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SANDY SANTOS DA FONSECA

24-EPIBRASSINOLIDE POSITIVELY MODULATES LEAF TISSUES AND ANTIOXIDANT SYSTEM ATTENUATING THE DELETERIOUS EFFECTS ON PHOTOSYNTHETIC MACHINERY IN RICE PLANTS UNDER SIMULATED ACID RAIN

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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

ABA	Abscisic acid
APX	Ascorbate peroxidase
BCD	Bulliform cell diameter
Ca	Calcium
CAR	Carotenoids
CAT	Catalase
$CdCl_2$	Cadmium chloride
Chl *a*	Chlorophyll a
Chl *b*	Chlorophyll b
C_i	Intercellular CO_2 concentration
CO_2	Carbon dioxide
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
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
H_2SO_4	Sulfuric acid
HNO_3	Nitric acid
K	Potassium
LAA	Leaf aerenchyma area

MDA	Malondialdehyde
Mg	Magnesium
MT	Mesophyll thickness
Na	Sodium
NaCl	Sodium chloride
NPQ	Nonphotochemical quenching
O_2^-	Superoxide
PDS	Polar diameter of the stomata
pH	Hydrogen potential
PHE	Phenanthrene
P_N	Net photosynthetic rate
P_N/C_i	Instantaneous carboxylation efficiency
POX	Peroxidase
PSII	Photosystem II
PYR	Pyrene
q_P	Photochemical quenching
RDM	Root dry matter
ROS	Reactive oxygen species
RUBISCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SAR	Simulated acid rain
SD	Stomatal density
SDM	Shoot dry matter
SF	Stomatal functionality
SOD	Superoxide dismutase
TD	Trichome density
TDM	Total dry matter
Total Chl	Total Chlorophyll
WUE	Water-use efficiency
Φ_{PSII}	Effective quantum yield of PSII photochemistry

SUMMARY

RESUMO

A chuva ácida causa danos significativos às plantas de arroz, afetando o maquinário fotossintético e o crescimento. O 24-epibrassinolídeo (EBR) é um esteroide orgânico usado em baixas concentrações que regula positivamente o crescimento das plantas e atenua os efeitos deletérios relacionados às mudanças ambientais. O objetivo deste estudo foi investigar se o tratamento exógeno com 24-epibrassinolídeo pode aliviar os efeitos negativos da chuva ácida simulada (SAR) e o possível mecanismo de tolerância envolvido, avaliando a fluorescência das clorofilas, as trocas gasosas, o sistema antioxidante e as variáveis anatômicas foliares. O experimento foi randomizado com quatro tratamentos, sendo duas chuvas ácidas simuladas (H_2SO_4 0 e 0,5 M, descritas como - SAR e + SAR, respectivamente) e duas concentrações de brassinoesteroides (EBR 0 e 100 nM, descritas como - EBR e + EBR, respectivamente). Nossos resultados mostraram que as plantas expostas à SAR sofreram interferências negativas; no entanto, plantas tratadas com EBR apresentaram benefícios na fluorescência das clorofilas, aliviando a fotoinibição no fotossistema II e protegendo contra os danos causados pelo desequilíbrio das espécies reativas de oxigênio. Além disso, o EBR promoveu aumentos nas trocas gasosas claramente relacionadas à regulação estomática, melhorando a captação e distribuição de CO_2 nos espaços intercelulares. Esta pesquisa revelou que o EBR atenuou os efeitos negativos da SAR, aumentando as atividades das enzimas antioxidantes (superóxido dismutase, catalase, ascorbato peroxidase e peroxidase), reduzindo os danos nas membranas dos tilacoides, confirmados pelo aumento de clorofilas e carotenoides. Finalmente, os efeitos do EBR observados em plantas sob SAR demonstram que esta substância modulou positivamente importantes estruturas anatômicas ligadas à proteção das folhas, aumentando a quantidade de tricomas, cera epicuticular e área de aerênquima. Esses resultados fornecem evidências de que o EBR conferiu tolerância em plantas de arroz expostas à SAR.

PALAVRAS-CHAVE: Aerênquima. Cera epicuticular. *Oryza sativa* L. Problema ambiental global. Rendimento quântico fotoquímico do PSII. Taxa fotossintética líquida.

ABSTRACT

Acid rain causes significant damages to rice plants, affecting photosynthetic machinery and growth. 24-epibrassinolide (EBR) is an organic steroid that used at low concentrations positively regulates plant growth and mitigates deleterious effects connected to environmental changes. The aim of this study was to investigate whether exogenous treatment with 24-epibrassinolide can alleviate the negative effects of the simulated acid rain (SAR) and the possible tolerance mechanism involved, evaluating the chlorophyll fluorescence, gas exchange, antioxidant system and leaf anatomical variables. The experiment was randomized with four treatments, being two simulated acid rain (0 and 0.5 M H_2SO_4, described as – SAR and + SAR, respectively) and two concentrations of brassinosteroids (0 and 100 nM EBR, described as – EBR and + EBR, respectively). Our results showed that plants exposed to SAR suffered negative interferences; however, EBR-treated plants presented benefits on chlorophyll fluorescence, alleviating the photoinhibition in the photosystem II and protection against damages caused by the imbalance of the reactive oxygen species. Additionally, EBR promoted increases in gas exchange clearly linked to stomatal regulation, improving uptake and distribution of CO_2 in intercellular spaces. This research revealed that EBR attenuated the negative effects of SAR, increasing the activities of the antioxidant enzymes (superoxide dismutase, catalase, ascorbate peroxidase and peroxidase), reducing the damages on tilacoid membrane, confirmed by increases in chlorophylls and carotenoids. Finally, the EBR effects observed in plants under SAR demonstrate that this substance positively modulated important anatomical structures linked to leaf protection, increasing the amount of trichomes, epicuticular wax and aerenchyma area. These results provide evidence that EBR conferred tolerance on rice plants exposed to SAR.

KEYWORDS: Aerenchyma. Effective quantum yield of PSII photochemistry. Global environmental problem. Epicuticular wax. Net photosynthetic rate. *Oryza sativa* L.

1. CONTEXTUALIZATION

Oriza sativa L. (rice) is one of the most cultivated and consumed grasses in the world. It contributes to reducing hunger and poverty and plays a fundamental role in the food security of more than half of the world population (LANKA, 2004). Rice ranks second as the most cultivated cereal in the world, with approximately 161 million hectares cultivated and production of about 756.5 million tons of husked grains (SOSBAI, 2018).

Rice is the most important food crop in Asia (CLAUSS et al., 2018), where most countries practice rice growing using traditional forms of cultivation, which are passed on through the generations (LIU, X et al., 2019). In addition, rice is an important energy source on the Asian continent, especially in China, considered the largest rice producer in the world, with more than 200 million tons of grains produced annually (ZHANG et al., 2019).

