Vascular cylinder diameter
WUE	Water-use efficiency
Zn	Zinc
Φ_{PSII}	Effective quantum yield of PSII photochemistry

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SUMMARY

RESUMO

A toxicidade de ferro (Fe) é um dos estresses abióticos mais frequentes no arroz, afetando de 15% a 30% da produção total. Os brassinosteróides (BRs), incluindo o 24-epibrassinolídeo (EBR), regulam a homeostase de íons e melhoram o sistema antioxidante. O objetivo desta pesquisa foi responder se o EBR pode contribuir para a tolerância das plantas de arroz expostas à toxicidade de Fe, avaliando as possíveis repercussões nas características anatômicas, concentrações de nutrientes, sistema antioxidante e trocas gasosas. O experimento foi randomizado com quatro tratamentos, incluindo duas concentrações de Fe (250 e 6250 Mm de Fe descritas como controle e toxicidade, respectivamente) e duas concentrações de brassinosteróides (0 e 10 nM EBR, descritas como - EBR e + EBR, respectivamente). Plantas expostas à toxicidade de Fe e tratadas com EBR apresentaram aumentos nas razões Mg^{2+}/Fe^{2+}, Mn^{2+}/Fe^{2+}, Cu^{2+}/Fe^{2+} e Zn^{2+}/Fe^{2+} na parte aérea de 83%, 89%, 103% e 120%, respectivamente, comparando com o mesmo tratamento sem EBR. Esta pesquisa revelou que o EBR mitigou os efeitos deletérios da toxicidade de Fe sobre plantas de arroz, modulando a área de aerênquima, na qual esta estrutura contribui para a formação de uma barreira oxidativa, imobilizando o Fe na superfície radicular. Concomitantemente, esse esteróide aumentou o conteúdo de outros metais, como magnésio, manganês, cobre e zinco, comprovando a influência relacionada à homeostase do metal, confirmada por aumentos nas razões de metais. Plantas sob toxicidade de Fe e tratadas com EBR apresentaram aumentos em todas as atividades enzimáticas avaliadas, superóxido dismutase, catalase, ascorbato peroxidase e peroxidase, mitigando os danos oxidativos e favorecendo a eliminação de espécies reativas de oxigênio. Finalmente, as ações do EBR minimizaram os impactos negativos induzidos pelo excesso de Fe na eficiência fotossintética líquida e na eficiência instantânea da carboxilação, sendo esses benefícios diretamente relacionados ao maior transporte de elétrons e densidade estomática e indiretamente ligados ao mecanismo de proteção exercido pelas enzimas antioxidantes no maquinário fotossintético. Portanto, nossos resultados indicam claramente que o EBR é capaz de conferir tolerância em plantas de arroz sob toxicidade de Fe.

KEYWORDS: 24-epibrassinolide. Catalase. Fotossíntese. *Oryza sativa*. Anatomia da raiz

ABSTRACT

Iron (Fe) toxicity is one of the most frequent abiotic stresses in rice, affecting from 15% to 30% of total production. Brassinosteroids (BRs), including the 24-epibrassinolide (EBR) regulate ion homeostasis and improve antioxidant system. The aim of this research was to answer if the EBR can contribute to tolerance of rice plants exposed to Fe toxicity, evaluating the possible repercussions on anatomical characteristics, nutrient concentrations, antioxidant system, and gas exchange. The experiment was randomized with four treatments, including two Fe supply (250 and 6250 Mm Fe described as control and toxicity, respectively) and two concentrations of brassinosteroids (0 and 10 nM EBR, described as – EBR and + EBR, respectively). Plants exposed to Fe toxicity and treated with EBR presented increases in Mg^{2+}/Fe^{2+}, Mn^{2+}/Fe^{2+}, Cu^{2+}/Fe^{2+} and Zn^{2+}/Fe^{2+} ratios in shoot of 83%, 89%, 103% and 120%, comparing with same treatment without EBR. This research revealed that the EBR mitigated the deleterious effects of the Fe toxicity on rice plants, modulating the aerenchyma area, in which this structure contribute to the formation of an oxidative barrier, Fe immobilization in root surface. Concomitantly, this steroid increased the contents of other metals, such as magnesium, manganese, copper and zinc, proving the influence related to metal homeostasis, confirmed by increases in metal ratios. Plants under Fe toxicity and treated with EBR presented increases in all enzyme activities evaluated, superoxide dismutase, catalase, ascorbate peroxidase and peroxidase, mitigating the oxidative damages and favoring the scavenging of reactive oxygen species. Finally, the EBR actions alleviated the negative impacts induced by Fe excess on net photosynthetic rate and carboxylation instantaneous efficiency, being these benefits directly related to higher electron transport and stomatal density and indirectly linked to protection mechanism exercised by the antioxidant enzymes on photosynthetic machinery. Therefore, our results clearly indicate that the EBR is able to confer tolerance in rice plants under Fe toxicity.

KEYWORDS: 24-epibrassinolide. Catalase. Photosynthesis. *Oryza sativa*. Root anatomy

1. CONTEXTUALIZATION

Rice is grown and consumed in all continents, among the 10 largest producing countries, eight belong to Asian continent, Brazil is in ninth position, contributing 1.7% of world production. In Mercosur, Brazil stands out, ranks 1st, with a historical average production of 12 million tons of rice (CONAB, 2018). The Rio Grande do Sul is the country's largest producer, with a production of 8.315.795 tons of rice, followed by Santa Catarina, the second largest domestic producer, with a production of 1.078.015 tons (IBGE, 2017).

Rice (*Oryza sativa* L.) is one of the most cultivated and consumed cereals in the world, can be grown in three different hydric conditions: dryland, moist soils and flood irrigation. In dryland cultivation, sowing is done on dry soil and its development and productivity are conditioned to climatic conditions. The cultivation in moist soils is carried out on soils partially drained and temporarily flooded during rainy periods. The irrigated cultivation, in turn, requires a permanent water depth during the vegetative phase until phase of physiological maturation. This third cropping system corresponds to the majority of the planted area in the world and contributes 75% of the world production of rice grains (ABDALLAH et al., 2018).

Iron (Fe), an essential nutrient for plants, is required for vital respiration processes in photosynthesis, where it participates in the transfer of electrons through reversible redox reactions, changing between Fe^{2+} and Fe^{3+} (CHEN et al., 2015). Several stages of metabolism of photosynthetic pigments are iron-dependent (BRIAT et al., 2010). However, if absorbed in excess, iron induces nutritional disturbances and oxidative damages that adversely affect crop production (STEIN et al., 2014).

Iron toxicity is one of most frequent abiotic stresses in rice crops under anaerobic conditions and in acid soils (STEIN et al., 2009). This toxicity tends to occur in soils that have been flooded for a long period of time because the redox potential reduction causes Fe^{3+} in soil minerals to be converted to Fe^{2+} (ELEC et al., 2013).

Brassinosteroids (BRs) are steroids that have significant growth promoting properties (BARTWAL et al., 2013; VARDHINI; ANJUM, 2015). Their role in protecting plants against environmental stresses has become the subject of much scientific research to clarify their mode of action and contribute greatly to their use in agricultural production (SHARMA et al., 2012).

BRs are associated with many physiological processes of plants. They act directly on cell elongation, cell expansion, differentiation of xylem, besides promoting the increase of

yield and biomass production, accelerate the maturation of plants, stimulate antioxidant enzymes activity against oxidative damage to cells and induce tolerance to biotic stresses and abiotic (JIROUTOVA et al., 2018; TONG; CHU, 2018).

Among abiotic stresses, BRs are effective in increasing resistance to high and low temperatures, drought, salinity and metals toxicity (ANWAR et al., 2018). There are reports of the positive effects of 24-epibrassinolide (EBR), an analog of the BRs, in the attenuation of drought damage (FAROOQ et al., 2009), low temperature (RAO et al., 2002), Fe deficiency (WANG et al., 2015), salinity (LARRÉ et al., 2014) and excess metals (SHARMA et al., 2016) in *Oryza sativa* plants.

In rice, EBR increased the temperature resistance between 1-5 ° C due to increased ATP production, proline levels and enzymatic activity, indicating that this growth regulator is involved in membrane stability and osmoregulation (RAO et al. ., 2002). Another fact that interests physiologists and biochemists is related to the potentiation of antioxidant enzymes in plants exposed to abiotic stresses (FARIDUDDIN et al., 2013).

Given the importance of rice to the world economy, it is important to know and understand new tools of possible management, such as the effect of the application of BRs on the physiological, biochemical and anatomical parameters in response to the negative interferences caused by Fe toxicity.

Considering this context, the aim of this research was to answer if the EBR can contribute to tolerance of rice plants exposed to Fe toxicity, evaluating the possible repercussions on anatomical characteristics, nutrient concentrations, antioxidant system, and gas exchange.

1. 1. literature review

1.1.1. General aspects, socioeconomic importance and international panorama of rice

The genus *Oryza* belongs to family Poaceae, has two cultivated species (*Oryza sativa* L. and *Oryza glaberrima* Steud) and 21 wild species distributed in the tropics of Africa, Asia, Americas and Australia (GAUT et al., 1999; AMMIRAJU et al., 2010; SHIVRAIN et al., 2010). The species *O. glaberrima* is cultivated in West Africa, while *O. sativa* is widely distributed world, being cultivated in all continents (SHIVRAIN et al., 2010).

Rice (*Oryza sativa* L.) is a hydrophilic species whose evolutionary process led adaptation to most varied edaphoclimatic conditions (KHUSH et al., 2005). It is characterized as one of the species that can germinate and develop in permanently soaked soils, due to

presence of aerenchyma in the stem and roots of plant, that allow the passage of oxygen from air for layer of the rhizosphere (YAMAUCHI et al., 2013).

Cereal of high economic, social and nutritional value, the rice is used as staple food for more than half of world population (AKHTAR et al., 2010). Approximately 150 million hectares of rice are crop annually in the world, producing 590 million tons, where more 75% this production comes from irrigated cropping system (ABDALLAH et al., 2018).

Asian continent is responsible for the cultivation of approximately 90% of all rice produced in the world, basically in the irrigated system, followed by American, African, European and Oceanic continents with 5%, 4%, 0.5% and 0 , 5% of world rice production, respectively (CONAB, 2015).

China is the world's largest rice producer, has production of 145 million tonnes, followed by India with production of 105 million tons (CONAB, 2018). Brazil is among the ten largest producers of the world, responsible for contributing 1.6% of this block. The country, with annual production of 11.5 million tons of rice, contributes with 82% of Mercosur production, followed by Uruguay, Argentina and, lastly, Paraguay, with less than 1% of the total group (CONAB, 2018).

Rio Grande do Sul, Brazil's largest rice producer, contributes 71% of the national total, producing 8 million tons in an area of little more 1 million hectares; Followed by Santa Catarina, second largest national producer, with average production of 1 million tons (IBGE, 2017). Of this production, in the 2017/18 crop, irrigated rice was responsible for producing about 1.4 million tons of the total of 1.9 million tons, approximately 74% of the harvest (CONAB, 2018). This fact shows the importance of irrigated agricultural systems for national production.

Traditionally, rice is one of the most consumed foods in Brazil, has annual consumption of 11.5 million tons (CONAB, 2017). The crop has high nutritional value, providing 20% of calories, 15% of proteins, minerals and fibers (LESTARI et al., 2016). The grains are an excellent source of energy due to high concentration of carbohydrate, 80% is formed by starch, the remaining constituent percentages are completed by proteins, lipids, vitamins and minerals (CONAB, 2015). In addition, the chain of this grain has an important cultural, social and economic role in the country, with emphasis on creating jobs and income for domestic economy.

1.1.2. Iron and its toxicity

1.1.2.1. Iron in the soil and its absorption by plants

Iron (Fe) is an essential micronutrient for virtually all organisms. It is absorbed by the plants preferentially in its ionic form Fe^{2+} (LÓPEZ-MILLÁN et al., 2013). In soil, iron is present in the oxidized form (trivalent, Fe^{+3}), which is little available to plants. Thus, with the scarcity of iron in well-ventilated soils, the plants developed two different absorption strategies.

Strategy I is based on the reduction of iron in its ferric (Fe^{3+}) to ferrous (Fe^{2+}) form. Initially an ATPase proton extrusion present in the root epidermal cells occurs (AUNG et al., 2018). The acidification of the medium increases iron solubilities in the rhizosphere and consequently reduces oxidized Fe^{3+} to the soluble Fe^{2+} form, through iron chelate reductase enzymes encoded by the FRO2 gene (Ferric Reductase Oxidase), after the reduction, the iron is transported inland of the cell by specific IRT1 proteins (Iron Regulated Transporter) (GIEHL et al., 2009).

Grasses generally use strategy II, based on iron chelation. These plants release compounds known as phytosiderophores (FS) from the mugineic acid family. The FS are synthesized from L-methionine via nicotianamine (NA), by the action of the enzymes nicotianamine synthase (NAS) and nicotianamine tranferase (NAAT) (HINDT; GUERINOT, 2012). SFs have high affinity for Fe^{3+} and bind efficiently to trivalent iron in the rhizosphere. Fe^{3+}-FS complexes are then transported to the plant roots by specific carrier proteins encoded by YSL (Yellow Stripe Like) genes (WANG et al., 2013).

Lowland or irrigated soils are subject to periodic changes between anoxic and oxidized conditions. Under conditions of anaerobic conditions and low pH, followed by the depletion of oxygen in the soil solution, it favors the reduction of Fe^{3+} to Fe^{2+} because microorganisms use this iron as electro-receptors in their respiration (BECKER; ASCH, 2015; SANTOS et al., 2018). This behavior increases the iron availability due to the high concentration of Fe^{2+} in the soil solution, facilitating its absorption by the plants.

The rice, despite being a gramineous and using strategy II (grasses), has a ferrous carrier, the OsIRT1, allowing the absorption of Fe^{2+} and Fe^{3+}-FS complex uptake by the OsYSL15 (KOBAYASHI; NISHIZAWA, 2012). However, rice has a low activity of iron chelating reductase, which suggests an adaptation to directly absorb Fe^{2+}, which is abundant in submerged and anaerobic conditions.

1.1.2.2. Importance, transport and toxicity of iron in plants

Iron (Fe), an essential nutrient for plants, is necessary for physiological processes in plants (KIM, GUERINOT, 2007). The main function of Fe in plants is to be an enzymatic component, where most participate in oxidation processes (KOBAYASHI; NISHIZAWA, 2012). The enzymes that act in the transfer of electrons use the Fe as cofactor of choice, being these enzymes involved in a variety of reversible redox reactions (FOURCROY et al., 2014), participates in the route of chlorophyll synthesis (ABADÍA et al., 2011), in the photosynthesis (LIU et al., 2017), DNA synthesis, respiration (BRIAT et al., 2015), biosynthesis of phytoriums (gibberellic acid, jasmonic acid and ethylene) in the production and the elimination of reactive oxygen species (EROs) (DIXON; STOCKWELL, 2014).

Once absorbed by the roots, the iron is loaded into the xylem and translocated to the upper part through the transpiratory flow (BECKER; ASCH, 2005). Iron is oxidized when released into the xylem vessels, being transported as complexes with organic acids, especially citrate, which is the major metal chelator in the xylem (KOBAYASHI; NISHIZAWA 2012). The absorption of iron by mesophyll cells also requires a reduction step after the ferric ion is released by the citrate molecule, suggesting the presence of a specific Fe^{2+} transporter located in the foliar cell plasmalema. The electron donor for iron reduction is NADPH, and this process appears to be strongly induced by the increase in the NADPH/$NADP^{+}$ ratio resulting from the photochemical step of photosynthesis. Once in the mesophyll, iron can be stored in the vacuoles or immobilized by the ferritin protein, which occurs mainly in plastids (ZANCANI et al., 2004).

Excess iron in the shoot can cause phytotoxicity and necrotic spots on the leaves, browning of the roots and inhibition of plant growth, cationic imbalance, causing nutritional disorder (AUDEBERT; FOFANA, 2009). Among the essential nutrients, the decrease in the absorption of P, K, Ca and Mg has been reported for rice cultivars sensitive to Fe (SILVEIRA et al., 2007). In addition, iron toxicity also potentiates oxidative stress, with increased production of reactive oxygen species, such as hydrogen peroxide, superoxide and hydroxyl radical, the latter considered more toxic to the cell (GILL; TUTEJA, 2010).

To support growth and prevent toxicity of cellular iron, plants rely on the ability to store and remove iron (BRIAT et al., 2007). In rice, two ferritin class proteins are reported, FER1 and FER2. Ferritin acts as a mechanism strongly involved in the tolerance of this metal, since it incorporates Fe in its structure, minimizing its reactivity (MAJERUS et al., 2007).

1.1.2.3. Fe toxicity damages in rice

Iron is an abundant element in nature, corresponds to 5% of the earth's crust. However, majority of the iron present in the soil is unavailable to the plants, forming insoluble complexes in the presence of oxygen under conditions of neutral or alkaline pH (CHEN et al., 2015). The amount of iron in the soil solution required for plant growth ranges from 10^{-9} to 10^{-4} M; However, anoxic soils with acidic pH increase the amount of iron available and contributing to the excessive absorption of this element by plants (GUIRENOT; YI, 1994).

The occurrence of Fe toxicity is caused by large Fe^{2+} concentrations in the soil solution and, consecutively, by presence of high iron levels in the plant tissues. The iron concentration, which precedes soil submersion rarely exceeds 0.1 mg L^{-1}, can reach, in acid soils, approximately 600 mg L^{-1} (PONNAMPERUMA et al., 1978). However, in extreme cases, values up to 5,000 mg L^{-1} already were detected (HANSEN; VAN BREEMEN, 1975). The Fe^{2+} concentrations in the soil solution capable of affect rice production range from 10 to >2000 mg L^{-1} (BECKER; ASCH, 2015).

Production losses in rice associated with iron toxicity are estimated at 15 to 20% (SCHMIDT et al., 2013). Some authors extend this production loss to 30% (BECKER; ASCH, 2005; LIU et al., 2016). However, total production losses in rice caused by excess of this element have already been described in the literature (AUDEBERT; SAHRAWAT, 2000; WINSLOW et al., 1989). This amplitude of production losses in rice crops occurs due to varieties of factors that interfere in the cultivation, such as the cultivar used, stage of development of the plant, iron levels present in the solution and crop management (AUDEBERT; FOFANA, 2009).

Rice cultivars with different levels of tolerance to iron toxicity and agronomic practices such as alternative planting, adequate water management, and application of fertilizers have been developed and used in areas of occurrence of iron excess toxicity (SCHMIDT et al., 2013; SURIYAGODA et al., 2016). In this context, the most efficient practice is the choice of resistant genotypes. However, because of the environments diversity that excess iron toxicity can occur, none these options is totally applicable or efficient (BECKER; ASCH, 2005). Thus, knowledge about the impact of iron excess on the physiology of rice plants becomes necessary for the crop.

1.1.3. Action of brassinosteroids

Brassinosteroids (BRs) constitute a class of approximately 70 polyhydroxy steroid derivatives that appear to be distributed throughout vegetable kingdom (SIDDIQUI et al.,

2018). The identification of endogenous steroid compounds from plants resulted of effort almost 30 years of research to identify new substances capable of promoting growth present in pollen extracts from different plant species (FARIDUDDIN et al., 2013).

The first reports of BRs were identified in a study using the *Brassica napus* pollen extract, where was observed that this substance promoted increases in stem elongation and cell division in bean internodes (OKLESTKOVA et al., 2015). After this study, was concluded that BRs are specific translocable organic compounds isolated from a plant capable of promoting measurable growth in another plant (CLOUSE, 2011).

As growth promoters, BRs has fundamental roles in the regulating a broad variety of plant development physiological processes (FRIDMAN; SAVALDI-GOLDSTEIN, 2013; BAJGUZ; PIOTROWSKA-NICZYPORUK, 2014; BRUYNE et al., 2014), including seed germination, cell elongation, photomorphogenesis, xylem differentiation, root and trunk growth, floral initiation, and flower and fruit development (JIROUTOVA et al., 2018; TONG; CHU, 2018).

These steroids as growth regulators are also capable of interacting with other hormones or act similar way to them and stimulating or inhibiting root growth (MAZORRA; NÚÑEZ, 2008). In addition, they are involved in the activation of plant protection mechanisms, especially against oxidative stress, structural changes and cell membranes permeability (ANURADHA; RAO, 2007).

Involved in protection mechanisms, the BRs are able confer tolerance to plant against several types of biotic and abiotic stresses (ANWAR et al., 2018) as stresses caused by cold (LI et al., 2015), high temperature (ZHANG et al., 2014), water deficit (JANECZKO et al., 2016), excess water, salinity (ARORA et al., 2008), pathogens (BRUYNE et al., 2014) and heavy metals toxicity (RAMAKRISHNA; RAO 2015).

The application of these growth regulators, such as 24-epibrinosinolide (EBR), a brassinosteroid analogue, can improve the plant's response to different types of stress, increasing in the mechanisms that confer tolerance to biotic and abiotic stresses.

REFERENCES

ABADÍA, J; VÁZQUEZ, S; RELLÁN-ÁLVAREZ, R; EL-JENDOUBI, H; ABADÍA, A; ÁLVAREZ-FERNÁNDEZ, A. Towards a knowledge-based correction of iron chlorosis. **Plant Physiology and Biochemistry**, v. 49, n. 5, p. 471-482, 2011.

ABDALLAH, A; ALZOHEIRY, A; BURKEY, K. Comparison of flooded and furrow-irrigated transplanted rice (*Oryza sativa* L.): farm-level perspectives. **Journal of Irrigation and Drainage Engineering**, v. 144, n. 9, p. 1-8, 2018.

AKHTAR, S; BHAT, M. A; WANI, S. A; BHAT, K. A; CHALKOO, S; MIR, M. R; WANI, S. A. Marker assisted selection in rice. **Journal of Phytology**, v. 2, n. 10, p. 66-81, 2010.

AMMIRAJU, J. S. S; FAN, C; YU, Y; SONG, X; CRANSTON, K. A; PONTAROLLI, A. C; LU, F; SANYAL, A; JIANG, N; RAMBO, T; CURRIE, J; COLLURA, K; TALAG, J; BENNETZEN, J. L; CHEN, M; JACKSON, S; WING, R. A. Spatiotemporal patterns of genome evolution in allotetraploid species of the genus *Oryza*. **The Plant Journal**, v. 63, n. 3, p. 430-442, 2010.

ANURADHA S; RAO S. S. R. The effect of brassinosteroids on radish (*Raphanus sativus* L.) seedlings growing under cadmium stress. **Plant Soil Environ**, v. 53, p. 465-472, 2007.

ANWAR, A; LIU, Y; DONG, R; BAI, L; YU, X; LI, Y. The physiological and molecular mechanism of brassinosteroid in response to stress: a review. **Biological Research**, v. 51, p. 46, 2018.

ARORA, N; BHARDWAJ, R; SHARMA, P; ARORA H. K. Effects of 28-homobrassinolide on growth, lipid peroxidation and antioxidative enzyme activities in seedlings of *Zea mays* L. under salinity stress. **Acta Physiologiae Plantarum**, v. 30, p. 833, 2008.

AUDEBERT, A.; SAHRAWAT, K. L. Mechanisms for iron toxicity tolerance in lowland rice. **Journal of Plant Nutrition**, v. 23, n. 11-12, p. 1877-1885, 2000.

AUDEBERT, A; FOFANA, M. Rice yield gap due to iron toxicity in west Africa. **Journal of Agronomy and Crop Science**, v. 195, n. 1, p. 66-76, 2009.

AUNG, M. S; KOBAYASHI, T; MASUDA, H; NISHIZAWA, N. K. Rice HRZ ubiquitin ligases are crucial for the response to excess iron. **Physiologia Plantarum**, v. 163, p. 282–296, 2018.

BAJGUZ, A; PIOTROWSKA-NICZYPORUK, A. Interactive effect of brassinosteroids and cytokinins on growth, chlorophyll, monosaccharide and protein content in the green alga *Chlorella vulgaris* (Trebouxiophyceae). **Plant Physiology and Biochemistry**, v. 80, p. 176-183, 2014.

BARTWAL, A; MALL, R; LOHANI, P; GURU, S. K; ARORA, S. Role of secondary metabolites and brassinosteroids in plant defense against environmental stresses. **Journal of Plant Growth Regulation**, v. 32, n. 1, p. 216–232, 2013.

BECKER, M.; ASCH, F. Iron toxicity in rice - conditions and management concepts. **Journal of Plant Nutrition and Soil Science**, v. 168, n. 4, p. 558-573, 2005.

BRIAT, J. F; CURIE, C; GAYMARD, F. Iron utilization and metabolism in plants. **Current Opinion in Plant Biology**, v. 10, n. 3, p. 276-282, 2007.

BRIAT, J.-F; DUBOS, C; GAYMARD, F. Iron nutrition, biomass production, and plant product quality. **Trends in Plant Science**, v. 20, n. 1, p. 33-40, 2015.

BRIAT, J. F; RAVET, K. ARNAUD, N; DUC, C; BOUCHEREZ, J; TOURAINE, B; CELLIER F; GAYMARD, F. New insights into ferritin synthesis and function highlight a link between iron homeostasis and oxidative stress in plants. **Annals of Botany**, v. 105, n. 5, p. 811–822, 2010.

BRUYNE, L; HÖFTE, M; VLEESSCHAUWER, D. Connecting Growth and Defense: The Emerging Roles of Brassinosteroids and Gibberellins in Plant Innate Immunity. **Molecular Plant**, 7, p. 943–959, 2014.

CHEN, L; DING, C; ZHAO, X; XU, JUNXU; MOHAMMAD, A. A; WANG, S; DING, Y. Differential regulation of proteins in rice (*Oryza sativa* L.) under iron deficiency. **Plan Cell Reports**, v. 34, n. 1 p. 83–96, 2015.

CLOUSE, S. D. Brassinosteroids. **The Arabidopsis Book**, v. 2011, n. 9, p. 3-28, 2011.

CONAB. Companhia nacional de abastecimento. A cultura do arroz. Brasília, p. 1-180, 2015.

CONAB. Companhia nacional de abastecimento. Acompanhamento da safra brasileira - Safra 2017/18. Brasília, v. 10, p. 1-178, 2018.

CONAB. Companhia nacional de abastecimento. Perspectivas para a agropecuária - Safra 2015/16. Brasília, v. 3, p. 1-130, 2015.

CONAB. Companhia nacional de abastecimento. Perspectivas para a agropecuária - Safra 2075/18. Brasília, v. 5, p. 1-130, 2017.

DIXON, S. J; STOCKWELL, B. R. The role of iron and reactive oxygen species in cell death. **Nature Chemical Biology**, v. 10, n. 1, p. 9-17, 2014.

ELEC, V; QUIMIO, C. A; MENDOZA, R; SAJISE, A. G. C; BEEBOUT, S. E. J; GERGORIO, G. B; SINGH, R. K. Maintaining elevated Fe^{2+} concentration in solution culture for the development of a rapid and repeatable screening technique for iron toxicity tolerance in rice (*Oryza sativa* L.). **Plant and Soil**, v. 372, n. 1-2, p. 253–264, 2013.

FARIDUDDIN, Q; KHALIL, R. R. A. E; MIR, B. A; YUSUF, M; AHMAD, A. 24-Epibrassinolide regulates photosynthesis, antioxidant enzyme activities and proline content of *Cucumis sativus* under salt and/or copper stress. **Environmental Monitoring and Assessment**. v. 185, n. 9, pp 7845–7856, 2013.

FAROOQ, M; WAHID, A; BASRA, S. M. A; ISLAM-UD-DIN. Improving water relations and gas Exchange with brassinosteroids in rice under drought stress. **Journal of Agronomy and Crop Science** ,v. 195, n. 4, p. 262-269, 2009.

FOURCROY, P; SISÓ-TERRAZA, P; SUDRE, D; SAVIRÓN, M; REYT, G; GAYMARD, F; BRIAT, J. F. Involvement of the ABCG37 transporter in secretion of scopoletin and derivatives by *Arabidopsis* roots in response to iron deficiency. **New Phytologist**, v. 201, n. 1, p. 155-167, 2014.

FRIDMAN, Y; SAVALDI-GOLDSTEIN, S. Brassinosteroids in growth control: How, when and where. **Plant Science**, v. 209, p. 24-31, 2013.

GAUT, B. S; PEEK, A. S; MORTON, B. R; CLEGG, M. T. Patterns of genetic diversification within the Adh gene family in the grass (Poaceae). **Society for Molecular Biology and Evolution**, v. 16, n. 8, p. 1086-1097, 1999.

GIEHL, R. F. H; MEDA, A. R; WIRÉN, N. V. Moving up, down, and everywhere: signaling of micronutrients in plants. **Current Opinion in Plant Biology**, v. 12, n. 3, p. 320-327, 2009.

GILL, S. S; TUTEJA, N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. **Plant Physiology and Biochemistry**, v. 48, n. 12, p. 909-930, 2010.

GUERINOT, M. L.; YI, Y. Iron: nutritious, noxious and not readily available. **Plant Physiology**, v. 104, n. 3, p. 815-820, 1994.

HANSEN K.; VAN BREEMEN N. Translocation of iron in sulfate acid soils: II Reduction and diffusion of dissolved ferrous iron. **Soil Science Society of Amarica Journal**, v. 39, n. 6, p. 1148-1153, 1975.

HINDT, M. N; GUERINOT, M. L. Getting a sense for signals: regulation of the plant iron deficiency response. **Biochimica et Biophysica Acta**, v. 1823, n. 9, p. 1521–1530, 2012.

IBGE. Instituto brasileiro de geografia e estatística. Levantamento sistemático da produção agrícola. Pesquisa mensal de previsão e acompanhamento das safras agrícolas no ano civil janeiro, v. 30, n. 1, p. 1-81, 2017.

JANECZKO, A; GRUSZKA, D; POCIECHA, E; DZIURKA, M; FILEK, M; JURCZYK, B; KALAJI, H. M; KOCUREK, M; WALIGÓRSKI, P. Physiological and biochemical

characterisation of watered and drought-stressed barley mutants in the HvDWARF gene encoding C6- oxidase involved in brassinosteroid biosynthesis. **Plant Physiology and Biochemistry**, v. 99, p. 126-141, 2016.

JIROUTOVA, P; OKLESTKOVA, J; STRNAD, M. Crosstalk between Brassinosteroids and Ethylene during Plant Growth and under Abiotic Stress Conditions. **International Journal of Molecular Sciences**, v. 19, n. 10, p. 3283, 2018.

KIM, S. A; GUERINOT, M. L. Mining iron: Iron uptake and transport in plants. **FEBS Letters**, v. 581, n. 12, p. 2273–2280, 2007.

KOBAYASHI, T; NISHIZAWA, N. K. Iron Uptake, Translocation, and Regulation in Higher Plants. **Anual Review of Plant Biology**, v. 63 p. 131–52, 2012.

KHUSH, G. S. What it will take to feed 5.0 Billion Rice consumers in 2030. **Plant Molecular Biology**, v. 59, n. 1, p. 1–6, 2005.

LARRÉ C. F; MARINI P; MORAES C. L; AMARANTE L; MORAES D. M. Influência do 24-epibrassinolídeo na tolerância ao estresse salino em plântulas de arroz. **Semina: Ciências Agrárias**, v. 35, n. 1, p. 67-76, 2014.

LESTARI, P; UTAMI, D. W; ROSDIANTI, I; SABRAN, M. Morphological variability of Indonesian rice germplasm and the associated SNP markers. **Emirates Journal of Food and Agriculture**, v. 28, n. 9, p. 660-670, 2016.

LI, J; YANG, P; GAN, Y; YU, J; XIE, J. Brassinosteroid alleviates chilling-induced oxidative stress in pepper by enhancing antioxidation systems and maintenance of photosystem II. **Acta Physiologiae Plantarum**, v. 37, n. 11, p. 222-232, 2015.

LIU, H.; SOOMRO, A.; ZHU, Y.; QIU, X.; CHEN, K.; ZHENG, T.; YANG, L.; XING, D.; XU, J. QTL underlying iron and zinc toxicity tolerances at seedling stage revealed by two sets of reciprocal introgression populations of rice (*Oryza sativa* L.). **The Crop Journal**, v. 4, n. 4, p. 280-289, 2016.

LIU, H; ZHANG, C; WANG, J; ZHOU, C; FENG, H; MAHAJAN, M. D; HAN, X. Influence and interaction of iron and cadmium on photosynthesis and antioxidative enzymes in two rice cultivars. **Chemosphere**, v. 171, p. 240-247, 2017.

LÓPEZ-MILLÁN, A. F; GRUSAK, M. A; ABADÍA, A; ABADÍA, J. Iron deficiency in plants: an insight from proteomic approaches. **Frontiers in Plant Science**, v. 4, n. 254, p. 1-8, 2013.

MAJERUS, V; BERTIN, P; SWENDEN, V; FORTEMPS, A; LOBRÉAUX, S; LUTTS, S. Organ-dependent responses of the African rice to short-term iron toxicity: ferritin regulation and antioxidative responses. **Biologia Plantarum**, v. 51, n. 2, p. 303-312, 2007.

MAZORRA L. M; NÚÑEZ M. Estado actual sobre el conocimiento de la biosintesis y los mecanismos moleculares de acción de los brassinosteroids em las plantas. **Cultivos Tropicales**, v. 29, n. 1, p. 91-105, 2008.

OKLESTKOVA J; RÁROVÁ, L; KVASNICA, M; STRNAD, M. Brassinosteroids: synthesis and biological activities. **Phytochemistry reviews**, v. 14, n. 6, p. 1053-1072, 2015.

PONNAMPERUMA, F. N. Electrochemical changes in submerged soils and the growth of rice. **Soils and Rice**, p. 421-441, 1978.

RAMAKRISHNA, B; RAO, S. R. R. Foliar application of brassinosteroids alleviates adverse effects of zinc toxicity in radish (*Raphanus sativus* L.) plants. **Protoplasma**, v. 252, n. 2, p. 665-677, 2015.

RAO S. S. R; VARDHINI, B. V; SUJATHA, E; ANURADHA, S. Brassinosteroids – A new class of phytohormones. **Current Science**, v. 82, n. 10, p. 1239-1245, 2002.