Brazil is also a major rice producer (RODRIGUES SILVEIRA et al., 2019), inclusive, it is estimated that the production of the 2019/20 harvest will be 10.5 million tons (CONAB, 2019). A large part of the Brazilian rice production is concentrated in the southern region of the country, which has the leadership of national production, with planted areas stabilized for over ten years and which, despite the time, maintain productivity of around 7,000 kg/ha (SOSBAI, 2018).

Rice is sensitive to acid rain, being more sensitive in the seedling stage than in the grain loading and filling stage (ZHANG, F. et al., 2018). Acid rain has become a very common environmental problem in Europe, America and more intensely in Asia, where acid precipitation is rich in sulfur and nitrogen (DUAN et al., 2016).

On the European continent, there is a strong environmental policy to reduce acid deposition, whose pH values are considered acidic and slightly acidic, ranging from 4.19 to 5.82 (KERESZTESI et al., 2019). In Brazil, with the increase of anthropogenic activities, acid rain continues to be a serious environmental threat (MARTINS et al., 2014).

Studies simulating acid rain conditions in plants have shown that simulated acid rain (SAR) affects photosynthetic performance with extensive reflections on chlorophyll fluorescence and many photosynthetic parameters (LIU, M., et al., 2019). In addition, acid rain over plants reduces the absorption of nutrients and biomass (LI; LIANG, 2019), increases the levels of reactive oxygen species, inhibits growth and photosynthesis (DEBNATH et al., 2018).

On the other hand, several studies with the phytohormone brassinosteroids (BRs), have pointed out that this is an interesting option for tolerance to abiotic stress in rice (KUTSCHERA; WANG, 2012; SHARMA; KAUR; PATI, 2017; FERRERO-SERRANO et al., 2019). Brassinosteroids are a class of naturally occurring steroids that play fundamental roles in the development and growth of plants, participating in processes of cell division, stomatal opening, photomorphogenesis (CASSON; HETHERINGTON, 2012) and grain filling (WU et al., 2008).

Additionally, studies have reported the beneficial action of phytohormones, such as BRs, attenuating environmental stress, which results in physiological and molecular changes in plants (SHARMA; KAUR; PATI, 2017; ZHANG et al., 2020), modulating the antioxidant defense system components (VARDHINI; ANJUM, 2015), inducing improvements in photosynthetic activity (AHAMMED et al., 2012; SIDDIQUI; AHMED; HAYAT, 2018; SIDDIQUI; HAYAT; BAJGUZ, 2018) and gas exchange (AHAMMED et al., 2012, 2013).

Since 2000, global rice production has declined significantly, indicating the need for support to increase its productivity; however, to achieve a sustainable increase in rice production and guarantee food security (LANKA, 2004), it is necessary to expand the line of knowledge to develop technologies so that rice cultivation can withstand different types of abiotic stresses, such as acid rain (LANKA, 2004; ROMERO; GATICA-ARIAS, 2019).

Thus, the aim of this study was to investigate whether exogenous treatment with EBR (an active form of BR) can mitigate the negative effects of SAR, giving tolerance to rice exposed to acidic water, evaluating the possible effects of this steroid on photosynthesis, exchange gas, antioxidant system, oxidant system, growth and leaf anatomy of rice plants.

1. 1. Literature review

1.1.1. Agricultural, food and socioeconomic importance of rice

Rice is an annual monocot that belongs to the Poaceae family and is considered a model plant (KHUSH, 2013; PETTKÓ-SZANDTNER et al., 2015; SAMO et al., 2017), consisting of leaves, stem, roots and reproductive organs (ATUNGULU; PAN, 2014) (ATUNGULU; PAN, 2014). This plant emit tillers, its leaves are arranged on the stem, one in each node alternately, its inflorescence is called panicle and its root system is branched, consisting of many thin and long roots (SOSBAI, 2018).

It is a plant adapted to aquatic environments, due to the presence of aerenchyma in the roots. Aerenchymas are tissues containing intercellular air spaces, essential for root oxygenation under hypoxia (MOHAMMED et al., 2019; LIU et al., 2020). In addition, it is a short days culture, so its vegetative cycle is short, where the optimal temperature range for its development is between 20-35°C (SOSBAI, 2018).

For this study rice seeds of the cultivar Puitá INTA CL™ were selected, which has the following characteristics: quality and high yield of grains (premium or noble grain), tolerance to herbicides of the imidazolinone group, high productivity, easy cultivation, short stature, leaves with trichomes and early cycle (SOSBAI, 2018).

Rice is an excellent source of carbohydrates, whose grains are predominantly rich in starch (AMAGLIANI et al., 2016; FAN et al., 2019), approximately 80-85%, 4-10% protein, 1% lipids and 10% moisture content (BALINDONG et al., 2018), being also a source of calcium, iron, zinc and vitamins B and E (ATUNGULU; PAN, 2014).

From the processing of grains, various products are made available on the market, such as: rice bran oil (rich in oleic acid), rice bran (LIU, X., et al, 2019), rice wine (TIAN et al., 2016), rice flour, brown rice flour (BAEK; LEE 2014), parboiled rice (MIR, SA et al., 2015), polished white rice (SALEH et al., 2019), brown rice (ATUNGULU; PAN 2014; XIA, et al., 2019), rice vinegar (PAZUCH et al., 2019), among other products of interest to the consumer.

It is considered a staple food for about 3.5 billion people (MUTHAYYA et al., 2014; XIA et al., 2019), being grown on all continents, except Antarctica (AMAGLIANI et al., 2017). In the world ranking, the two largest rice producers are China and India, respectively, followed by other countries such as Indonesia, Bangladesh, Vietnam, Thailand, Myanmar, Philippines and Brazil (SOSBAI, 2018).

In Brazil, the five regions that divide the country cultivate rice. In the 2019 harvest, the production in grains with husks in the southern region was 8.369,037 tons, North 944.367 tons, Center-west 577.721 tons, Northeast 314.090 tons and Southeast 55.259 tons (IBGE, 2019). In the southern region, the state of Rio Grande do Sul leads the grain supply within Brazil and Latina America, accounting for more than 80% of total production (IBGE, 2019; RODRIGUES SILVEIRA et al., 2019). In the northern region, the states of Tocantins (647.900 tons), Rondônia (125.800 tons) and Pará (94.676 tons) stand out, in increasing order of rice production, respectively (IBGE, 2019).