SANTOS, F. I. C; MARINI, N; SANTOS, R. S; HOFFMAN, B. S. F; ALVES-FERREIRA, M; OLIVEIRA, A. C. Selection and testing of reference genes for accurate RT-qPCR in rice seedlings under iron toxicity. **Plos One**, v. 13, n. 3, p. 1-13, 2018.

SCHMIDT, F.; FORTES, M. A.; WESZ, J.; BUSS, G. L.; SOUSA, R. O. Impacto do manejo da água na toxidez por ferro no arroz irrigado por alagamento. **Revista Brasileira de Ciência do Solo**, v. 37, n. 5, p. 1226-1235, 2013.

SHARMA, P; JHA, A. B; DUBEY, R. S; PESSARAKLI, M. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. **Journal of Botany**, v. 47, p. 172-177, 2012.

SHARMA, P; KUMAR, A; BHARDWAJ, R. Plant steroidal hormone epibrassinolide regulate – Heavy metal stress tolerance in *Oryza sativa* L. by modulating antioxidant defense expression. **Enviromental and Experimental Botany**, v. 122, p. 1-9, 2016.

SHIVRAIN, V. K; BURGOS, N. R; AGRAMA, H. A; LAWTON-RAUH, A; LU, B; SALES, M. A; BOYETT, V; GEALY, D. R; MOLDENHAUER K. A. K. Genetic diversity of weedy red rice (*Oryza sativa*) in Arkansas. **Weed Research**, v. 50, n. 4, p. 289-302, 2010.

SIDDIQUI, H; AHMED, K. B. M; HAYAT, S. Comparative effect of 28-homobrassinolide and 24-epibrassinolide on the performance of different components influencing the photosynthetic machinery in *Brassica juncea* L. **Plant Physiology and Biochemistry**, v. 129, p. 198-212, 2018.

SILVEIRA, V. C; OLIVEIRA, A. P; SPEROTTOL, R A; ESPINDOLA, L. S; AMARAL, L; DIAS, J. F; CUNHA, J. B; FETTL, J. P. Influence of iron on mineral status of two rice (*Oryza sativa* L.) cultivars. **Brazilian Journal of Plant Physiology**, v. 19, n. 2, p. 127-139, 2007.

STEIN, R. J; DUARTE, G. L; SPOHR, M. G; LOPES, S. I. G; FETT, J. P. Distinct physiological responses of two rice cultivars subjected to iron toxicity under field conditions. **Annals of Applied Biology**, v. 154, n. 2, p. 269-277, 2009.

STEIN, R. J; LOPES, S. I. G; FETT, J. P. Iron toxicity in field-cultivated rice: contrasting tolerance mechanisms in distinct cultivars. **Theoretical and Experimental Plant Physiology**, v. 26, n. 2, p. 135-146, 2014.

SURIYAGODA, L. D. B.; SIRISENA, D. N.; SOMAWEERA, K. A. T. N.; DISSANAYAKE, A.; COSTA, W. A. J. M.; LAMBERS, H. Incorporation of dolomite reduces iron toxicity, enhances growth and yield, and improves phosphorus and potassium nutrition in lowland rice (*Oryza sativa* L). **Plant and Soil**, v. 410, n. 1-2, p. 299-312, 2016.

TONG, H; CHU, C. Functional specificities of brassinosteroid and potential utilization for crop improvement. **Trends in Plant Science**, v. 23, n. 11, p. 1016-1028, 2018.

VARDHINI, B. V; ANJUM, N. A. Brassinosteroids make plant life easier under abiotic stresses mainly by modulating major components of antioxidant defense system. **Fronties in Enviromental Science**, v. 2, n. 67, p. 1-16, 2015.

WANG, B; LI, G; ZHANG, W. H. Brassinosteroids are involved in Fe homeostasis in rice (*Oryza sativa* L.). **Journal of Experimental Botany**, v. 66, n. 9, p. 2749–2761, 2015.

WANG, N; CUI, Y; LIU, Y; FAN, H; DU, J; HUANG, Z; YUAN, Y; WU, H; LING, H. Q. Requirement and functional redundancy of Ib subgroup bHLH proteins for iron deficiency responses and uptake in *Arabidopsis thaliana*. **Molecular Plant**, v. 6, n. 2, p. 503-513, 2013.

WINSLOW, M. D.; YAMAUCHI, M.; ALLURI, K.; MASAJO, T. M. Reducing iron toxicity in rice with resistant genotype and ridge planting. **Agronomy Journal**, v. 81, n. 3, p. 458-460, 1989.

YAMAUCHI, T; SHIMAMURA, S; NAKAZONO, M; MOCHIZUKI, T. Aerenchyma formation in crop species: a review. **Field Crops Research**, v. 152, p. 8-16, 2013.

ZANCANI, M; PERESSON, C; BIROCCIO, A; FEDERICI, G; URBANI, A; MURGIA, I; MICALI, F; VIANELLO, A; MACRÌ, F. Evidence for the presence of ferritin in plant mitochondria. **European Journal of Biochemistry**, v. 271, n. 18, p. 3657-3664, 2004.

ZHANG, Y. P; HE, J; YANG, S. J; CHEN, Y. Y. Exogenous 24-epibrassinolide ameliorates high temperature-induced inhibition of growth and photosynthesis in *Cucumis melo*. **Biologia Plantarum**, v. 58, n. 2, p. 311-318, 2014.

MANUSCRIPT
FORMATTED TO PLANT GROWTH REGULATION

Page title

Iron tolerance modulated by brassinosteroids is triggered enhancing aerenchyma area and metal homeostasis, coupled to benefits on ROS scavenging and CO_2 assimilation in rice plants

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Author contribution statement

AKSL was the advisor of this project, planning all phases of this research. LBAT conducted the experiment in the greenhouse and performed physiological, biochemical and morphological determinations, while BRSS measured anatomical parameters and BLB performed nutritional determinations and helped in drafting the manuscript and in interpreting the results.

Iron tolerance modulated by brassinosteroids is triggered enhancing aerenchyma area and metal homeostasis, coupled to benefits on ROS scavenging and CO_2 assimilation in rice plants

Abstract

Iron (Fe) toxicity is one of the most frequent abiotic stresses in rice, affecting from 15% to 30% of total production. Brassinosteroids (BRs), including the 24-epibrassinolide (EBR) regulate ion homeostasis and improve antioxidant system. The aim of this research was to answer if the EBR can contribute to tolerance of rice plants exposed to Fe toxicity, evaluating the possible repercussions on anatomical characteristics, nutrient concentrations, antioxidant system, and gas exchange. The experiment was randomized with four treatments, including two Fe supply (250 and 6250 Mm Fe described as control and toxicity, respectively) and two concentrations of brassinosteroids (0 and 10 nM EBR, described as – EBR and + EBR, respectively). Plants exposed to Fe toxicity and treated with EBR presented increases in Mg^{2+}/Fe^{2+}, Mn^{2+}/Fe^{2+}, Cu^{2+}/Fe^{2+} and Zn^{2+}/Fe^{2+} ratios in shoot of 83%, 89%, 103% and 120%, comparing with same treatment without EBR. This research revealed that the EBR mitigated the deleterious effects of the Fe toxicity on rice plants, modulating the aerenchyma area, in which this structure contribute to the formation of an oxidative barrier, Fe immobilization in root surface. Concomitantly, this steroid increased the contents of other metals, such as magnesium, manganese, copper and zinc, proving the influence related to metal homeostasis, confirmed by increases in metal ratios. Plants under Fe toxicity and treated with EBR presented increases in all enzyme activities evaluated, superoxide dismutase, catalase, ascorbate peroxidase and peroxidase, mitigating the oxidative damages and favoring the scavenging of reactive oxygen species. Finally, the EBR actions alleviated the negative impacts induced by Fe excess on net photosynthetic rate and carboxylation instantaneous efficiency, being these benefits directly related to higher electron transport and stomatal density and indirectly linked to protection mechanism exercised by the antioxidant enzymes on photosynthetic machinery. Therefore, our results clearly indicate that the EBR is able to confer tolerance in rice plants under Fe toxicity.

Keywords 24-epibrassinolide ● catalase ● photosynthesis ● *Oryza sativa* ● root anatomy

Abbreviations

APX	Ascorbate peroxidase
BCD	Bulliform cell diameter
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
EDS	Equatorial diameter of the stomata
EL	Electrolyte leakage
ETAb	Epidermis thickness from abaxial leaf side
ETAd	Epidermis thickness from adaxial leaf side
ETR	Electron transport rate
ETR/P_N	Ratio between the apparent electron transport rate and net photosynthetic rate
EXC	Relative energy excess at the PSII level
F_0	Minimal fluorescence yield of the dark-adapted state
Fe	Iron
F_m	Maximal fluorescence yield of the dark-adapted state
F_v	Variable fluorescence
F_v/F_m	Maximal quantum yield of PSII photochemistry
g_s	Stomatal conductance
H_2O_2	Hydrogen peroxide
K	Potassium
LAA	Leaf aerenchyma area
MDA	Malondialdehyde
Mg	Magnesium
Mn	Manganese
MT	Mesophyll thickness
NPQ	Nonphotochemical quenching
O_2^-	Superoxide
PDS	Polar diameter of the stomata
P_N	Net photosynthetic rate
P_N/C_i	Instantaneous carboxylation efficiency
POX	Peroxidase
PSII	Photosystem II

q_P	Photochemical quenching
RAA	Root aerenchyma area
RCD	Root cortex diameter
RDM	Root dry matter
RMD	Root metaxylem diameter
RDT	Root endodermis thickness
RET	Root epidermis thickness
ROS	Reactive oxygen species
RUBISCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RXT	Root exodermis thickness
SD	Stomatal density
SDM	Shoot dry matter
SF	Stomatal functionality
SOD	Superoxide dismutase
TD	Trichome density
TDM	Total dry matter
Total Chl	Total Chlorophyll
VCD	Vascular cylinder diameter
WUE	Water-use efficiency
Zn	Zinc
Φ_{PSII}	Effective quantum yield of PSII photochemistry

Introduction

Rice (*Oryza sativa* L.) is one of the most cultivated and consumed cereals in the world, being an important source of carbohydrates to the two thirds of the world population (Parthasarathi et al. 2018). About 75% of this production is carried out under irrigated conditions, predominantly the flood irrigation system (Abdallah et al. 2018).

Iron (Fe) toxicity is one of the most frequent abiotic stresses in rice cultivations in flooded conditions and in acid soils (Stein et al. 2009). Rice yield losses associated with Fe toxicity generally affect from 15% to 30% of total production (Becker and Asch 2005; Stein et al. 2009). However, in case of severe toxicity can occur complete failure of the cultivation (Audebert and Sahrawat 2000). The yield loss levels vary between rice cultivars, stage of development of the plant, degree of Fe toxicity and crop management, in relation water control and mineral fertilization (Audebert and Fofana 2009).

In the soil, the Fe is oxidized (trivalent, Fe^{3+}), as oxides and hydroxides of Fe, which are few available to plants. However, in irrigated soils characterized by flooded conditions and low pH, there is a microbial reduction of Fe^{3+} to Fe^{2+} (Crestani et al. 2009; Santos et al. 2017). This reduction occurs because anaerobic microorganisms use Fe^{3+} as electro acceptors in their respiration, increasing the availability of Fe^{2+} in the soil solution (Becker and Asch 2005).

Fe is the essential micronutrient most absorbed by the plants, participates in several physiological and metabolic processes, such as chlorophyll synthesis, respiration, redox reactions and electron transfer (Chen et al. 2015; Rout and Sahoo 2015). However, the excessive absorption of this element by the plant can induce negative changes on metabolism (Briat et al. 2007), including oxidative stress, disorders linked to photosynthetic machinery and imbalance nutritional (Silveira et al. 2007; Pereira et al. 2013a; Wu et al. 2017).

The appearance of Fe symptoms involves the excessive uptake of Fe^{2+} by the roots and their translocation via xylem flow to the leaves, where the accumulation of this element maximizes the production of radicals that cause damages in essential organelles, like as chloroplasts, mitochondria and peroxisomes (Gill and Tuteja 2010). In general, the symptoms in leaf are characterized by purple, reddish-brown or yellow spots, resulting in lower growth rates of root and shoot, few tillering and low fertility of spikelets (Sahrawat 2004; Audebert and Fofana 2009).

A possible solution to damages occasioned by the Fe toxicity in plants may be the exogenous application of 24-epibrasinolide (EBR). EBR is a bioactive form of brassinosteroids (BRs) frequently used in experimental scale in low concentrations (Vardhini and Anjum 2015). The brassinosteroids (BRs) are polyhydroxy steroids, with multiple actions and possibilities on growth and development (Bartwal et al. 2013; Bajguz and Piotrowska-Niczyporuk 2014; Vardhini and Anjum 2015). In addition, this steroid also confer tolerance to plants against biotic and abiotic stresses (Krishna 2003; Anwar et al. 2018).

Positive effects induced by the EBR in plants under metallic stresses suggest that this steroid can mitigate the Fe toxicity. Fariduddin et al. (2015) described benefits on gas exchange in *Brassica juncea* plants exposed Mn toxicity. Research conducted by Ramakrishna and Rao (2015) with *Raphanus sativus* plants under Zn stress detected reduction of the oxidative stress intrinsically connected with lower accumulations of reactive oxygen species (ROS). Li et al. (2016) evaluating *Solanum lycopersicum*

seedlings submitted to Zn oxide nanoparticles found higher efficiency of the antioxidant system, being clearly linked to increases in activities of antioxidant enzymes.

Our hypothesis considered that the Fe toxicity causes negative interferences on plant metabolism. In other hand, the EBR exercise multiple roles contributing to ion homeostasis (Oliveira et al. 2019), improving anatomical responses (Maia et al. 2018), stimulating the antioxidant enzymes (Lima and Lobato 2017) and higher CO_2 fixation (Lima et al. 2018). Therefore, the aim of this research was to answer if the EBR can contribute to tolerance of rice plants exposed to Fe toxicity, evaluating the possible repercussions on anatomical characteristics, nutrient concentrations, antioxidant system, and gas exchange.

Materials and Methods

Location and growth conditions

The experiment was performed at the Campus of Paragominas of the Universidade Federal Rural da Amazônia, Paragominas, Brazil (2°55' S, 47°34' W). The study was conducted in a greenhouse with the temperature and humidity controlled. The minimum, maximum, and median temperatures were 22, 30 and 25.1 °C, respectively. The relative humidity during the experimental period varied between 60% and 80%.

Plants, containers and acclimation

Four-days-old seedlings of *Oryza sativa* L. cv. Puitá INTA CL™ were selected (similar aspects and sizes) and placed in 0.5-L pots (10cm in height and 8cm in diameter). All plants were cultivated under hydroponic conditions. A modified Hoagland and Arnon (1950) solution was used for nutrients, with the ionic strength beginning at 50% (6^{th} day) and later modified to 100% after two days (8^{th} day). After this period, the nutritive solution remained at total ionic strength.

Experimental design

The experiment was randomized with four treatments, including two Fe supply (250 and 6250 mM Fe described as control and toxicity, respectively) and two concentrations of brassinosteroids (0 and 10 nM EBR, described as – EBR and + EBR, respectively). Nine replicates for each one of the four treatments were conducted yielding a total of 36 experimental units used in the experiment, with three plants in each unit.

24-epibrassinolide (EBR) preparation and application

Ten-day-old seedlings were treated with 24-epibrassinolide (EBR) or Milli-Q water via root (containing a proportion of ethanol that was equal to that used to prepare the EBR solution) during 20 days (days 10–30 after the start of the experiment). The 0 and 10 nM EBR (Sigma-Aldrich, USA) solutions were prepared by dissolving the solute in ethanol followed by dilution with Milli-Q water [ethanol:water (v/v) = 1:10,000] (Ahammed et al. 2013).

Plant conduction and Fe application

Three plants per pot was used to examine the plant parameters. The plants received the following macro- and micronutrients contained in the nutrient solution: 8.75 mM KNO_3, 7.5 mMCa $(NO_3)_2 \cdot 4H_2O$, 3.25 mM $NH_4H_2PO_4$, 1.5 mM $MgSO_4 \cdot 7H_2O$, 62.50 µM KCl, 31.25 µM H_3BO_3, 2.50 µM $MnSO_4 \cdot H_2O$, 2.50 µM $ZnSO_4 \cdot 7H_2O$, 0.63 µM $CuSO_4 \cdot 5H_2O$ and 0.63 µM $NaMoO_4 \cdot 5H_2O$. For Fe treatments, $FeCl_2$ was used at concentrations of 250 mM (control) and 6250 mM (toxicity) applied over 15 days (days 15–30 after the start of the experiment). During the study, the nutrient solutions were changed at 07:00 h at 3-day intervals, with the pH adjusted to 5.5 using HCl or NaOH. On day 30 of the experiment, physiological and morphological parameters were measured for all plants, and leaf tissues were harvested for anatomical, biochemical and nutritional analyses.