Rice is the staple food of most Brazilians, contributing 67% and 90% of the total food intake (CIMINELLI et al., 2017). In addition, rice farming generates hundreds of jobs and guarantees the income of many families, linked to family farming or commercial agriculture (SOSBAI, 2018). It is cultivated in two types of ecosystems, the first is called upland rice or "*sequeiro*" rice, common to the northeast region and the second, it is called lowland or "*várzeas*" rice, typical of the southern region (KATSURAYAMA et al., 2018; GARCIA et al., 2011).

Rice is sensitive to climate changes, which have negative influences on its phenological processes, characterized by the shortening of the period of growth and flowering (BAI et al., 2019). According to Godfray et al. (2010) the effects of climate change threaten food security, with the need to reevaluate our actions on the environment to ensure food production in a balanced and sustainable way.

1.1.2. Deleterious effects of acid rain

1.1.2.1. Acid rain: Origin and consequences in the environment

Acid rain is a global environmental problem and is considered one of the three most serious environmental disasters in the world, it has its origin in human activities (XU et al., 2015; SHU et al., 2019). It is mainly caused by the dissolution of sulfur dioxide and nitrogen oxides in the atmosphere, and has negative effects on ecosystems (LIU et al., 2017).

Sulfur and nitrogen deposits come from industrial, urban, agricultural, vehicular, marine and natural sources such as volcanic activity, for example (GONZALEZ; ARISTIZÁBAl, 2012; CHO et al., 2019; CHEN et al., 2020). Significant increases in these emissions have been accompanied by environmental monitoring programs around the world, which detected air quality degradation and the formation of acid rain rich in sulfuric and nitric acids (GONZALEZ; ARISTIZÁBAl, 2012; HAN et al., 2019; CHEN et al., 2020).

The term “acid rain” emerged in the XIX century, and refers to the acidic components found in rain, snow, particles, gases and steam, being considered acid when the pH value is less than 5 (FORNARO, 2006; BURNS et al., 2016). These acidic precipitations cause great losses, such as: acidification of soils and water sources, inhibition of forest growth, corrosion in buildings and damage to human health (BURNS et al., 2016; MALTZ et al., 2019).

Some regions of the planet receive acid precipitation in their territories more frequently, such as America, Europe and Asia. In these territories, this environmental problem is aggravated because there is intense economic growth, accompanied by a high number of vehicles and industrial activity, which results in daily emission of large amounts of pollutants into the atmosphere (WANG et al., 2014; LIU et al., 2017).

In Brazil, the climatic conditions are favorable for the formation of acid precipitation, the summer is usually hot and rainy, besides the dispersion of several pollutants by the industrial nuclei (FACCHINI CERQUEIRA et al., 2014) in the industrialized urban regions. In these nuclei, particles released in the air are from burning fossil fuels, urban waste incineration, mining and burning (SOUZA et al., 2010).

Acid rains that occur in the Limeira region (São Paulo), southeastern Brazil, have as pollution sources the agricultural emissions from sugarcane and orange plantations, releasing Ca^{2+}, NH^{4+}, Na^{+} and HCO^{3-} ions and anthropogenic sources from greenhouse gases, vehicles, burning and mining by releasing ions SO_4^{2-}, NO^{3-}, Cl^{-}, K^{+}, Mg^{2+} and HCO^{3-} (MARTINS et al., 2019).

In Manaus, a city located in the north of Brazil, the occurrence of acid rain is a phenomenon that occurs due to rapid population growth, deforestation, fires, industrial areas, followed by the increased demand for fossil fuels, generating rain rich in H_2SO_4 and HNO_3 (HONÓRIO; HORBE; SEYLER, 2010).

1.1.2.2. Effects of simulated acid rain on photosynthetic apparatus and plant oxidative system

Acid rain is considered an important abiotic stress factor in plants (JU et al., 2017) because it can affect different growth and development stages, leading to potential economic losses (DU et al., 2017). Several studies have contributed to the understanding of the effects caused by acid rain on plants, from simulated acid rain (SAR) experiments with different pH values (DU et al., 2017; JU et al., 2017; DEBNATH et al., 2018; LIANG; ZHANG, 2018; MALTZ et al., 2019; SHU et al., 2019).

One of these studies reported negative influences promoted by SAR in *Pisum sativum* L. leaves, as damages to the photosynthetic apparatus, revealed in transmission electron microscopy; the SAR destroyed the chloroplast membrane system, causing ultra-structural changes in this organelle (POLISHCHUK et al., 2016).

Damages to leaf tissues, caused by SAR, reduces the levels of photosynthetic pigments, impairs stomatal conductance (SUN et al., 2012; KHALID et al., 2018), causes negative disturbances in the light capture centers of the photosystems, affecting the metabolic activities of plants, in particular the efficiency of the Calvin Cycle, and the integrity of cells, that is greatly impaired by increases in reactive oxygen species (ROS) (YANG et al., 2018).

The generation of ROS is a natural process, which results from plant metabolic activity, but plants efficiently eliminate these radicals through their antioxidant defense system (VARDHINI; ANJUM, 2015). However, SAR can break the balance that exists between the antioxidant defense system and the plant's oxidative system, leading to the excessive accumulation of ROS, which decreases the activity of the antioxidant system, causing oxidative stress (SUN et al., 2012; CHEN et al., 2013; REN et al., 2018).

DEBNATH et al. (2018) reported that severe SAR stress in *Solanum lycopersicum* L., resulted in increases in ROS, especially ROS hydrogen peroxide, intensifying the damage of lipid peroxidation of membranes and malondialdehyde levels. Some of these damages are evidenced by changes in the color of the leaves, such as chlorosis, necrosis and leaf deformations, but the intensity of these damages depends on the time of exposure and the pH value of the SAR, to which the plants are submitted (SUN et al., 2012; CHEN et al., 2013; DEBNATH et al., 2018).

Finally, Liang and Zhang (2018) reported indirect effects caused by SAR in *Glycine max* L., which included a decrease in root dry biomass. Different pH values were tested in *Horsfieldia hainanensis* Merr. Then, the results revealed that the decrease in the pH value severely or slightly inhibited the growth of leaves, stem and root (HUANG et al., 2019).

1.1.3. Action of brassinosteroids

The discovery of “new hormones” called “brassins” was reported by Mitchell et al. (1970), who after performing procedures with extracts obtained from pollen from *Brassica napus* L., obtained an oily product, which they submitted to the bioassay of the common bean (*Phaseolus vulgaris* L.) second internode and observed a marked elongation in the region where the product was applied, which presented different characteristics from the gibberellins.

Subsequently, a study conducted by Grove et al. (1979) was of paramount importance for determining the chemical structure of the oily product identified by Mitchell et al. (1970). Grove et al. (1979) isolated this oily compound, which promoted the growth of plants, taking the pollen extract of *B. napus* L. as raw material and based on X-ray analysis, they determined its chemical structure, biological activity and named it brassinolide. According to Grove et al. (1979), when the brassinolide was applied to the second internode of the bean, it promoted an intense elongation of the plant tissue when compared to plants without treatment.

After the publication by Mitchell et al. (1970) and Grove et al. (1979), other studies appeared in the 1990s, which contributed to the fact that brassinosteroids (BRs) were finally recognized as plant steroid hormones. Since then, more than 69 BRs have been identified in different plant species; it is believed that they are synthesized in compartments of the endoplasmic reticulum, its basic structure is a steroidal lactone, differentiated by having 27, 28 or 29 carbons in its structure (BAJGUZ, 2011; KUTSCHERA; WANG, 2012; SAKAMOTO et al., 2012; YOKOTA et al., 2017; VUKASINOVIC; RUSSINOVA, 2018).

Brassinosteroids are currently considered the sixth class of plant hormones, they are polyhydroxylated steroids present in all plant species, where they act in important growth and development processes (SIDDIQUI; HAYAT; BAJGUZ, 2018; PERES et al., 2019). BRs are also known for their positive effects on plants under different types of stresses, biotic or abiotic ones, activating different stress tolerance mechanisms (AHANGER et al., 2018).

These steroids are received in the plasma membrane by the receptor kinase BRASSINOSTEROID INSENSITIVE 1 (BRI1) (NAKAMURA et al., 2017; PERES et al., 2019), and contribute to several physiological, biochemical and molecular responses in plants (ALI, 2017), endogenously, such as division, elongation and differentiation of various types of cells (DU et al., 2019; PLANAS-RIVEROLA, et al., 2019), improve the post-harvest quality of fruits and flower buds (YAO et al., 2017; BAGHEL et al., 2019), root development (WEI; LI, 2016), leaf senescence, flowering (YANG et al., 2011; YIN et al., 2019) and plant morphogenesis (ZHENG et al., 2019).

Brassinosteroids decrease the loss of photosynthetic pigments, help to overcome stomatal limitations and increase the efficiency of carbon fixation, the efficiency of PSII and the photosynthetic rate, respectively (SIDDIQUI; AHMED; HAYAT, 2018; SIDDIQUI; HAYAT; BAJGUZ, 2018; SHU et al., 2016). In rice, BRs act on height, fertility, tillers angle, filling, size and shape of the grains (KUTSCHERA; WANG 2012; FERRERO-SERRANO et al., 2019). In abiotic stress conditions in grasses, the BRs participate in cell wall remodeling (RAO et al., 2017).

In addition, the ability of BRs to mitigate different origins of abiotic stresses has been evidenced in several studies (YH et al., 2012; WU et al., 2014; REYES GUERRERO et al., 2015; POCIECHA, et al., 2017, WU et al., 2017; XIA et al., 2018). In these works, the use of the brassinosteroids analog called 24-epibrassinolide (EBR), which is an active form of brassinosteroids, is quite frequent (VARDHINI; ANJUM, 2015).

24-epibrassinolide at low concentrations regulates plant growth and at higher doses increases plant resistance to stress (ASGHARI; ZAHEDIPOUR 2016). Exogenous applications of EBR in plants have shown positive regulation for the expression of gene routes, mainly the expression of the enzymes Catalase (CAT), Superoxide dismutase (SOD), Peroxidase (POX) and Ascorbate Peroxidase (APX), which are enzymes of antioxidant defense system (VARDHINI; ANJUM, 2015; SHARMA et al., 2018).

In a study conducted by Zhong et al. (2020), EBR improved tolerance to lead (Pb) through the antioxidant system, with a reduction in the accumulation of reactive oxygen species in *Festuca arundinacea* Schreb. providing efficient phytoremediation to heavy metal. In addition, leaf sprays of EBR had a protective effect on plants of *O. sativa* L. submitted to salt stress by sodium chloride (NaCl), reinforcing the antioxidant system and growth parameters (REYES GUERRERO et al., 2015).

24-epibrassinolide increased the chlorophyll content, density and stomas length in *Vitis vinife*ra L. under water stress, alleviating the photosynthetic limitations caused by water stress (WANG et al., 2015). Ahammed et al. (2013) reported increased biomass, photosynthetic capacity and photoinhibition relief, respectively, in *S. lycopersicum* L. exposed to persistent organic pollutants, suggesting that EBR protected plants from further damage caused by pollutants.

Considering what was mentioned before, it is of utmost importance to investigate measures that can alleviate any negative interference caused by acid rain on plants, especially on rice. And at this point, EBR is an excellent alternative to be explored, because it promotes multiple benefits in plant physiology of plants under different types of abiotic stresses (RAMAKRISHNA; RAO, 2012; FARIDUDDIN et al., 2013; WANG et al., 2017; MAIA; SILVA; LOBATO, 2018); RIBEIRO; SILVA; LOBATO, 2019). In view of this, our hypothesis was based on the damage caused by acid rain on plant metabolism and on the beneficial role of EBR in plants under various types of stress, investigating whether exogenous treatment with EBR can mitigate the negative effects of SAR.

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Page title

24-epibrassinolide positively modulates leaf tissues and antioxidant system attenuating the deleterious effects on photosynthetic machinery in rice plants under simulated acid rain

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AKSL was the advisor of this research. SSF conducted the experiment in the greenhouse and performed physiological, biochemical and morphological determinations, while BRSS measured anatomical parameters.

24-epibrassinolide positively modulates leaf tissues attenuating the deleterious effects on photosynthetic machinery in rice plants under simulated acid rain

Abstract

Acid rain causes significant damages to rice plants, affecting photosynthetic machinery and growth. 24-epibrassinolide (EBR) is an organic steroid that used at low concentrations positively regulates plant growth and mitigates deleterious effects connected to environmental changes. The aim of this study was to investigate whether exogenous treatment with 24-epibrassinolide can alleviate the negative effects of the simulated acid rain (SAR) and the possible tolerance mechanism involved, evaluating the chlorophyll fluorescence, gas exchange, antioxidant system and leaf anatomical variables. The experiment was randomized with four treatments, being two simulated acid rain (0 and 0.5 M H_2SO_4, described as – SAR and + SAR, respectively) and two concentrations of brassinosteroids (0 and 100 nM EBR, described as – EBR and + EBR, respectively). Our results provide that plants exposed to SAR suffered negative interferences; however, EBR-treated plants presented benefits on chlorophyll fluorescence, alleviating the photoinhibition in the photosystem II and protection against damages caused by the imbalance of the reactive oxygen species. Additionally, EBR promoted increases in gas exchange clearly linked to stomatal regulation, improving uptake and distribution of CO_2 in intercellular spaces. This research revealed that EBR attenuated the negative effects of SAR, increasing the activities of the antioxidant enzymes (superoxide dismutase, catalase, ascorbate peroxidase and peroxidase), reducing the damages on tilacoid membrane, confirmed by increases in chlorophylls and carotenoids. Finally, the EBR effects observed in plants under SAR demonstrate that this substance positively modulated important anatomical structures linked to leaf protection, increasing the amount of trichomes, epicuticular wax and aerenchyma area. These results provide evidence that EBR conferred tolerance on rice plants exposed to SAR.