Measurement of chlorophyll fluorescence

The minimal fluorescence yield of the dark-adapted state (F_0), the maximal fluorescence yield of the dark-adapted state (F_m), the variable fluorescence (F_v), the maximal quantum yield of PSII photochemistry (F_v/F_m), the effective quantum yield of PSII photochemistry (Φ_{PSII}), the photochemical quenching coefficient (q_P), the nonphotochemical quenching (NPQ), the electron transport rate (ETR), the relative energy excess at the PSII level (EXC) and the ratio between the electron transport rate and the net photosynthetic rate (ETR/P_N) were determined using a modulated chlorophyll fluorometer (model OS5p; Opti-Sciences). Chlorophyll fluorescence was measured in fully expanded leaves under light. Preliminary tests determined the location of the leaf, the part of the leaf and the time required to obtain the greatest F_v/F_m ratio; therefore, the acropetal third of leaves that were in the middle third of the plant and adapted to the dark for 30 min was used in the evaluation. The intensity and duration of the saturation light pulse were 7,500 µmol m^{-2} s^{-1} and 0.7 s, respectively.

Evaluation of gas exchange

The net photosynthetic rate (P_N), transpiration rate (E), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) were evaluated using an infrared gas analyser (model LCPro$^+$; ADC BioScientific). These parameters were measured at the adaxial surface of fully expanded leaves that were collected from the middle region of the plant. The water-use efficiency (WUE) was estimated according to Ma et al. (2004), and the instantaneous carboxylation efficiency (P_N/C_i) was calculated using the formula described by Aragão et al. (2012). Gas exchange was evaluated in all plants under constant conditions of CO_2 concentration, photosynthetically active radiation, air-flow rate and temperature in a chamber at 360 µmol mol^{-1} CO_2, 800 µmol photons m^{-2} s^{-1}, 300 µmol s^{-1} and 28 °C, respectively, between 10:00 and 12:00 h.

Measurements of anatomical parameters

Samples were collected from the middle region of the leaf limb of fully expanded leaves of the third node and roots 5 cm from the root apex. Subsequently, all collected botanical material was fixed in FAA 70 for 24 hours and dehydrated in ethanol and embedded in historesin Leica^{TM} (Leica, Nussloch, Germany).Transverse sections with a thickness of 5µmwere obtained with a rotating microtome (model Leica RM 2245, Leica Biosystems), stained with toluidine blue(O'Brien et al. 1964). For stomatal

characterization, the epidermal impression method was used according to Segatto et al. (2004). The slides were observed and photomicrographed under an optical microscope (Motic BA 310, Motic Group Co. LTD.) coupled to a digital camera (Motic 2500, Motic Group Co., LTD.). The images were analysed with Motic plus 2.0 previously calibrated with a micrometre slide of the manufacturer. The anatomical parameters evaluated were polar diameter of the stomata (PDS), equatorial diameter of the stomata (EDS), epidermis thickness from adaxial leaf side (ETAd), epidermis thickness from abaxial leaf side (ETAb), mesophyll thickness (MT), leaf aerenchyma area (LAA), bulliform cell diameter (BCD), and trichome density (TD). In both leaf faces, the stomatal density (SD) was calculated as the number of stomata per unit area and the stomatal functionality (SF) as the ratio PDS/EDS according to Castro et al. (2009). In root samples, the root epidermis thickness (RET), root exodermis thickness (RDT), root endodermis thickness (RDT), root cortex thickness (RCT), root aerenchyma area (RAA), vascular cylinder diameter (VCD) and root metaxylem diameter (RMD) were measured.

Extraction of antioxidant enzymes, superoxide and soluble proteins

Antioxidant enzymes (SOD, CAT, APX and POX), superoxide and soluble proteins were extracted from leaf tissues according to the method of (Badawi et al. 2004). The extraction mixture was prepared by homogenizing 500 mg of fresh plant material in 5 ml of extraction buffer, which consisted of 50 mM phosphate buffer (pH 7.6), 1.0 mM ascorbate and 1.0 mM EDTA. Samples were centrifuged at 14,000 × *g* for 4 min at 3 °C, and the supernatant was collected. Quantification of the total soluble proteins was performed using the method described by (Bradford 1976). Absorbance was measured at 595 nm, using bovine albumin as a standard.

Superoxide dismutase assay

For the SOD assay (EC 1.15.1.1), 2.8 ml of a reaction mixture containing 50 mM phosphate buffer (pH 7.6), 0.1 mM EDTA, 13 mM methionine (pH 7.6), 75 µM NBT, and 4 µM riboflavin was mixed with 0.2 ml of supernatant. The absorbance was then measured at 560 nm (Giannopolitis and Ries 1977).One SOD unit was defined as the amount of enzyme required to inhibit 50% of the NBT photoreduction. The SOD activity was expressed in unit mg^{-1} protein.

Catalase assay

For the CAT assay (EC 1.11.1.6), 0.2 ml of supernatant and 1.8 ml of a reaction mixture containing 50 mM phosphate buffer (pH 7.0) and 12.5 mM hydrogen peroxide were mixed, and the absorbance was measured at 240 nm (Havir and McHale 1987).The CAT activity was expressed in µmol H_2O_2 mg^{-1} protein min^{-1}.

Ascorbate peroxidase assay

For the APX assay (EC 1.11.1.11), 1.8 ml of a reaction mixture containing 50 mM phosphate buffer (pH 7.0), 0.5 mMascorbate, 0.1 mM EDTA, and 1.0 mM hydrogen peroxide was mixed with 0.2 ml of supernatant, and the absorbance was measured at 290 nm (Nakano and Asada 1981).The APX activity was expressed in µmol AsA mg^{-1} protein min^{-1}.

Peroxidase assay

For the POX assay (EC 1.11.1.7), 1.78 ml of a reaction mixture containing 50 mM phosphate buffer (pH 7.0) and 0.05 % guaiacol was mixed with 0.2 ml of supernatant, followed by addition of 20 µL of 10 mM hydrogen peroxide. The absorbance was then measured at 470 nm (Cakmak and Marschner 1992).The POX activity was expressed in µmol tetraguaiacol mg^{-1} protein min^{-1}.

Determination of superoxide concentration

For the determination of O_2^-, 1 ml of extract was incubated with 30 mM phosphate buffer [pH 7.6] and 0.51 mM hydroxylamine hydrochloride for 20 min at 25 °C. Then, 17 mM sulphanilamide and 7 mM α-naphthylamine were added to the incubation mixture for 20 min at 25 °C. After the reaction, ethyl ether was added in the identical volume and centrifuged at 3,000 × *g* for 5 min. The absorbance was measured at 530 nm (Elstner and Heupel 1976).

Extraction of nonenzymatic compounds

Nonenzymatic compounds (H_2O_2 and MDA) were extracted as described by Wu et al. (2006). Briefly, a mixture for extraction of H_2O_2 and MDA was prepared by homogenizing 500 mg of fresh leaf materials in 5 mL of 5% (w/v) trichloroacetic acid. Then, the samples were centrifuged at 15,000 x g for 15 min at 3°C to collect the supernatant.

Determination of hydrogen peroxide concentration

To measure H_2O_2, 200 µL of supernatant and 1800 µL of reaction mixture (2.5 mM potassium phosphate buffer [pH 7.0] and 500 mM potassium iodide) were mixed, and the absorbance was measured at 390 nm (Velikova et al. 2000).

Quantification of malondialdehyde concentration

MDA was determined by mixing 500 µL of supernatant with 1,000 µL of the reaction mixture, which contained 0.5% (w/v) thiobarbituric acid in 20% trichloroacetic acid. The mixture was incubated in boiling water at 95°C for 20 min, with the reaction terminated by placing the reaction container in an ice bath. The samples were centrifuged at 10,000 × g for 10 min, and the absorbance was measured at 532 nm. The nonspecific absorption at 600 nm was subtracted from the absorbance data. The MDA–TBA complex (red pigment) amount was calculated based on the method of Cakmak and Horst (1991), with minor modifications and using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Determination of electrolyte leakage

Electrolyte leakage was measured according to the method of Gong et al. (1998) with minor modifications. Fresh tissue (200 mg) was cut into pieces 1 cm in length and placed in containers with 8 mL of distilled deionised water. The containers were incubated in a water bath at 40°C for 30 min, and the initial electrical conductivity of the medium (EC_1) was measured. Then, the samples were boiled at 95°C for 20 min to release the electrolytes. After cooling, the final electrical conductivity (EC_2) was

measured (Gong et al. 1998). The percentage of electrolyte leakage was calculated using the formula EL (%) = (EC_1/EC_2) x 100.

Determination of photosynthetic pigments

The chlorophyll and carotenoid determinations were performed with 40 mg of leaf tissue. The samples were homogenized in the dark with 8 mL of 90% methanol (Nuclear). The homogenate was centrifuged at 6,000 × g for 10 min at 5°C. The supernatant was removed, and chlorophyll *a* (Chl *a*) and *b* (Chl *b*), carotenoid (Car) and total chlorophyll (total Chl) contents were quantified using a spectrophotometer (model UV-M51; Bel Photonics), according to the methodology of Lichtenthaler and Buschmann (2001).

Determining of Fe and nutrients

Samples with 100 mg of milled samples were weighed in 50-mL conical tubes (Falcon™, Corning, Mexico) and pre-digested (48 h) with 2 ml of sub boiled HNO_3 (DST 1000, Savillex, USA). After, 8 ml of a solution containing 4 ml of H_2O_2 (30% v/v, Synth, Brasil) and 4 ml of ultra-pure water (Milli-Q System, Millipore, USA) were added, and the mixture was transferred to a Teflon digestion vessel, closed and heated in a block digester (EasyDigest®, Analab, France) according to the following program: i) 100°C for 30 min; ii) 150°C for 30 min; iii) 130°C for 10 min; iv) 100°C for 30 min and; and v) left to cool. The volume was made to 50 mL with ultra-pure water, and iridium was used as an internal standard at 10 μg l^{-1}. The determination of Fe, K, Ca, Mg, Mn, Cu and Zn was carried out using an inductively coupled plasma mass spectrometer (ICP-MS 7900, Agilent, USA). Certified reference materials (NIST 1570a and NIST 1577c) were run in each batch for quality control purposes. All found values were in agreement with certified values.

Measurements of morphological parameters

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

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.

Results

EBR minimized the Fe contents in plants exposed to toxicity

The Fe contents in the root and shoot of rice plants exposed to Fe toxicity were significantly increased (Table 1). However, plants submitted to $FeCl_2$ (6250 mM Fe) and application of EBR (10 nM) had significant reductions of 17% and 40% in root and shoot, respectively, when compared to equal treatment without EBR.

Pretreatment with EBR induced higher protection against Fe excess

Plants exposed to Fe toxicity suffered declines in the values of RET, RXT, RDT, RCD, VCD, RMD and RAA (Fig. 1). Plants treated with EBR and exposed to Fe toxicity presented increases of 18%, 24%, 24%, 10%, 33%, 20% and 70% in RET, RXT, RDT, RCD, VCD, RMD and RAA (Table 2), respectively, when compared to equal treatment without EBR.

EBR regulate positively the metal homeostasis and contents of nutrients

Fe toxicity caused negative changes on nutrient contents in root and shoot (P<0.05). In other hand, plants treated with EBR and submitted to Fe toxicity presented increases in contents of K, Ca, Mg, Mn, Cu and Zn in shoot of 2%, 13%, 10%, 14%, 22% and 30% (Table 3), respectively, while in the root the increments were of 4%, 17%, 11%, 29%, 34% and 24%, respectively, comparing with similar treatment without steroid. In relation to metal ratios, plants exposed to Fe^{2+} and treated with EBR presented increases in Mg^{2+}/Fe^{2+}, Mn^{2+}/Fe^{2+}, Cu^{2+}/Fe^{2+} and Zn^{2+}/Fe^{2+} ratios in shoot of 83%, 89%, 103% and 120% (Table 4), respectively, while in the root the increments were of 99%, 67%, 100% and 101%, respectively, comparing with same treatment without EBR.

Improvements in the capture of light energy induced by the EBR

The Fe toxicity promoted an increase in F_0 and decreases in F_m, F_v and F_v/F_m (Fig. 2). However, the treatment Fe toxicity + EBR had a significant reduction of F_0 (26%) and increase in F_m, F_v and F_v/F_m of 6%, 25% and 18%, respectively, when compared to the same treatment without EBR. In addition, plants subjected to Fe toxicity suffered decreases in Φ_{PSII}, q_P and ETR and increases in NPQ, EXC and ETR/P_N (Table 5). However, plants submitted to 10 nM EBR and exposed to 6250 mM Fe had significant increases of 27%, 55% and 28% for Φ_{PSII}, q_P and ETR, respectively, and reductions in NPQ (27%), EXC (3%) and ETR/P_N (9%), if compared to the same treatment in the absence of EBR.

EBR attenuated the non-stomatal limitations in plants exposed to Fe toxicity

The Fe toxicity provoked significant reductions in the values of P_N, g_s, WUE and P_N/C_i and increase in C_i values (Table 6). However, treatment with 6250 mM Fe + 10 nM EBR showed increases of 41%, 4%, 74% and 45% in P_N, g_s, WUE and P_N/C_i and significant decreases of 19% and 4% in E and C_i, when compared to equal treatment without EBR.

Stomatal performance modulated by EBR

Fe toxicity caused significant increases in TD, PDS and EDS and negatively altered SD and SF values on both leaf faces (adaxial and abaxial), with exception to TD presents only in adaxial face (Fig. 3 and Table 7). However, plants receiving 10 nM of EBR combined with 6250 mM Fe had increases of 8% and 4% in SD and SF (abaxial), respectively, and reduction in TD (13%), PDS (8%) and EDS (12%) on the adaxial face, compared to the same treatment without EBR.

EBR positively modulated the leaf anatomy

Plants submitted to Fe toxicity presented significant decreases for ETAd, ETAb, LAA and BCD (Fig. 3 and Fig. 4). However, plants exposed to Fe toxicity + EBR had increases in ETAd, ETAb, MT, LAA and BCD and LAA of 10%, 18%, 4%, 21% and 9% (Table 8), respectively, relative to the same treatment without EBR application.

Benefits on the antioxidant system induced by EBR

The activities of antioxidant enzymes (SOD, CAT, APX and POX) were significantly increased in response to Fe toxicity (Fig. 5). The application of EBR in plants exposed to Fe^{3+}toxicity provoked increases (P<0.05) on the activities of these enzymes in 25%, 20%, 9% and 10% for SOD, CAT, APX and POX, respectively, in relation to equal treatment without EBR.

Steroid promoted decreases in the ROS concentrations and stress indicators

Plants under Fe toxicity presented significant increments on the oxidant compounds (Fig. 6). However, plants exposed to Fe toxicity + EBR had significant reductions in O_2^-, H_2O_2, MDA and EL of 31%, 28%, 15% and 44%, respectively, when compared to treatment under absence of EBR.

Maintenance of membrane integrity of chloroplasts after treatment with EBR

Significant decreases in Chl *a*, Chl *b* and Total Chl values and increases in ratio Chl *a*/Chl *b* and Total Chl/Car were observed in plants exposed to Fe toxicity (Table 9). In contrast, plants exposed to Fe toxicity + EBR presented increases of 21%, 20%, 20%, 44% and 1% for Chl *a*, Chl *b*, Total Chl, Car and Ratio Chl *a*/Chl *b*, respectively, and a decrease in ratio Total Chl/Car (15%), when compared to plants exposed to Fe toxicity without EBR.

EBR reduced the deleterious effects caused by Fe toxicity on biomass

The growth was significantly restricted after the exposition to Fe toxicity (Fig. 7). Interestingly control plants with EBR presented significant increases of 40%, 24% and 33% for SDM, RDM and TDM, respectively, In relation to plants exposed to Fe toxicity and treated with 10 nM of EBR showed significant increases in RDM (36%) and TDM (35%), when compared to equal treatment without steroid.

Discussion

Increases in Fe contents of root and shoot of rice plants confirm the effectiveness of the toxicity occasioned in this study. The treatment with 10 nM EBR minimized the toxic effects, reducing the Fe contents in plant tissues. Decreases in Fe contents in both tissues after application of EBR can be related to the higher synthesis of phytochelatins (PC) (Anwar et al. 2018). PC act in the mechanism of detoxification of heavy metals, in which are peptides responsible by the chelating of metal ions forming complexes in the cytoplasm of cells (Bajguz and Hayat 2009; Bajguz 2010). Choudhary et al. (2012) evaluating the effects of EBR and spermidine (Spd) on phytohormonal and physiological strategies capable to confer tolerance in *Raphanus sativus* seedlings during Cr-induced stress (1.2 mM K_2CrO_4)

found that the pre-treatment with EBR (1 nM) promoted increase in the PC concentration and a significant decrease in Cr content, when compared to the same treatment without EBR.

EBR promoted increases in RET, RXT, RDT, RCD and RAA of plants exposed to Fe toxicity, suggesting that the increases related to thickness and diameter of these tissues can contribute as a barrier, decreasing the Fe transport in excess. The aerenchyma are gaseous spaces that longitudinally lead the O_2 from the leaves to the roots (Yamauchi et al. 2013), and these structures contribute to the formation of an oxidative barrier in the rhizosphere that will oxidize Fe^{2+} to Fe^{3+}, resulting in the formation and accumulation of deposits of $Fe(OH)_3$ that will be immobilized (Becker and Asch 2005). Additionally, the exodermis and endodermis are tissues that act in the protection and selectivity of the root, contributing in the apoplastic immobilization of Fe absorbed by the roots (Enstone et al. 2003; Pereira et al. 2014). Therefore, plants treated with EBR must have higher protection against Fe excess, increasing Fe retention in root tissues and decreasing their transport to the shoot. Stein et al. (2014) evaluating the Fe toxicity tolerance mechanisms using three contrasting *Oryza sativa* cultivars (two tolerant and one susceptible to Fe excess) quantified the concentrations of total iron, intracellular iron and apoplastic iron present in the roots, detecting concentrations oscillating from 86 until 94% of iron precipitated in the apoplastic spaces of the root.

EBR attenuated the stress caused by the Fe toxicity and increased contents of macronutrients (K, Ca and Mg) and micronutrients (Mn, Cu and Zn). These results demonstrate that EBR regulate positively metal homeostasis, confirmed by increases in Mg^{2+}/Fe^{2+}, Mn^{2+}/Fe^{2+}, Cu^{2+}/Fe^{2+} and Zn^{2+}/Fe^{2+} ratios. Fe can be absorbed and transported by the plants using specific transporters, such as OsYSL2, OsNRAMP5 and DMA, that act concomitantly in the transport of other metals, such as Zn, Cu and Mn (Koike et al. 2004; Ishimaru et al. 2012; Aung et al. 2018). Majerus et al. (2007) working with *Oryza glaberrima* plants of 14 day-old exposed to high Fe concentration (500 mg dm^{-3} $FeSO_4$) for 3 days aiming to identify the responses in three organs (root, sheath and laminae) in the regulation of ferritin and antioxidant responses verified that the Fe excess promoted reductions in K and Mg contents in all the organs. Silveira et al. (2007) studying the nutritional status and mechanisms involved in two contrasting cultivars of *Oryza sativa* (sensitive and tolerant to Fe toxicity) submitted to three supplements of Fe (0, 6.5 or 500 mg L^{-1} of $FeSO_4$, referring to deficiency, control and excess of Fe, respectively) observed decreases in Mn contents in root and shoot of both cultivars, when exposed to Fe excess.

The application of EBR (10 nM) reduced the effects of Fe toxicity, minimizing the negative effects on F_0 and F_v/F_m. The reduction of F_0 after application of EBR is associated to the increase in the proportion of oxidized quinone (Q_A), increasing the efficiency of the capture of light energy in the PSII reaction center (Li et al. 2015). Frequently F_0 is altered due to environmental stresses, such as Fe excess, limiting the PSII performance. Positive effects of the EBR on F_v/F_m are directly related to the increase in F_v. Pinto et al. (2016) detected increase in the F_0 and reduction in F_v/F_m evaluating a sensitive *Oryza sativa* cultivar treated with 7 mM Fe-EDTA during seven days. Other study conducted by Ahammed et al. (2013) reported an increase in F_v/F_m after spray with 0.1 µM EBR in *Solanum lycopersicum* plants under stress induced by 100 µM $CdCl_2$.

Plants exposed to Fe toxicity and treated with EBR presented increases in Φ_{PSII}, q_P and ETR values, which can be explained by the positive effects of EBR on F_0, F_m and F_v/F_m. These results confirm

the improvements in the capture of light energy by open PSII reaction centers and in the quantum yield of electron transport (Rivero et al. 2010; Zhang et al. 2013). In addition, increases in ETR will stimulate the production of ATP and NADPH (Kumari et al. 2017), contributing to maximization in P_N. Thussagunpanit et al. (2015) studied the mechanisms of EBR action in *Oryza sativa* plants under thermal stress (40/30 °C day/night) and reported after application of 10^{-8} M of EBR increases in q_P and Φ_{PSII} of 102 and 111%, respectively.

The treatment with EBR promoted reductions in NPQ, EXC and ETR/P_N of plants exposed to Fe toxicity, revealing that this steroid occasioned a decrease in the dissipation of excitation energy in the form of heat. The decrease in NPQ coupled with the decrease in EXC demonstrated that the EBR minimized the photochemical damages on the PSII (Silva et al. 2012). Moreover, the reduction of ETR/P_N in plants under EBR application indicates that the excess of electrons was less used in secondary processes, such as photorespiration, nitrogen metabolism or Mehler reaction (Silva et al. 2011). Müller et al. (2017) observed increases of NPQ studying ecophysiological responses in four cultivars of *Oryza sativa* exposed to 7 mM $FeSO_4$-EDTA.

Plants exposed to Fe toxicity and treated with EBR had increases in P_N and g_s values. These increases are related to the benefits promoted by the EBR on photosynthetic apparatus, corroborated by maximizations detected in Φ_{PSII} and ETR. Concomitantly, decreases in E after application of EBR are intrinsically related to prevention mechanism to reduce the excessive absorption of Fe (Pereira et al. 2013b). Research conducted by Hayat et al. (2007) obtained increases in P_N evaluating *Brassica juncea* plants sprayed with 0.01 µM of HBL and exposed to four Cd concentrations (0, 50, 100 and 150 µM of $CdCl_2$). Fariduddin et al. (2013) observed that the foliar application of 0.01 µM EBR attenuated the losses caused in P_N e g_s of *Cucumis sativus* plants exposed to Cu stress (100 mg kg^{-1} of sand).

The EBR action mitigated the negative effects related to Fe^{2+} excess on C_i, proving that the EBR attenuated the non-stomatal limitations, such as verified increases in P_N and WUE (our study) and P_N (Shu et al. 2016). EBR interference increasing the activity of the RUBISCO enzyme were found by Pociecha et al. (2017), contributing to a higher assimilation of CO_2 in the Calvin cycle (Yu et al. 2004). Additionally, increases in WUE are attributed to increase in P_N and reduction of E, being intrinsically related to EBR effects on the photosynthetic machinery and stomatal form, respectively, both observed in this study. Hayat et al. (2012) described that the application of BR (10^{-8} M EBL and HBL) in *Solanum lycopersicum* plants under Cd stress (3, 6, 9, 12 mg kg^{-1} of $CdCl_2$) promoted positive effects on WUE, alleviating the effects caused by this metal. (Lima et al. 2019) evaluating gas exchange and photochemical efficiency in *Eucalyptus urophylla* plants submitted to Fe deficiency had significant reductions of C_i after treatment with EBR.

EBR alleviated the effects of the Fe toxicity on stomatal characteristics (SD, TD, PDS, EDS and SF). Increases in SD and SF revealed that EBR improved stomatal performance, corroborating with the increase in g_s previously observed in this study. This fact demonstrates that the EBR can maximize the gas exchange, improving the diffusion of CO_2 into the leaves. The influence on SF, PDS and EDS variables proves that the EBR interferes on shape of the stomata. The shape of the stomata is directly related with functionality (Martins et al. 2018). The greater stomatal functionality is characterized by stomata more elliptic (Martins et al. 2015). In addition, the reduced size of the stomata allows that the

ostiole to remain open without significant losses of water via transpiration. The lower transpiration can decrease the Fe translocation from xylem to shoot. Asch et al. (2005) detected that the low vapor pressure deficit significantly reduced the symptoms linked to Fe toxicity (1000 mg L^{-1} of Fe^{2+}) in two *Oryza sativa* genotypes.

The application of EBR (10 nM) promoted increases in ETAd, ETAb, MT, LAA and BCD. Higher values of ETAd and ETAb in plants treated with EBR must be explained by the increases in WUE, because the epidermis is a surface tissue intrinsically related to water use, avoiding excessive losses during the transpiration process (Javelle et al. 2011). EBR stimulates essential processes to growth and development, such as cell division and elongation (Jiroutova et al. 2018) and improves the stability and integrity of membranes (Shahbaz and Ashraf 2007), contributing to the increases in MT and BCD. Akhtar et al. (2018) examined morphological and physiological aspects related to osmoregulation in six populations of *Typha domingensis* under waterlogged conditions combined with four Ni concentrations (0, 50, 100 and 150 mg Ni kg^{-1} soil) verified increases in LAA in the all populations when exposed to Ni, comparing with control treatment.

Plants under Fe toxicity and treated with EBR presented increases in SOD, CAT, APX and POX, indicating that the EBR promoted benefits on the antioxidant system. The action of this steroid increasing the activities of these enzymes clearly contributes to attenuation of the oxidative damages and ROS homeostasis (Hasan et al. 2011; Sharma et al. 2016). Under stress conditions, these enzymes work in process of ROS detoxification, which are substances highly toxics to plant metabolism (Liu et al. 2009; Dalyan et al. 2018). *Oryza sativa* plants with tolerant to Fe under stress (200-400µM Fe^{2+}) exhibit higher enzymatic activity (POD, CAT and SOD), when compared to non-tolerant plants (Zhang et al. 2011). Our result suggests that the EBR can increase the tolerance against oxidative damages induced by excess Fe, specifically due to the increase in the activities of the antioxidant enzymes. Studies with other metals have demonstrated that the application of BRs promoted increases in the activities of these enzymes, such as *Raphanus sativus* plants under stress caused by Zn (Ramakrishna and Rao 2015), and *Brassica juncea* plants under Mn toxicity (Fariduddin et al. 2015).

The treatment with EBR promoted decreases in the O_2^- and H_2O_2 concentrations of the plants submitted to Fe toxicity, being related to the increase of the activities of the antioxidant enzymes, positively induced by the EBR. The SOD enzyme is the first line of defense of plants to combat the accumulation of O_2^-, in which it is responsible for the dismutation of O_2^- in H_2O_2 and O_2 (Yusuf et al. 2011). Subsequently, the CAT, APX and POX enzymes act on the H_2O_2 reducing in H_2O and O_2 (Arora et al. 2008; Gill and Tuteja 2010). Li et al. (2016) evaluating oxidative compounds and antioxidant enzymes in *Solanum lycopersicum* seedlings under application of EBR and exposed to Zn oxide nanoparticles (0, 10, 20, 50 and 100 mg L^{-1} ZnO NPs) also observed reductions in H_2O_2 after the application of 5 nM of EBR.

The application of 10 nM of EBR in plants exposed to Fe toxicity promoted reductions in MDA and EL. This result can be explained by decreases in O_2^- and H_2O_2 levels combined with increases in antioxidant enzyme activities previously described. Overproduction of ROS must cause damages to essential biomolecules present in plant cells, such as lipids and proteins (Sharma et al. 2012). The oxidation of fatty acids and membrane proteins increase the permeability of the membrane, promoting

ruptures and consequently reducing the membrane integrity (Jakubowska and Janicka 2017). In addition, H_2O_2 can produce the hydroxyl radical (OH^-) in the presence of Fe^{2+}, in which this reaction catalyzed by the Fe will contribute to lipid peroxidation. Wu et al. (2017) studying the mechanisms of tolerance to Fe toxicity in two contrasting *Oryza sativa* genotypes detected significant increases in MDA concentrations in both genotypes when exposed to Fe (1000 mg L^{-1} $FeSO_4$) toxicity.

Plants treated with 10 nM EBR had increases in photosynthetic pigments (Chl *a*, Chl *b*, Total chl and Car), evidencing that the EBR attenuated the damages caused to chloroplast membranes, confirmed by the lower concentrations of MDA and EL, besides clearly to mitigate the accumulation of ROS, such as O_2^- and H_2O_2. In addition, the maintenance of membrane integrity of chloroplasts can be confirmed by increases in ETR, because the chlorophylls play an important role in the transport of PSII electrons (Wang et al. 2016). Our study also detected that the EBR increased the content of Mg, which is an integral element of the chlorophyll molecule, improving the synthesis of these pigments (Ali et al. 2008; Yang et al. 2015). Dufey et al. (2009) evaluating the morphological and physiological behavior of *Oryza sativa* plants in relation to tolerance to Fe toxicity detected that IR64 and Azucena cultivars and its recombinant inbred lines (RILs) suffered reductions in chlorophyll concentrations when supplemented with 250 mg L^{-1} $FeSO_4$.

The EBR application reduced the deleterious effects caused by the Fe toxicity on plant biomass (RDM and TDM). The benefits of the EBR on growth are related to increases in anatomical characteristics of specific tissues, mitigating Fe toxicity (RAA and LAA), promoting higher absorption and accumulation of other nutrients (RMD and VCD), and homeostasis with other metals with equal valence, such as Mg, Mn, Cu and Zn. These responses are confirmed by increases in chlorophyll fluorescence (Φ_{PSII} and ETR) and gas exchanges (P_N and WUE), reduction of oxidative damages to membranes and photosynthetic pigments that are clearly associated with the benefits of antioxidant metabolism, mainly SOD and CAT enzymes. Quinet et al. (2012) evaluating the gene expression and responses biochemical, physiological and morphological of *Oryza sativa* plants exposed to Fe toxicity (125 mg L^{-1} $FeSO_4$ by three weeks) detected significant reductions in shoot (31%) and root (26%), when compared to the control.

Conclusion

This research revealed that the EBR mitigated the deleterious effects of the Fe toxicity on rice plants, modulating the aerenchyma area, in which this structure contribute to the formation of an oxidative barrier, Fe immobilization in root surface. Concomitantly, this steroid increased the contents of other metals, such as magnesium, manganese, copper and zinc, proving the influence related to metal homeostasis, confirmed by increases in metal ratios. Plants under Fe toxicity and treated with EBR presented increases in all enzyme activities evaluated, superoxide dismutase, catalase, ascorbate peroxidase and peroxidase, mitigating the oxidative damages and favoring the scavenging of reactive oxygen species. Finally, the EBR actions alleviated the negative impacts induced by Fe excess on net photosynthetic rate and carboxylation instantaneous efficiency, being these benefits directly related to higher electron transport and stomatal density and indirectly linked to protection mechanism exercised by

the antioxidant enzymes on photosynthetic machinery. Therefore, our results clearly indicate that the EBR is able to confer tolerance in rice plants under Fe toxicity.

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Conflict of interest

The authors declare that they have no competing interests.

References

Abdallah A, Alzoheiry A, Burkey K (2018) Comparison of flooded and furrow-irrigated transplanted rice (Oryza sativa L.): Farm-level perspectives. J Irrig Drain Eng 144:1–8 . doi: 10.1061/(ASCE)IR.1943-4774.0001337

Ahammed GJ, Choudhary SP, Chen S, et al (2013) Role of brassinosteroids in alleviation of phenanthrene–cadmium co-contamination-induced photosynthetic inhibition and oxidative stress in tomato. J Exp Bot 64:199–213 . doi: 10.1093/jxb/ers323

Akhtar N, Hameed M, Hamid A, et al (2018) Effects of nickel toxicity on morphological and physiological aspects of osmoregulation in Typha domingensis (Typhaceae) populations. Limnology 19:185–197 . doi: 10.1007/s10201-017-0529-8

Ali B, Hasan SA, Hayat S, et al (2008) A role for brassinosteroids in the amelioration of aluminium stress through antioxidant system in mung bean (Vigna radiata L. Wilczek). Environ Exp Bot 62:153–159 . doi: 10.1016/j.envexpbot.2007.07.014

Anwar A, Liu Y, Dong R, et al (2018) The physiological and molecular mechanism of brassinosteroid in response to stress: a review. Biol Res 51:1–15 . doi: 10.1186/s40659-018-0195-2

Aragão RM, Silva EN, Vieira CF, Silveira JAG (2012) High supply of NO3 − mitigates salinity effects through an enhancement in the efficiency of photosystem II and CO2 assimilation in Jatropha curcas plants. Acta Physiol Plant 34:2135–2143 . doi: 10.1007/s11738-012-1014-y

Arora N, Bhardwaj R, Sharma P, Arora HK (2008) Effects of 28-homobrassinolide on growth, lipid peroxidation and antioxidative enzyme activities in seedlings of Zea mays L. under salinity stress. Acta Physiol Plant 30:833–839 . doi: 10.1007/s11738-008-0188-9

Asch F, Becker M, Kpongor DS (2005) A quick and efficient screen for resistance to iron toxicity in lowland rice. J Plant Nutr Soil Sci 168:764–773 . doi: 10.1002/jpln.200520540

Audebert A, Fofana M (2009) Rice yield gap due to iron toxicity in West Africa. J Agron Crop Sci 195:66–76 . doi: 10.1111/j.1439-037X.2008.00339.x

Audebert A, Sahrawat KL (2000) Mechanisms for iron toxicity tolerance in lowland rice. J Plant Nutr 23:1877–1885 . doi: 10.1080/01904160009382150

Aung MS, Masuda H, Kobayashi T, Nishizawa NK (2018) Physiological and transcriptomic analysis of responses to different levels of iron excess stress in various rice tissues. Soil Sci Plant Nutr. doi: 10.1080/00380768.2018.1443754

Badawi GH, Yamauchi Y, Shimada E, et al (2004) Enhanced tolerance to salt stress and water deficit by overexpressing superoxide dismutase in tobacco (Nicotiana tabacum) chloroplasts. Plant Sci 166:919–928 . doi: 10.1016/j.plantsci.2003.12.007

Bajguz A (2010) An enhancing effect of exogenous brassinolide on the growth and antioxidant activity in Chlorella vulgaris cultures under heavy metals stress. Environ Exp Bot 68:175–179 . doi: 10.1016/j.envexpbot.2009.11.003

Bajguz A, Hayat S (2009) Effects of brassinosteroids on the plant responses to environmental stresses. Plant Physiol Biochem 47:1–8 . doi: 10.1016/j.plaphy.2008.10.002