Keywords Aerenchyma ● Effective quantum yield of PSII photochemistry ● Global environmental problem ● Epicuticular wax ● Net photosynthetic rate ● *Oryza sativa* L.

Abbreviations

APX	Ascorbate peroxidase
BCD	Bulliform cell diameter
CAR	Carotenoids
CAT	Catalase
Chl *a*	Chlorophyll a
Chl *b*	Chlorophyll b
C_i	Intercellular CO_2 concentration
CO_2	Carbon dioxide
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
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
H_2SO_4	Sulfuric acid
HNO_3	Nitric acid
K	Potassium
LAA	Leaf aerenchyma area
MDA	Malondialdehyde
Mg	Magnesium
MT	Mesophyll thickness
NaCl	Sodium chloride
NPQ	Nonphotochemical quenching
O_2^-	Superoxide
PDS	Polar diameter of the stomata
pH	Hydrogen potential
PHE	Phenanthrene
P_N	Net photosynthetic rate
P_N/C_i	Instantaneous carboxylation efficiency
POX	Peroxidase

PSII	Photosystem II
PYR	Pyrene
q_P	Photochemical quenching
RDM	Root dry matter
ROS	Reactive oxygen species
RUBISCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SAR	Simulated acid rain
SD	Stomatal density
SDM	Shoot dry matter
SF	Stomatal functionality
SOD	Superoxide dismutase
TD	Trichome density
TDM	Total dry matter
Total Chl	Total Chlorophyll
WUE	Water-use efficiency
Φ_{PSII}	Effective quantum yield of PSII photochemistry

Introduction

Rice is considered a plant model, being a gramineous sensitive to climate change, often exerting negative interferences on phenological stages, such as growth and flowering (Bai et al. 2019). Rice grains represent an important source of calories in the human diet, responsible by about 20% of the calorie amount consumed by more than 50% of the world population (Amb and Ahluwalia 2016), rich in starch (Fan et al. 2019), containing approximately 80-85% carbohydrates, 4-10% protein and 1% lipids (Balindong et al. 2018).

Effects of climate change represents a serious threat to food security (Godfray et al. 2010), because these global changes negatively interfere on agriculture, in which the acid rain is considered one of the more recurrent environmental problems in the world (Xu et al. 2015; Shu et al. 2019). Acid rain is a form of precipitation that contains acidic components, being caused by the dissolution of sulfur dioxide and nitrogen oxides in the atmosphere, and negatively impacting the ecosystems (Liu et al. 2017). In Brazil, the climatic conditions are favorable for the formation of acid precipitation, in which the summer is usually hot and rainy, besides the dispersion of several pollutants connected to industrial activity (Facchini Cerqueira et al. 2014), utilization of fossil fuel, urban waste incineration, mining and and forest burning (Souza et al. 2010).

Plants exposed to acid rain frequently suffers direct and indirect effects. Directly interfering on leaves, causing overproduction of reactive oxygen species (Sun et al. 2012), reduction of photosynthetic pigments (Chen et al. 2013; Liang and Zhang 2018), damages to chloroplast structure (Wen et al. 2011), negative impact on of thylakoid ultrastructure (Polishchuk et al. 2016), photosynthetic rate (Khalid et al. 2018) and leaf growth inhibition (Debnath et al. 2018b). In other words, biochemical, physiological and anatomical modifications in leaf provoke in leaf color changes from green to yellow or white, with curled and wrinkled aspects (Liang et al. 2018). Indirectly the acid rain affects the root and soil, more specifically reducing the root growth (Liu et al. 2018), soil microbial activity (Wang et al. 2014; Wei et al. 2017) and nutrient losses in soil (Wen et al. 2013).

Brassinosteroids (BRs) are polyhydroxylated steroids that can contribute significantly to food safety, because they have benefits connected to physiological, biochemical and molecular responses (Ali 2017), stimulating division, elongation and differentiation in plant cells (Planas-Riverola et al. 2019). Other interesting roles of these substances are associated with the mitigation of detrimental changes to plant metabolism, activating different tolerance mechanisms (Ahanger et al. 2018). Since its discovery, more than 62 molecules have been identified, which are active even at very low concentrations, highlighting the 24-epibrassinolide (EBR), used in this study aiming to alleviate the effects of the acid rain simulated (SAR) (Kanwar et al. 2017).

EBR is a BRs analog very active, in which used at low concentrations positively regulates plant growth (Asghari and Zahedipour 2016) and maintains plant homeostasis under stress conditions, contributing to adaptations to environmental changes (Peres et al. 2019). Fariduddin et al. (2013) described that EBR acted improving the antioxidant system and growth in *Cucumis sativus* exposed high NaCl and Cu concentrations. Yang et al. (2019)reported that the application of EBR helped maintain the integrity of chloroplast and tilacoid structures, improving the stomatal opening in *C. sativus* under toxicity by cinnamic acid. Additionally, Ahammed et al. (2012) verified that EBR induced improvement in stomatal conductance in *Solanum lycopersicum* under phenanthrene stress.

Our hypothesis was based on the damages of acid rain on plant metabolism and beneficial roles of EBR (more active BR) in plants subjected to several stresses, such as salinity (Reyes Guerrero et al. 2015; Wu et al. 2017), heavy metals (Hasan et al. 2011; Bajguz 2011; Santos et al. 2018), organic pollutants (Ahammed et al. 2013b), pesticide phytotoxicity (Wang et al. 2017; Sharma et al. 2017), high temperature (Wu et al. 2014), low temperature (Pociecha et al. 2017; Xia et al. 2018) and drought (Li et al. 2012; Lima and Lobato 2017; Pereira et al. 2019). Therefore, the aim of this study was to investigate whether exogenous treatment with 24-epibrassinolide can alleviate the negative effects of the simulated acid rain (SAR) and the possible tolerance mechanism involved, evaluating the chlorophyll fluorescence, gas exchange, antioxidant system and leaf anatomical variables.

Materials and Methods

Location and growth conditions

The experiment was performed of the Universidade Federal Rural da Amazônia, Paragominas, Brazil (2°55' S, 47°34' W). The study was conducted in a greenhouse with the temperature and humidity controlled. The minimum, maximum, and median temperatures were 23, 29 and 25.6°C, respectively. The relative humidity during the experimental period varied between 60% and 80%.