Bajguz A, Piotrowska-Niczyporuk A (2014) Interactive effect of brassinosteroids and cytokinins on growth, chlorophyll, monosaccharide and protein content in the green alga Chlorella vulgaris (Trebouxiophyceae). Plant Physiol Biochem 80:176–183 . doi: 10.1016/j.plaphy.2014.04.009

Bartwal A, Mall R, Lohani P, et al (2013) Role of secondary metabolites and brassinosteroids in plant defense against environmental stresses. J Plant Growth Regul 32:216–232 . doi: 10.1007/s00344-012-9272-x

Becker M, Asch F (2005) Iron toxicity in rice - conditions and management concepts. J Plant Nutr Soil Sci 168:558–573 . doi: 10.1002/jpln.200520504

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254 . doi: 10.1016/0003-2697(76)90527-3

Briat JF, Curie C, Gaymard F (2007) Iron utilization and metabolism in plants. Curr Opin Plant Biol 10:276–282 . doi: 10.1016/j.pbi.2007.04.003

Cakmak I, Horst WJ (1991) Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (Glycine max). Physiol Plant 83:463–468 . doi: 10.1111/j.1399-3054.1991.tb00121.x

Cakmak I, Marschner H (1992) Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves. Plant Physiol 98:1222–1227 . doi: 10.1104/pp.98.4.1222

Castro EM, Pereira FJ, Paiva R (2009) Histologia Vegetal: estrutura e função dos órgãos vegetativos. Lavras

Chen L, Ding C, Zhao X, et al (2015) Differential regulation of proteins in rice (Oryza sativa L.) under iron deficiency. Plant Cell Rep 34:83–96 . doi: 10.1007/s00299-014-1689-1

Choudhary SP, Kanwar M, Bhardwaj R, et al (2012) Chromium stress mitigation by polyamine-brassinosteroid application involves phytohormonal and physiological strategies in Raphanus sativus L. PLoS One 7:e33210 . doi: 10.1371/journal.pone.0033210

Crestani M, Silva JAG, Souza VQ, et al (2009) Irrigated rice genotype performance under excess iron stress in hydroponic culture. Crop Breed Appl Biotechnol 9:87–95 . doi: 10.12702/1984-7033.v09n01a12

Dalyan E, Yüzbaşıoğlu E, Akpinar I (2018) Effect of 24-epibrassinolide on antioxidative defence system against lead-induced oxidative stress in the roots. Russ J Plant Physiol 65:570–578 . doi: 10.1134/S1021443718040118

Dufey I, Hakizimana P, Draye X, et al (2009) QTL mapping for biomass and physiological parameters linked to resistance mechanisms to ferrous iron toxicity in rice. Euphytica 167:143–160 . doi: 10.1007/s10681-008-9870-7

Elstner EF, Heupel A (1976) Inhibition of nitrite formation from hydroxylammoniumchloride: A simple assay for superoxide dismutase. Anal Biochem 70:616–620 . doi: 10.1016/0003-2697(76)90488-7

Enstone DE, Peterson CA, Ma F (2003) Root endodermis and exodermis: structure, function, and responses to the environment. J Plant Growth Regul 21:335–351 . doi: 10.1007/s00344-003-0002-2

Fariduddin Q, Ahmed M, Mir BA, et al (2015) 24-Epibrassinolide mitigates the adverse effects of manganese induced toxicity through improved antioxidant system and photosynthetic attributes in Brassica juncea. Environ Sci Pollut Res 22:11349–11359 . doi: 10.1007/s11356-015-4339-4

Fariduddin Q, Khalil RRAE, Mir BA, et al (2013) 24-Epibrassinolide regulates photosynthesis, antioxidant enzyme activities and proline content of Cucumis sativus under salt and/or copper stress. Environ Monit Assess 185:7845–7856 . doi: 10.1007/s10661-013-3139-x

Giannopolitis CN, Ries SK (1977) Superoxide dismutases: I. Occurrence in higher plants. Plant Physiol 59:309–314

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem 48:909–930 . doi: 10.1016/j.plaphy.2010.08.016

Gong M, Li Y-J, Chen S-Z (1998) Abscisic acid-induced thermotolerance in maize seedlings is mediated by calcium and associated with antioxidant systems. J Plant Physiol 153:488–496 . doi: 10.1016/S0176-1617(98)80179-X

Hasan SA, Hayat S, Ahmad A (2011) Brassinosteroids protect photosynthetic machinery against the cadmium induced oxidative stress in two tomato cultivars. Chemosphere 84:1446–1451 . doi: 10.1016/j.chemosphere.2011.04.047

Havir EA, McHale NA (1987) Biochemical and developmental characterization of multiple forms of catalase in tobacco leaves. Plant Physiol 84:450–455 . doi: 10.1104/pp.84.2.450

Hayat S, Ali B, Hasan SA, Ahmad A (2007) Brassinosteroid enhanced the level of antioxidants under cadmium stress in Brassica juncea. Environ Exp Bot 60:33–41 . doi: 10.1016/j.envexpbot.2006.06.002

Hayat S, Alyemeni MN, Hasan SA (2012) Foliar spray of brassinosteroid enhances yield and quality of Solanum lycopersicum under cadmium stress. Saudi J Biol Sci 19:325–335

Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil, 2nd edn. California Agricultural Experiment Station

Ishimaru Y, Takahashi R, Bashir K, et al (2012) Characterizing the role of rice NRAMP5 in manganese, iron and cadmium transport. Sci Rep 2:1–8 . doi: 10.1038/srep00286

Jakubowska D, Janicka M (2017) The role of brassinosteroids in the regulation of the plasma membrane H+-ATPase and NADPH oxidase under cadmium stress. Plant Sci 264:37–47 . doi: 10.1016/j.plantsci.2017.08.007

Javelle M, Vernoud V, Rogowsky PM, Ingram GC (2011) Epidermis: the formation and functions of a fundamental plant tissue. New Phytol 189:17–39 . doi: 10.1111/j.1469-8137.2010.03514.x

Jiroutova P, Oklestkova J, Strnad M (2018) Crosstalk between brassinosteroids and ethylene during plant growth and under abiotic stress conditions. Int J Mol Sci 19:1–13 . doi: 10.3390/ijms19103283

Koike S, Inoue H, Mizuno D, et al (2004) OsYSL2 is a rice metal-nicotianamine transporter that is regulated by iron and expressed in the phloem. Plant J 39:415–424 . doi: 10.1111/j.1365-313X.2004.02146.x

Krishna P (2003) Brassinosteroid-mediated stress responses. J Plant Growth Regul 22:289–297 . doi: 10.1007/s00344-003-0058-z

Kumari J, Udawat P, Dubey AK, et al (2017) Overexpression of SbSI-1, a nuclear protein from Salicornia brachiata confers drought and salt stress tolerance and maintains photosynthetic efficiency in transgenic tobacco. Front Plant Sci 8:1215 . doi: 10.3389/fpls.2017.01215

Li J, Yang P, Gan Y, et al (2015) Brassinosteroid alleviates chilling-induced oxidative stress in pepper by enhancing antioxidation systems and maintenance of photosystem II. Acta Physiol Plant 37:1–11 . doi: 10.1007/s11738-015-1966-9

Li M, Ahammed GJ, Li C, et al (2016) Brassinosteroid ameliorates zinc oxide nanoparticles-induced oxidative stress by improving antioxidant potential and redox homeostasis in tomato seedling. Front Plant Sci 7:1–13 . doi: 10.3389/fpls.2016.00615

Lichtenthaler HK, Buschmann C (2001) Chlorophylls and carotenoids: Measurement and characterization by UV-VIS spectroscopy. In: Current Protocols in Food Analytical Chemistry. John Wiley & Sons, Inc., Hoboken, NJ, USA, pp 431–438

Lima JV, Lobato AKS (2017) Brassinosteroids improve photosystem II efficiency, gas exchange, antioxidant enzymes and growth of cowpea plants exposed to water deficit. Physiol Mol Biol Plants 23:59–72 . doi: 10.1007/s12298-016-0410-y

Lima MDR, Barros Junior UO, Batista BL, Lobato AKS (2018) Brassinosteroids mitigate iron deficiency improving nutritional status and photochemical efficiency in Eucalyptus urophylla plants. Trees 32:1681–1694 . doi: 10.1007/s00468-018-1743-7

Lima MDR, Barros Junior UO, Batista BL, Lobato AKS (2019) Brassinosteroids mitigate iron deficiency improving nutritional status and photochemical efficiency in Eucalyptus urophylla plants. Trees - Struct Funct 32:1681–1694 . doi: 10.1007/s00468-018-1743-7

Liu Y, Zhao Z, Si J, et al (2009) Brassinosteroids alleviate chilling-induced oxidative damage by enhancing antioxidant defense system in suspension cultured cells of Chorispora bungeana. Plant Growth Regul 59:207–214 . doi: 10.1007/s10725-009-9405-9

Ma JF, Mitani N, Nagao S, et al (2004) Characterization of the silicon uptake system and molecular mapping of the silicon transporter gene in rice. Plant Physiol 136:3284–3289 . doi: 10.1104/pp.104.047365

Maia CF, Silva BRS, Lobato AKS (2018) Brassinosteroids positively modulate growth: physiological, biochemical and anatomical evidence using two tomato genotypes contrasting to dwarfism. J Plant Growth Regul 37:1–14 . doi: 10.1007/s00344-018-9802-2

Majerus V, Bertin P, Lutts S (2007) Effects of iron toxicity on osmotic potential, osmolytes and

polyamines concentrations in the African rice (Oryza glaberrima Steud.). Plant Sci 173:96–105 . doi: 10.1016/j.plantsci.2007.04.003

Martins JPR, Santos ER, Rodrigues LCA, et al (2018) Effects of 6-benzylaminopurine on photosystem II functionality and leaf anatomy of in vitro cultivated Aechmea blanchetiana. Biol Plant 62:793–800 . doi: 10.1007/s10535-018-0822-3

Martins JPR, Verdoodt V, Pasqual M, De Proft M (2015) Impacts of photoautotrophic and photomixotrophic conditions on in vitro propagated Billbergia zebrina (Bromeliaceae). Plant Cell Tissue Organ Cult 123:121–132 . doi: 10.1007/s11240-015-0820-5

Müller C, Silveira SF da S, Daloso D de M, et al (2017) Ecophysiological responses to excess iron in lowland and upland rice cultivars. Chemosphere 189:123–133 . doi: 10.1016/j.chemosphere.2017.09.033

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22:867–880

O'Brien TP, Feder N, McCully ME (1964) Polychromatic staining of plant cell walls by toluidine blue O. Protoplasma 59:368–373

Oliveira VP, Lima MDR, Silva BRS, et al (2019) Brassinosteroids confer tolerance to salt stress in Eucalyptus urophylla plants enhancing homeostasis, antioxidant metabolism and leaf anatomy. J Plant Growth Regul In press:19 . doi: 10.1007/s00344-018-9870-3

Parthasarathi T, Vanitha K, Mohandass S, Vered E (2018) Evaluation of drip irrigation system for water productivity and yield of rice. Agron J 110:2378–2389 . doi: 10.2134/agronj2018.01.0002

Pereira EG, Oliva MA, Rosado-Souza L, et al (2013a) Iron excess affects rice photosynthesis through stomatal and non-stomatal limitations. Plant Sci 201–202:81–92 . doi: 10.1016/j.plantsci.2012.12.003

Pereira EG, Oliva MA, Rosado-Souza L, et al (2013b) Iron excess affects rice photosynthesis through stomatal and non-stomatal limitations. Plant Sci 201–202:81–92 . doi: 10.1016/j.plantsci.2012.12.003

Pereira EG, Oliva MA, Siqueira-Silva AI, et al (2014) Tropical rice cultivars from lowland and upland cropping systems differ in iron plaque formation. J Plant Nutr 37:1373–1394 . doi: 10.1080/01904167.2014.888744

Pinto SDS, Souza AE De, Oliva MA, Pereira EG (2016) Oxidative damage and photosynthetic impairment in tropical rice cultivars upon exposure to excess iron. Sci Agric 73:217–226 . doi: http://dx.doi.org/10.1590/0103-9016-2015-0288

Pociecha E, Dziurka M, Waligórski P, et al (2017) 24-Epibrassinolide pre-treatment modifies cold-induced photosynthetic acclimation mechanisms and phytohormone response of perennial Ryegrass in cultivar-dependent manner. J Plant Growth Regul 36:618–628 . doi: 10.1007/s00344-016-9662-6

Quinet M, Vromman D, Clippe A, et al (2012) Combined transcriptomic and physiological approaches reveal strong differences between short- and long-term response of rice (Oryza sativa) to iron toxicity. Plant Cell Environ 35:1837–1859 . doi: 10.1111/j.1365-3040.2012.02521.x

Ramakrishna B, Rao SSR (2015a) Foliar application of brassinosteroids alleviates adverse effects of zinc toxicity in radish (Raphanus sativus L.) plants. Protoplasma 252:665–677 . doi: 10.1007/s00709-

014-0714-0

Ramakrishna B, Rao SSR (2015b) Foliar application of brassinosteroids alleviates adverse effects of zinc toxicity in radish (Raphanus sativus L.) plants. Protoplasma 252:665–677 . doi: 10.1007/s00709-014-0714-0

Rivero RM, Gimeno J, Deynze A Van, et al (2010) Enhanced cytokinin synthesis in tobacco plants expressing PSARK: IPT prevents the degradation of photosynthetic protein complexes during drought. Plant Cell Physiol 51:1929–1941 . doi: 10.1093/pcp/pcq143

Rout GR, Sahoo S (2015) Role of Iron in Plant Growth and Metabolism. Rev Agric Sci 3:1–24 . doi: 10.7831/ras.3.1

Sahrawat KL (2004) Iron toxicity in wetland rice and the role of other nutrients. J Plant Nutr 27:1471–1504 . doi: 10.1081/PLN-200025869

Santos RS, Araujo AT, Pegoraro C, Oliveira AC (2017) Dealing with iron metabolism in rice: from breeding for stress tolerance to biofortification. Genet Mol Biol 40:312–325 . doi: 10.1590/1678-4685-gmb-2016-0036

Segatto FB, Bisognin DA, Benedetti M, et al (2004) A technique for the anatomical study of potato leaf epidermis. Ciência Rural 34:1597–1601 . doi: 10.1590/S0103-84782004000500042

Shahbaz M, Ashraf M (2007) Influence of exogenous application of brassinosteroid on growth and mineral nutrients of wheat (Triticum Aestivum L.) under saline conditions. Pakistan J Bot 39:513–522

Sharma P, Jha AB, Dubey RS, Pessarakli M (2012) Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. J Bot 2012:1–26 . doi: 10.1155/2012/217037

Sharma P, Kumar A, Bhardwaj R (2016) Plant steroidal hormone epibrassinolide regulate – Heavy metal stress tolerance in Oryza sativa L . by modulating antioxidant defense expression. Environ Exp Bot 122:1–9 . doi: 10.1016/j.envexpbot.2015.08.005

Shu S, Tang Y, Yuan Y, et al (2016) The role of 24-epibrassinolide in the regulation of photosynthetic characteristics and nitrogen metabolism of tomato seedlings under a combined low temperature and weak light stress. Plant Physiol Biochem 107:344–353 . doi: 10.1016/j.plaphy.2016.06.021

Silva EN, Ribeiro RV, Ferreira-Silva SL, et al (2011) Salt stress induced damages on the photosynthesis of physic nut young plants. Sci Agric 68:62–68 . doi: 10.1590/S0103-90162011000100010

Silva EN, Ribeiro R V, Ferreira-Silva SL, et al (2012) Coordinate changes in photosynthesis, sugar accumulation and antioxidative enzymes improve the performance of Jatropha curcas plants under drought stress. Biomass and Bioenergy 45:270–279 . doi: 10.1016/j.biombioe.2012.06.009

Silveira VC, Oliveira AP, Sperotto RA, et al (2007) Influence of iron on mineral status of two rice (Oryza sativa L.) cultivars. Brazilian J Plant Physiol 19:127–139 . doi: 10.1590/S1677-04202007000200005

Steel RG., Torrie JH, Dickey DA (2006) Principles and procedures of statistics: a biometrical approach, 3rd edn. Academic Internet Publishers, Moorpark

Stein RJ, Duarte GL, Spohr MG, et al (2009) Distinct physiological responses of two rice cultivars subjected to iron toxicity under field conditions. Ann Appl Biol 154:269–277 . doi: 10.1111/j.1744-

7348.2008.00293.x

Stein RJ, Lopes SIG, Fett JP (2014) Iron toxicity in field-cultivated rice: contrasting tolerance mechanisms in distinct cultivars. Theor Exp Plant Physiol 26:135–146 . doi: 10.1007/s40626-014-0013-3

Thussagunpanit J, Jutamanee K, Kaveeta L, et al (2015) Comparative effects of brassinosteroid and brassinosteroid mimic on improving photosynthesis, lipid peroxidation, and rice seed set under heat stress. J Plant Growth Regul 34:320–331 . doi: 10.1007/s00344-014-9467-4

Vardhini B V., Anjum NA (2015) Brassinosteroids make plant life easier under abiotic stresses mainly by modulating major components of antioxidant defense system. Front Environ Sci 2:1–16 . doi: 10.3389/fenvs.2014.00067