Plants, containers, acclimation and maintenance

Four-days-old seedlings of *Oryza sativa* (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% (8^{th} day) and later modified to 100% after two days (10^{th} day). After this period, the nutritive solution remained at total ionic strength. The plants received the following macro- and micronutrients contained in the nutrient solution: 8.75 mM KNO_3, 7.5 mM Ca $(NO_3)_2 \cdot 4H_2O$, 3.25 mM $NH_4H_2PO_4$, 1.5 mM $MgSO_4 \cdot 7H_2O$, 62.50 µM KCl, 31.25 µM H_3BO_3, 2.50 µM $MnSO_4 \cdot H_2O$, 2.50 µM $ZnSO_4 \cdot 7H_2O$, 0.63 µM $CuSO_4 \cdot 5H_2O$ and 0.63 µM $NaMoO_4 \cdot 5H_2O$ and 250.0 $NaEDTAFe \cdot 3H_2O$. 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.

24-epibrassinolide (EBR) preparation and application

The 0 and 100 nM EBR (Sigma-Aldrich, USA) solutions were prepared by dissolving the solute in ethanol followed by dilution with Milli-Q water [ethanol:water (v/v) = 1:10,000] (Ahammed et al. 2013a). Ten-day-old seedlings were sprayed with 24-epibrassinolide (EBR) or Milli-Q water (containing a proportion of ethanol that was equal to that used to prepare the EBR solution) during 20 days (days 10–30 after the start of the experiment).

Simulated acid rain preparation and application

The stock solution of the SAR was prepared based in studies that addressed the characteristics and trends of acid precipitation found in Brazil, in cities such as São Paulo, Cuiabá, Minas Gerais and Manaus, where rainfall composition is acid in dry periods due to industrial activity, burning of coal and use of fossil fuels (Honório et al. 2010; Dias et al. 2012; Facchini Cerqueira et al. 2014), and previous tests in rice plants. To simulate the simulated acid rain (SAR), H_2SO_4 0.5 M (pH 3.0) or Milli-Q water (pH 6.0) was sprayed two times (26^{th} and 28^{th} day after the start of the experiment). On day 30 of the experiment, physiological and morphological parameters were measured for all plants, and leaf tissues were harvested for anatomical and biochemical analyses.

Experimental design

The experiment was randomized with four treatments, being two simulated acid rain (0 and 0.5 M H_2SO_4, described as – SAR and + SAR, respectively) and two concentrations of brassinosteroids (0 and 100 nM EBR, described as – EBR and + EBR, respectively). Twelve replicates for each one of the four treatments were conducted, yielding 48 experimental units used in the experiment, with three plants in each unit, used to examine the plant parameters.

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.

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) and quantified in spectrophotometer (model UV-M51; Bel Photonics). 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 mM ascorbate, 0.1 mM EDTA, and 1.0 mM hydrogen peroxide was mixed with 0.2 ml of supernatant, and the absorbance was measured at 290 nm (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 (hydrogen peroxide and malondialdehyde) were extracted as described by (Wu et al. 2006). Briefly, a mixture for extraction of hydrogen peroxide and malondialdehyde 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).

Measurements of morphological parameters

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

Measurements of anatomical parameters

Samples were collected from the middle region of the leaf limb of fully expanded leaves. Subsequently, all collected botanical material was fixed in FAA 70 for 24 hours and dehydrated in ethanol and embedded in historesin Leica™ (Leica, Nussloch, Germany). Transverse sections with a thickness of 5 μm were obtained with a rotating microtome (model Leica RM 2245, Leica Biosystems), stained with toluidine blue (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). Wax extraction (EW) was based on the recommendations of Damato et al. (2017) with modifications. In individual pre-weighed flasks, 1 cm^2 fragments of the middle third of the leaf were immersed in 2 mL chloroform for 30 seconds. The obtained extract was placed in a water bath at 60°C until the total evaporation of chloroform and then weighed. Wax quantification was expressed by the amount of wax per unit leaf area (mg cm^{-2}).

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), using the Assistat software. Standard deviations were calculated for each treatment.

Results

Maintenance of membrane integrity of chloroplasts after treatment with EBR

The photosynthetic pigments presented decreases in Chl *a*, Chl *b*, Total Chl and Car and increases in Chl *a*/Chl *b* and Total Chl/Car ratios in plants exposed to SAR (Table 1). However, the addition of EBR caused significant improvements in Chl *a*, Chl *b*, Total Chl and Car levels of 11%, 63%, 21% and 57%, respectively, and contributed to significant decreases in Chl *a*/Chl *b* and Total Chl/Car of 32% and 22%, respectively.

Improvements in the capture of light energy induced by the EBR

Plants submitted to SAR presented negative interferences on the chlorophyll fluorescence, resulting in an increase in F_0 and reductions in F_v/F_m, F_m and F_v (Fig. 1). However, treatment using SAR combined with EBR provided increases in F_v/F_m of 4% and not significant increases ($P \geq 0.05$) in F_m, F_v of 3% and 7%, respectively, and a decrease in F_0 of 8%, when compared to treatment with SAR (+ SAR) in the absence of EBR (-EBR). In addition, plants exposed to SAR suffered reductions in Φ_{PSII}, ETR and q_P and increases in NPQ, EXC and ETR/P_N (Table 1). Treatment + SAR and + EBR had increases in Φ_{PSII}, ETR and q_P of 30%, 24% and 13%, respectively, and reductions in NPQ and EXC of 23% and 2%, respectively, when compared to the same treatment without EBR. In relation to ETR/P_N, the treatments without and with EBR did not have significant modifications.

EBR positively modulated stomatal anatomy

SAR caused significant increases in PDS and EDS values (adaxial and abaxial faces), reductions ($P \geq 0.05$) in SD and SF values (adaxial and abaxial faces) and decrease in TD (adaxial face) (Table 2). However, the treatment under SAR combined with EBR presented increases ($P \geq 0.05$) of 2% and 4% in SD (abaxial) and SF (abaxial) and 8% in TD, respectively, and reductions in PDS (4%) and EDS (10%) on the adaxial face, compared to the same treatment without EBR.

EBR attenuated the non-stomatal limitations in plants exposed to SAR

The SAR promoted changes in gas exchange, with significant reductions in P_N, P_N/C_i, g_s and WUE, besides increases in C_i and E $P \geq 0.05$ (Table 1). In contrast, treatment with SAR and EBR had significant increases in P_N, P_N/C_i, g_s and WUE corresponding to 29%, 39%, 15% and 25%, respectively, and reductions of 6% and 3% in C_i and E ($P \geq 0.05$), respectively, when compared to treatment + SAR and – EBR.

EBR positively modulated the leaf anatomy

Plants submitted to SAR presented decreases for ETAd ($P \geq 0.05$), ETAb, EWL, MT, LAA and BCD. However, plants exposed to SAR and EBR had increases of 5%, 9%, 48%, 14%, 100% and 12% in ETAd ($P \geq 0.05$), ETAb, EWL, MT, LAA and BCD, respectively, comparing with same treatment without EBR (Fig. 2, Fig. 3 and Table. 3).

Benefits on the antioxidant system induced by EBR

The activities of antioxidant enzymes increased after spraying with SAR (Fig. 4). Significant increases in SOD, CAT, APX and POX of plants sprayed with SAR and EBR were 12%, 17%, 17% and 33%, respectively, when compared to the similar treatment without EBR.

EBR promoted decreases in the ROS concentrations and stress indicators

The oxidant compounds (O_2^-, H_2O_2) and stress indicators (EL and MDA) increased in plants under SAR stress (Fig. 5). However, in the combination + SAR and + EBR the O_2^-, H_2O_2, MDA and EL levels decreased significantly of 18%, 22%, 13% and 26%, respectively, in relation to treatment + SAR and – EBR.

EBR reduced the deleterious effects caused by SAR on roots

The growth increased in SDM and decreased in RDM and TDM ($P \geq 0.05$) in plants exposed to SAR (Fig. 6). Treatment + SAR and + EBR had additive effects on SDM, RDM and TDM of 20%, 11% and 18%, respectively. While the treatment - SAR and + EBR showed increases in the SDM, RDM and TDM of 22%, 15% and 20%, respectively, when compared to equal treatment without the EBR.

Discussion

EBR application attenuated the impact promoted by the SAR on photosynthetic pigments (Chl *a*, Chl *b*, Total Chl and Car), due to a combined action. Our results suggested that EBR mitigated the damages on tilacoid membranes (Dobrikova et al. 2014) and improved the synthesis of the enzymes involved in chlorophyll production (Siddiqui et al. 2018b) confirmed by reductions in ROS and increase in Total Chl. Debnath et al. (2018) evaluating two *Solanum lycopersicum* cultivars under SAR spray (pH 2.5 and 3.5) for 20 days detected reductions in Chl *a*, Chl *b*, Total Chl and Car. Ramlall et al. (2015) described that leaves exposed to SAR lose the green pigmentation and develop yellow spots, which, to acording Hu et al. (2019) may be associated with a reduction in the nutrient content in tissues, especially magnesium (Mg^{2+}). Peng et al. (2019) studying the Mg^{2+} remobilization in *Oryza sativa* found that deficiency of this element causes a reduction in biosynthesis and/or pigments degradation, because Mg^{2+} is essential for the stability of the chlorophyll molecule, its lack induces chlorosis, found in this study. Siddiqui et al. (2018a) studying two forms of BRs (EBR and HBL) reported that EBR application was efficient and increased the photosynthetic pigments, probably due to increases in Mg^{2+} and K^{+} contents, favoring the light assimilation and stomatal mechanism. Study conducted by Ahmad et al. (2018) found that the leaf application of EBR (10 M^{-6}) in *Cicer arietinum* plants treated with 15 and 30 μM $HgCl_2$ increased Chl *a*, Chl *b*, Total Chl and Car levels, exerting important role in light absorption and maintenance of plant growth.

Plants sprayed with EBR and subjected to SAR had mitigated interferences on F_0, F_v, F_m and F_v/F_m, in which these results demonstrate the potential of EBR to alleviate damages linked to photosynthetic apparatus due to control the photoinhibition and positively to stimulate the photoprotection (Fang et al. 2019). Photoinhibition is intensified by the increased ROS, which can damage the photosynthetic apparatus, especially the PSII (Gururani et al. 2015), so the balance between uptake and distribution of light energy is essential to maintain the adequate electron flow, avoiding imbalances that can lead to overproduction of reactive oxygen species (ROS) (Derks et al. 2015), provoking damages on electron acceptor (PQ) from PSII (Czarnocka and Karpiński 2018). In general, the F_v/F_m and F_0 are sensitive to environmental stresses and decreases in F_v/F_m cause negative impacts on the functioning of PSII reaction centers (Liang et al. 2015). Debnath et al. (2018a) reported increase in F_0 (25%) and reduction in F_v/F_m (3%) evaluating *S. lycopersicum* plants sprayed with 20 mL SAR containing sulfuric acid (H_2SO_4) and nitric acid (HNO_3) under pH 2.5 during 17 days. Our results corroborate the study conducted by Li et al. (2015) detecting lower photoinhibition after EBR application (0.1 μM), being connected to increase in F_v/F_m, improving the excitation energy distribution and antioxidant system to neutralize ROS in *Capsicum annuum* seedlings under chilling stress.

EBR also had positive effects on fluorescence parameters, confirmed by increases in Φ_{PSII}, ETR and q_P, being probably minimized the damages on electron transport chain, corroborated by the positive effects promoted by the EBR on F_0, F_v, F_m and F_v/F_m, previously described in this study. Increments obtained after EBR spray on q_P and Φ_{PSII} with broad reflections on electron transport (ETR), NADPH and ATP productions will be metabolized in the Calvin Cycle (Jiang et al. 2012a, b). Leaf treatment with EBR resulted in reductions in NPQ and EXC of plants exposed to SAR, demonstrating the effectiveness of this steroid, improving the heat dissipation linked to excessive energy, protecting the plants against damages caused by the ROS (Goss and Lepetit 2015). Chen et al. (2013) found decreases in Φ_{PSII}, ETR and q_P of *Liquidambar formosana* and *Schima superba* plants sprayed with 5 mL of three SAR types (H_2SO_4, HNO_3 and H_2SO_4/HNO_3 pH 3.0) for 4 consecutive days, suggesting damages to the electron transport chain in PSII. Additionally, Yang et al. (2018) evaluating the gene expression in *Glycine max* in relation to SAR (mixture of HNO_3 and H_2SO_4, pH 2.5) described inhibition of the metabolic activities linked to light-dependent reactions in PSII, directly affecting the efficiency of the Cycle Calvin. Ahammed et al. (2012) investigating the effects of the exogenous EBR (50 nM and 5 nM) on *S. lycopersicum* grown under hydroponic conditions and under different stress levels for phenanthrene (PHE) and pyrene (PYR) (0, 10, 30, 100 and 300 μM) reported increases in q_P and Φ_{PSII}.