Velikova V, Yordanov I, Edreva A (2000) Oxidative stress and some antioxidant systems in acid rain-treated bean plants protective role of exogenous polyamines. Plant Sci 151:59–66 . doi: 10.1016/S0168-9452(99)00197-1

Wang J, Fang Z, Cheng W, et al (2016) Higher concentrations of nanoscale zero-valent iron (nZVI) in soil induced rice chlorosis due to inhibited active iron transportation. Environ Pollut 210:338–345 . doi: 10.1016/j.envpol.2016.01.028

Wu LB, Ueda Y, Lai SK, Frei M (2017) Shoot tolerance mechanisms to iron toxicity in rice (Oryza sativa L.). Plant Cell Environ 40:570–584 . doi: 10.1111/pce.12733

Wu Q-S, Xia R-X, Zou Y-N (2006) Reactive oxygen metabolism in mycorrhizal and non-mycorrhizal citrus (Poncirus trifoliata) seedlings subjected to water stress. J Plant Physiol 163:1101–1110 . doi: 10.1016/j.jplph.2005.09.001

Yamauchi T, Shimamura S, Nakazono M, Mochizuki T (2013) Aerenchyma formation in crop species: A review. F Crop Res 152:8–16 . doi: 10.1016/j.fcr.2012.12.008

Yang M, Tan L, Xu Y, et al (2015) Effect of low pH and aluminum toxicity on the photosynthetic characteristics of different fast-growing Eucalyptus vegetatively propagated clones. PLoS One 10:1–16 . doi: 10.1371/journal.pone.0130963

Yu JQ, Huang LF, Hu WH, et al (2004) A role for brassinosteroids in the regulation of photosynthesis in Cucumis sativus. J Exp Bot 55:1135–1143 . doi: 10.1093/jxb/erh124

Yusuf M, Fariduddin Q, Ahmad A (2011) 28-Homobrassinolide mitigates boron induced toxicity through enhanced antioxidant system in Vigna radiata plants. Chemosphere 85:1574–1584 . doi: 10.1016/j.chemosphere.2011.08.004

Zhang Y, Zheng GH, Liu P, et al (2011) Morphological and physiological responses of root tip cells to Fe2+ toxicity in rice. Acta Physiol Plant 33:683–689 . doi: 10.1007/s11738-010-0590-y

Zhang YP, Zhu XH, Ding HD, et al (2013) Foliar application of 24-epibrassinolide alleviates high-temperature-induced inhibition of photosynthesis in seedlings of two melon cultivars. Photosynthetica 51:341–349 . doi: 10.1007/s11099-013-0031-4

Figures

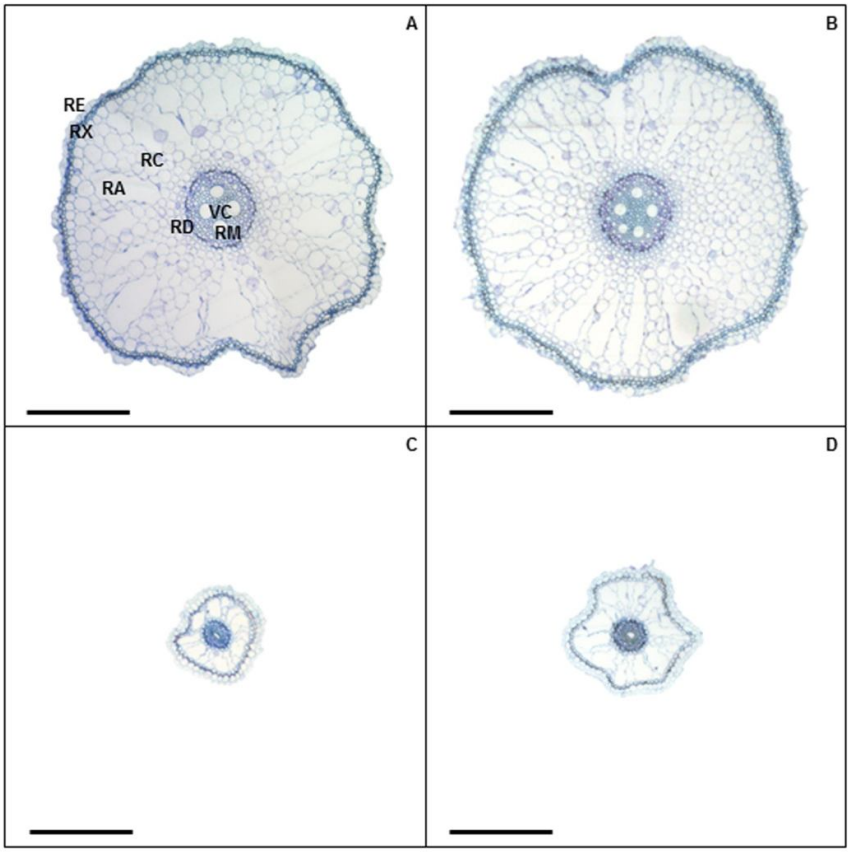

Fig. 1. Root cross sections in rice plants treated with EBR and exposed to Fe toxicity. Control / − EBR (A), Control / + EBR (B), Fe toxicity / − EBR (C) and Fe toxicity / + EBR (D). Legends: RE = Root epidermis;RX = Root exodermis; RC = Root cortex; RA = Root aerenchyma; RD= Root endodermis;VC = Vascular cylinder; RM = Root metaxylem. Bars: 200 µm.

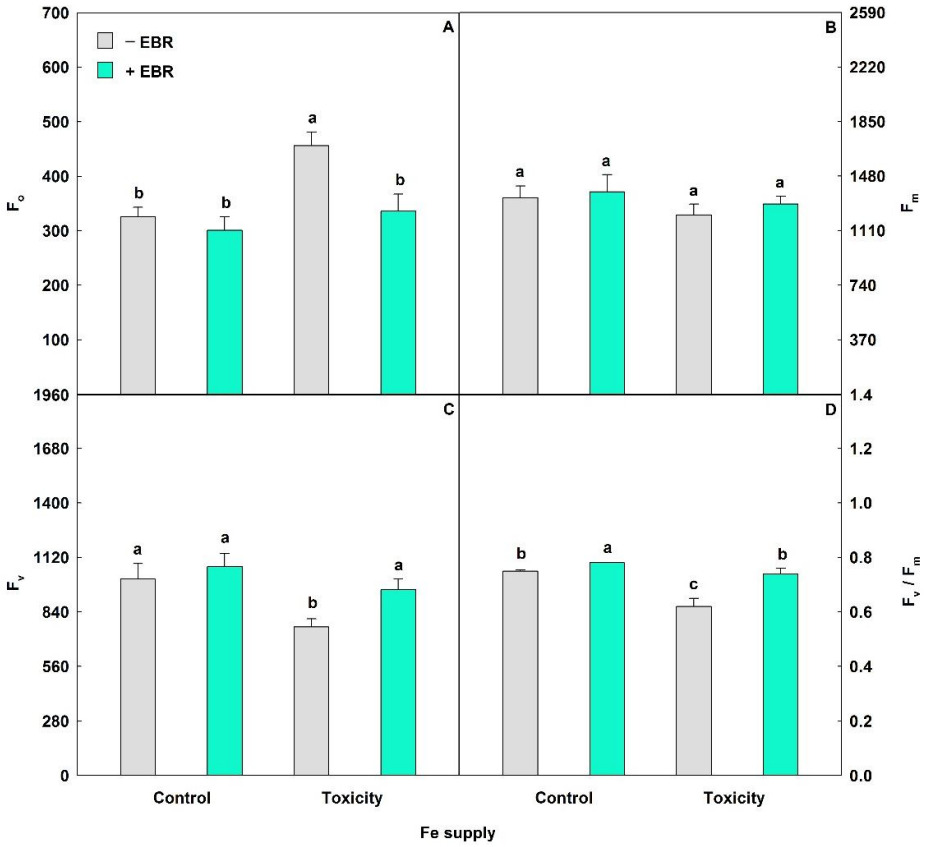

Fig. 2. Minimal fluorescence yield of the dark-adapted state (F_0), maximal fluorescence yield of the dark-adapted state (F_m), variable fluorescence (F_v) and maximal quantum yield of PSII photochemistry (F_v/F_m) in rice plants treated with EBR and exposed to Fe toxicity. Bars with different letters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

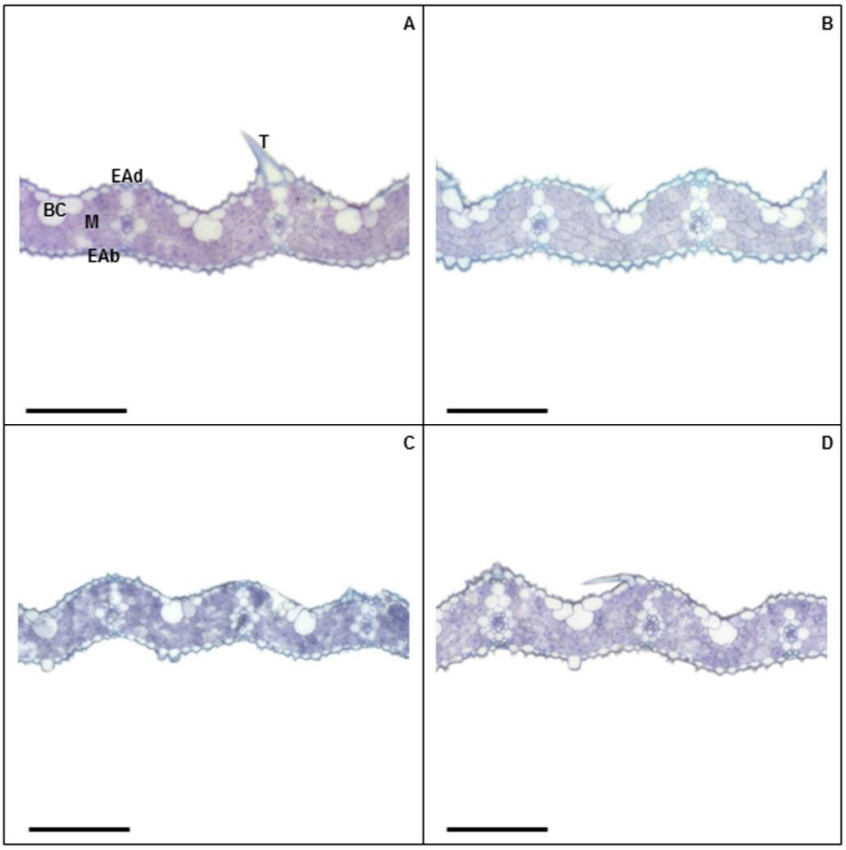

Fig. 3. Leaf cross sections in rice plants treated with EBR and exposed to Fe toxicity. Control / − EBR (A), Control / + EBR (B), Fe toxicity / − EBR (C) and Fe toxicity / + EBR (D). Legends:EAd = adaxial epidermis; EAb = Adaxial epidermis; BC = Bulliform cell; M = Mesophyll; T = Trichome. Bars: 100 μm.

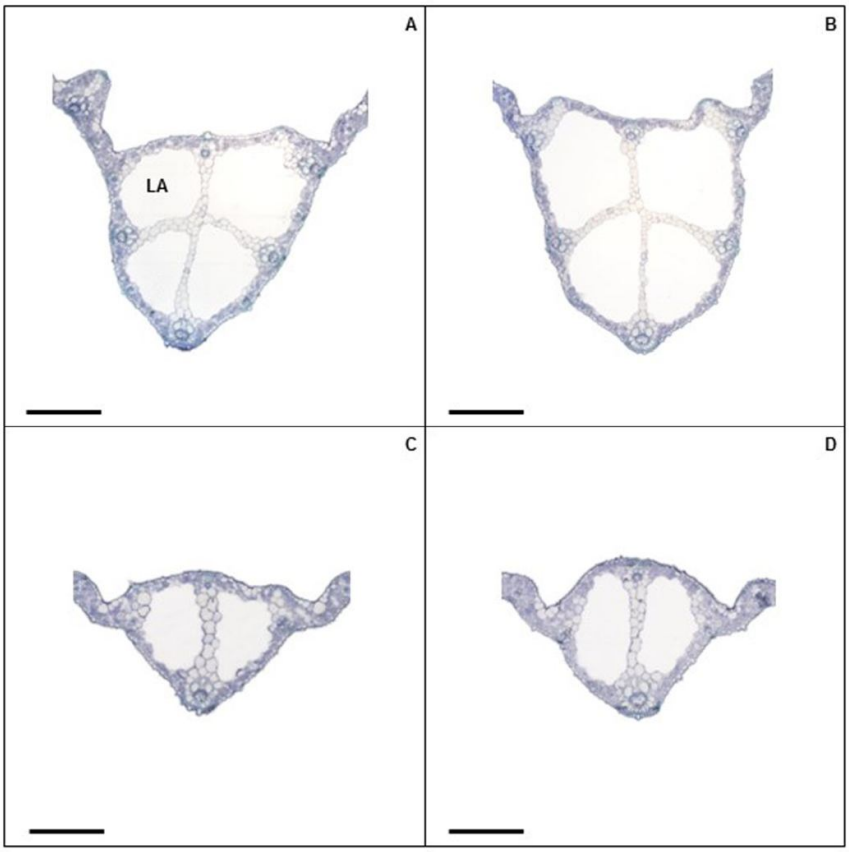

Fig. 4. Leaf main nervure in rice plants treated with EBR and exposed to Fe toxicity. Control / − EBR (A), Control / + EBR (B), Fe toxicity / − EBR (C) and Fe toxicity / + EBR (D). Legends: LA = leaf aerenchyma. Bars: 400 µm.

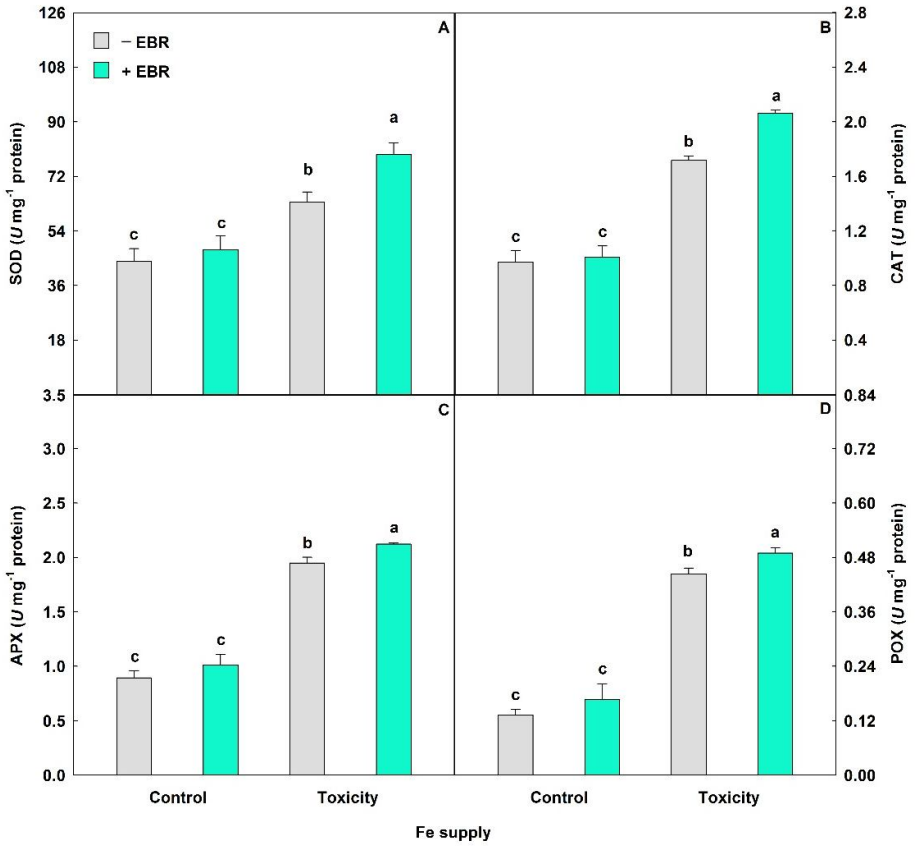

Fig. 5. Activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POX) in rice plants treated with EBR and exposed to Fe toxicity. Bars with different letters indicate significant differences from the Scott-Knott test (P<0.05). Bars corresponding to means from five repetitions and standard deviations.

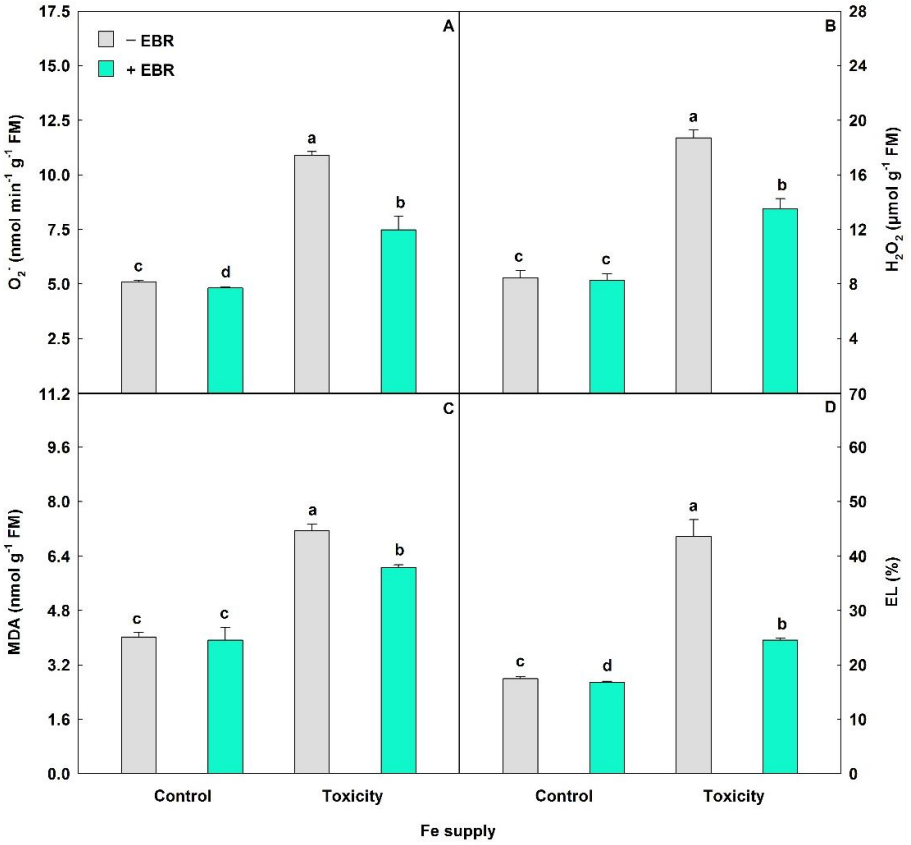

Fig. 6. Superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and electrolyte leakage (EL) in rice plants treated with EBR and exposed to Fe toxicity. Bars with different letters indicate significant differences from the Scott-Knott test (P<0.05). Bars corresponding to means from five repetitions and standard deviations.

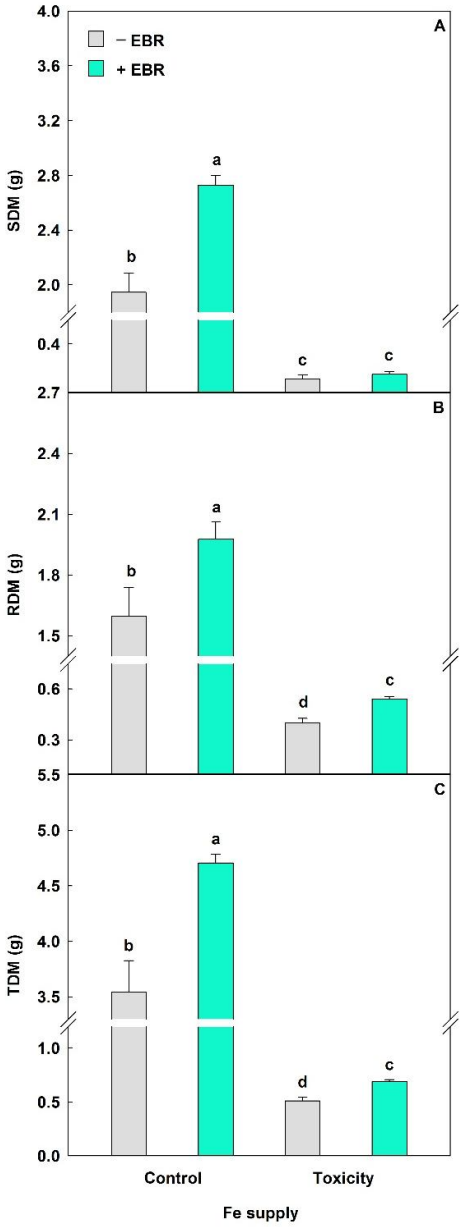

Fig. 7. Shoot dry matter (SDM), root dry matter (RDM) and total dry matter (TDM) in rice plants treated with EBR and exposed to Fe toxicity. Bars with different letters indicate significant differences from the Scott-Knott test (P<0.05). Bars corresponding to means from five repetitions and standard deviations.

Tables

Table 1. Fe contents in rice plants treated with EBR and exposed to Fe toxicity.

Fe supply	EBR	Fe in root (µg g DM^{-1})	Fe in shoot (µg g DM^{-1})
Control	−	547.1 ± 18.7c	62.2 ± 2.1c
Control	+	559.7 ± 18.3c	62.9 ± 4.1c
Toxicity	−	2257.3 ± 48.7a	275.6 ± 15.4a
Toxicity	+	1864.0 ± 41.9b	166.1 ± 15.6b

Fe = Iron. Columns with different letters indicate significant differences from the Scott-Knott test (P<0.05). Values described corresponding to means from five repetitions and standard deviations

Table 2. Root anatomy in rice plants treated with EBR and exposed to Fe toxicity.

Fe supply	EBR	RET (µm)	RXT (µm)	RDT (µm)	RCD (µm)	RAA (mm^2)	VCD (µm)	RMD (µm)
Control	−	17.81 ± 1.44b	24.52 ± 0.68b	12.86 ± 0.27b	296.97 ± 8.52b	0.05 ± 0.00b	153.87 ± 15.14b	36.81 ± 3.51a
Control	+	26.33 ± 1.10a	28.82 ± 2.42a	13.67 ± 0.32a	322.03 ± 12.15a	0.08 ± 0.01a	205.52 ± 20.30a	38.94 ± 2.09a
Toxicity	−	11.20 ± 0.88d	17.67 ± 1.11d	8.80 ± 0.71d	103.30 ± 10.27c	0.03 ± 0.00d	69.77 ± 9.06c	14.75 ± 1.39c
Toxicity	+	13.16 ± 0.91c	21.85 ± 1.27c	10.89 ± 0.34c	113.73 ± 7.18c	0.04 ± 0.00c	83.78 ± 8.21c	25.06 ± 2.46b

RET = Root epidermis thickness; RXT = Root exodermis thickness; RDT = Root endodermis thickness; RCD = Root cortex diameter; RAA = Root aerenchyma area; VCD = Vascular cylinder diameter; RMD = Root metaxylem diameter. Columns with different letters indicate significant differences from the Scott-Knott test ($P<0.05$).Values described corresponding to means from five repetitions and standard deviations.

Table 3. Nutrient contents in rice plants treated with EBR and exposed to Fe toxicity.

Fe supply	EBR	K (mg g DM^{-1})	Ca (mg g DM^{-1})	Mg (mg g DM^{-1})	Mn (µg g DM^{-1})	Cu (µg g DM^{-1})	Zn (µg g DM^{-1})
Contents in root							
Control	−	44.83 ± 1.09b	4.05 ± 0.04b	4.62 ± 0.11b	128.5 ± 1.2b	6.83 ± 0.36c	47.47 ± 0.99b
Control	+	47.86 ± 0.92a	4.41 ± 0.22a	5.18 ± 0.36a	139.4 ± 1.7a	8.57 ± 0.41a	53.19 ± 2.45a
Toxicity	−	33.30 ± 0.44c	3.24 ± 0.06d	2.69 ± 0.23c	73.9 ± 4.8d	5.57 ± 0.26d	25.83 ± 1.54d
Toxicity	+	34.66 ± 1.87c	3.78 ± 0.20c	2.98 ± 0.13c	95.7 ± 3.9c	7.44 ± 0.11b	31.95 ± 2.56c
Contents in shoot							
Control	−	35.20 ± 2.68b	4.52 ± 0.29b	3.12 ± 0.27b	191.9 ± 7.9a	12.12 ± 0.66b	35.76 ± 0.89a
Control	+	40.24 ± 2.04a	6.39 ± 0.36a	4.06 ± 0.28a	197.3 ± 10.9a	13.63 ± 0.61a	37.86 ± 2.45a
Toxicity	−	28.01 ± 0.58c	3.58 ± 0.18d	1.66 ± 0.11c	99.1 ± 5.1c	8.32 ± 0.57d	13.88 ± 2.20c
Toxicity	+	28.71 ± 1.00c	4.04 ± 0.14c	1.82 ± 0.15c	112.9 ± 6.9b	10.15 ± 0.49c	18.06 ± 1.15b

K = Potassium; Ca = Calcium; Mg = Magnesium; Mn = Manganese; Cu = Copper; Zn = Zinc. Columns with different letters indicate significant differences from the Scott-Knott test (P<0.05).Values described corresponding to means from five repetitions and standard deviations.

Table 4. Metal ratios in rice plants treated with EBR and exposed to Fe toxicity.

Fe supply	EBR	Mg^{2+}/Fe^{2+}	Mn^{2+}/Fe^{2+}	Cu^{2+}/Fe^{2+}	Zn^{2+}/Fe^{2+}
Ratios in root					
Control	−	0.008 ± 0.001a	0.23 ± 0.02a	0.012 ± 0.001b	0.09 ± 0.01a
Control	+	0.009 ± 0.001a	0.25 ± 0.02a	0.015 ± 0.001a	0.10 ± 0.01a
Toxicity	−	0.001 ± 0.000c	0.03 ± 0.00c	0.002 ± 0.000d	0.01 ± 0.00c
Toxicity	+	0.002 ± 0.000b	0.05 ± 0.00b	0.004 ± 0.001c	0.02 ± 0.00b
Ratios in shoot					
Control	−	0.050 ± 0.004b	3.09 ± 0.11a	0.195 ± 0.009b	0.57 ± 0.03a
Control	+	0.065 ± 0.003a	3.14 ± 0.19a	0.217 ± 0.010a	0.60 ± 0.05a
Toxicity	−	0.006 ± 0.000d	0.36 ± 0.02c	0.030 ± 0.003d	0.05 ± 0.00c
Toxicity	+	0.011 ± 0.001c	0.68 ± 0.04b	0.061 ± 0.004c	0.11 ± 0.02b

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

Table 5. Chlorophyll fluorescence in rice plants treated with EBR and exposed to Fe toxicity.

Fe supply	EBR	Φ_{PSII}	q_P	NPQ	ETR (µmol m^{-2} s^{-1})	EXC (µmol m^{-2} s^{-1})	ETR/P_N
Control	−	0.19 ± 0.010a	0.39 ± 0.023a	0.20 ± 0.025c	28.45 ± 1.50a	0.74 ± 0.018a	2.61 ± 0.12c
Control	+	0.21 ± 0.018a	0.40 ± 0.020a	0.19 ± 0.013c	30.35 ± 3.03a	0.74 ± 0.017a	2.36 ± 0.11d
Toxicity	−	0.15 ± 0.014b	0.22 ± 0.019c	0.44 ± 0.016a	22.05 ± 2.08b	0.76 ± 0.020a	3.13 ± 0.09a
Toxicity	+	0.19 ± 0.009a	0.34 ± 0.024b	0.32 ± 0.027b	28.26 ± 1.34a	0.74 ± 0.017a	2.85 ± 0.05b

Φ_{PSII} = Effective quantum yield of PSII photochemistry; q_P = Photochemical quenching coefficient; NPQ = Nonphotochemical quenching; ETR = Electron transport rate; EXC = Relative energy excess at the PSII level; ETR/P_N = Ratio between the electron transport rate and net photosynthetic rate. Columns with different letters indicate significant differences from the Scott-Knott test (P<0.05).Values described corresponding to means from five repetitions and standard deviations.

Table 6. Gas exchange in rice plants treated with EBR and exposed to Fe toxicity.

Fe supply	EBR	P_N (μmol m^{-2} s^{-1})	E (mmol m^{-2} s^{-1})	g_s (mol m^{-2} s^{-1})	C_i (μmol mol^{-1})	WUE (μmol $mmol^{-1}$)	P_N/C_i (μmol m^{-2} s^{-1} Pa^{-1})
Control	−	10.99 ± 1.00b	2.89 ± 0.07a	0.37 ± 0.021a	298 ± 11b	3.79 ± 0.23c	0.037 ± 0.003b
Control	+	13.00 ± 0.92a	2.80 ± 0.21a	0.38 ± 0.023a	274 ± 8c	4.65 ± 0.28a	0.047 ± 0.003a
Toxicity	−	7.04 ± 0.64c	2.93 ± 0.20a	0.24 ± 0.023b	319 ± 6a	2.41 ± 0.13d	0.022 ± 0.002d
Toxicity	+	9.90 ± 0.34b	2.36 ± 0.15b	0.25 ± 0.021b	305 ± 3b	4.20 ± 0.10b	0.032 ± 0.001c

P_N = Net photosynthetic rate; E = Transpiration rate; g_s = Stomatal conductance; C_i = Intercellular CO_2 concentration; WUE = Water-use efficiency; P_N/C_i = Carboxylation instantaneous efficiency. Columns with different letters indicate significant differences from the Scott-Knott test (P<0.05).Values described corresponding to means from five repetitions and standard deviations.

Table 7. Stomatal characteristics in rice plants treated with EBR and exposed to Fe toxicity.

Fe supply	EBR	SD (stomata per mm^2)	TD (trichome per mm^2)	PDS (µm)	EDS (µm)	SF
Adaxial face						
Control	−	455.76 ± 14.55a	41.28 ± 3.88c	11.70 ± 0.60c	17.85 ± 0.40c	0.66 ± 0.01b
Control	+	465.26 ± 14.83a	33.63 ± 3.30d	11.41 ± 0.91c	16.67 ± 0.38d	0.69 ± 0.01a
Toxicity	−	373.60 ± 6.85c	68.80 ± 4.54a	14.20 ± 0.46a	22.87 ± 0.89a	0.62 ± 0.05b
Toxicity	+	395.83 ± 7.03b	59.62 ± 3.59b	13.05 ± 0.50b	20.22 ± 0.68b	0.65 ± 0.02b
Abaxial face						
Control	−	431.87 ± 17.67a	*	12.14 ± 0.37b	21.31 ± 0.69c	0.57 ± 0.01a
Control	+	458.64 ± 19.53a	*	10.67 ± 0.58c	17.72 ± 0.56d	0.60 ± 0.03a
Toxicity	−	366.41 ± 5.70c	*	13.36 ± 0.56a	25.61 ± 0.89a	0.52 ± 0.02b
Toxicity	+	397.49 ± 8.36b	*	12.30 ± 0.45b	22.82 ± 0.57b	0.54 ± 0.01b

SD = Stomatal density; TD = Trichome density; PDS = Polar diameter of the stomata; EDS = Equatorial diameter of the stomata; SF = Stomatal functionality. * = Absence in abaxial face. Columns with different letters indicate significant differences from the Scott-Knott test (P<0.05).Values described corresponding to means from five repetitions and standard deviations.

Table 8. Leaf anatomy in rice plants treated with EBR and exposed to Fe toxicity.

Fe supply	EBR	ETAd (μm)	ETAb (μm)	MT (μm)	LAA (mm^2)	BCD (μm)
Control	−	8.67 ± 0.53a	8.80 ± 0.40a	51.01 ± 1.57b	0.53 ± 0.02b	29.51 ± 1.54a
Control	+	9.57 ± 0.74a	9.55 ± 0.65a	55.50 ± 2.34a	0.75 ± 0.04a	31.41 ± 1.66a
Toxicity	−	6.93 ± 0.55b	6.59 ± 0.37c	47.57 ± 2.74b	0.19 ± 0.00d	23.57 ± 1.27b
Toxicity	+	7.59 ± 0.44b	7.80 ± 0.46b	49.29 ± 1.51b	0.23 ± 0.01c	25.78 ± 1.56b

ETAd = Epidermis thickness from adaxial leaf side; ETAb = Epidermis thickness from abaxial leaf side; MT = Mesophyll thickness; LAA = Leaf aerenchyma area; BCD = Bulliform cell diameter. Columns with different letters indicate significant differences from the Scott-Knott test ($P<0.05$). Values described corresponding to means from five repetitions and standard deviations.

Table 9. Photosynthetic pigments in rice plants treated with EBR and exposed to Fe toxicity.

Fe supply	EBR	Chl *a* (mg g^{-1} FM)	Chl *b* (mg g^{-1} FM)	Total Chl (mg g^{-1} FM)	Car (mg g^{-1} FM)	Ratio Chl *a*/Chl *b*	Ratio Total Chl/Car
Control	−	19.63 ± 0.77a	5.55 ± 0.24b	25.18 ± 0.45b	0.74 ± 0.05a	3.57 ± 0.20b	30.38 ± 1.83b
Control	+	20.63 ± 0.90a	6.14 ± 0.28a	26.78 ± 0.64a	0.78 ± 0.07a	3.40 ± 0.22b	34.48 ± 1.35a
Toxicity	−	14.69 ± 0.61c	3.67 ± 0.26d	18.37 ± 0.59d	0.55 ± 0.05b	4.02 ± 0.13a	34.32 ± 1.01a
Toxicity	+	17.73 ± 0.86b	4.39 ± 0.22c	22.12 ± 0.87c	0.79 ± 0.07a	4.05 ± 0.21a	29.10 ± 1.72b

Chl *a* = Chlorophyll *a*; Chl *b* = Chlorophyll *b*; Total chl = Total chlorophyll; Car = Carotenoids. Columns with different letters indicate significant differences from the Scott-Knott test ($P<0.05$).Values described corresponding to means from five repetitions and standard deviations.