EBR alleviated the negative effects induced by SAR on stomatal characteristics (SD, TD, PDS, EDS, and SF). The increases in SD and SF confirm the efficacy of EBR mitigating the limitations on stomatal movements during abiotic stress situations (Wang et al. 2015). This steroid regulates the *PAG1* and *SPCH* genes involved in development and opening of stomatal (Fuentes et al. 2012; Casson and Hetherington 2012; Yang et al. 2014; Chen et al. 2019), contributing to increases verified in gas exchange, more specifically g_s, WUE and *E*. The reductions detected in PDS and EDS in plants exposed to SAR and EBR aiming to reduce the uptake of SAR by the stomata, maintaining the CO_2 influx and H*2*O efflux during gas exchange. The EBR promoted reductions in stomatal dimensions (PDS and EDS), with reflections on DS. This results reveal a strategy to minimize the entry of SAR by the ostioles (Daszkowska-Golec and Szarejko 2013; Martins et al. 2015; Woltering and Paillart 2018) and to maintain gas exchange without significant CO_2 losses during photosynthesis (P_N) and transpiration process (*E*) (Lawson and Blatt 2014; Bertolino et al. 2019).

In relation to trichomes, increases in TD after EBR spray contributed to the mechanism of leaf protection against damages caused by SAR, acting as the first line of defense in this organ (Liu et al. 2019), in which this structure positively modulates the transpiration (*E*) and reduces leaf exposure to ultraviolet radiation (Kim 2019). Additionally, Galdon-Armero et al. (2018) described that plants with a higher proportion of trichomes have higher WUE, when compared to the numbers of trichomes and stomata. Dias et al. (2010) studying the SAR effects (H_2SO_4 at pH 6.0 and 3.0) on *Phaseolus vulgaris* plants for 10 days reported that SAR at pH 3.0 decreased the number, length and eliminated the apex of the trichomes, also causing decreases in the number of stomata and inadequate functioning of photosynthetic machinery.

Plants pretreated with EBR and sprayed with SAR had beneficial changes in gas exchange, confirmed by increases in P_N, g_s and WUE. In general, these increases are related to actions promoted by EBR on photosynthetic apparatus, more specifically improving the efficiency of PSII, being corroborated with our results obtained in Φ_{PSII}, ETR and q_P. In parallel, the increases in P_N, g_s and WUE has been reported that the EBR improves stomatal regulation, reducing C_i, verified in our results with plants treated with EBR, reinforcing its roles linked to supplementation and distribution of CO_2 in intercellular spaces, despite damages caused by SAR in leaf tissue (Khamsuk et al. 2018). Decrease in P_N after SAR is related to the decline in g_s, or lower Rubisco enzyme activity (Ahammed et al. 2013b), resulting in a reduction in WUE, observed in our study. A study conducted by Yuan et al. (2017) found that leaf pretreatment with BRs (0.1 mg L^{-1}) in two *Setaria italica* cultivars herbicide-sprayed promoted increases in P_N of 27-31%, as well as these authors reported increases in Chl *a*, Chl *b* and Car, contributing to the increase in P_N. Ahammed et al. (2012a) evaluating the effects of EBR (0.1 μM) in *S. lycopersicum* plants exposed to intoxication by phenanthrene (300 μM PHE), being verified that the EBR acted improving the tolerance, inducing increases in P_N and g_s. Rady (2011) found that the EBR spray (5 μM) on *P. vulgaris* seedlings exposed to NaCl (150 mM) and/or $CdCl_2$ (1 mM Cd^{2+}) increased WUE, providing benefits on water relation.

EBR application in plants under SAR stimulated increases in ETAd, ETAb, EWL, MT, LAA and BCD, suggesting that this steroid minimized the stress caused by SAR, due to modulation of leaf anatomical structures with protective papers (Zhiponova et al. 2013). ETAd and ETAb make up protective epidermal layers of plant organs, which added to the cuticle and trichomes soften water losses, regulate gas exchange, control temperature and light input (Glover et al. 2016; Nguyen et al. 2018; Trivedi et al. 2019), the results obtained in these variables are corroborated with the improvements promoted by EBR in WUE, *E* and P_N in this study. Andrade and Silva (2017) reported damage to the epicuticular wax adaxial epidermis region, sagging and epidermal cell changes in *Paubrasilia echinata* and *Libidibia ferrea* leaves sprayed daily (15 minutes) with SAR containing H_2SO_4 (pH 3.0; deionized water pH 6.0) for 10 days, but contrary to our results on ETAd under SAR, these authors reported increases in adaxial leaf tissue thickness in *Libidibia ferrea*.

In addition, EBR minimized epicuticular wax (EWL) loss and buliform cell turgence (BCD), confirming internal improvements in water retention in the leaves, which were slightly curled after spraying with SAR. Additionally, the increases in MT and LAA are related to the fact that EBR acted to improve mesophyll and aerenchyma cells, increasing membrane integrity and CO_2 utilization, because BRs also play important roles in mediating remodeling of cell wall in grasses under abiotic stress (Rao and Dixon 2017), this corroborating with our results in C_i, SD, SF, PDS and EDS. Ju et al. (2017) evaluating SAR-sprayed *O. sativa* plants (mixture of H_2SO_4 and HNO_3) under four pH values (2.0, 3.0, 4.0 and 6.5) for 7 days, detected slight damage to mesophyll structures under SAR pH 3.0 and severe destruction to mesophyll cells under SAR pH 2.0 when compared to the control treatment (pH 6.5).

EBR attenuated the negative effects induced by SAR, which were confirmed by increases in antioxidant enzyme activities (SOD, CAT, APX and POX). Increments of these enzymes suggest that this steroid improved antioxidant metabolism in plants exposed to SAR, reducing ROS and minimizing oxidative damages to plant cells (Sharma et al. 2012; Xia et al. 2018). Ahammed et al. (2017) described that EBR stimulates antioxidant metabolism, in which the enzymes SOD, CAT, APX, and POX are responsible for the plant defense system against overproduction of ROS during abiotic stresses (Gautam et al. 2017).

SOD is the first enzyme in this antioxidant defense line, acting on O_2^- dismutation to H_2O_2, subsequently CAT, APX and POX will convert H_2O_2 into H_2O and O_2, alleviating the excess ROS in plants (Zhou et al. 2018). Ren et al. (2018) investigating the mechanisms involved in *Oriza sativa* tolerance to SAR under three pH (3.5, 3.0 and 2.5) found that SAR under pH 3.5 stimulated and promoted positive modulation of SOD, CAT and POX, reducing the oxidative damages. In other hand, plants submitted to SAR pH 3.0 or 2.5, these enzymes could not control the excess of MDA, O_2^- and H_2O_2, causing intense oxidative damages. Sharma et al. (2016) reported positive modulation linked to gene transcription of antioxidant enzyme precursors and significant increases in the activities of antioxidant enzymes (SOD, CAT, APX), after EBR treatments (0.1 μM, 0.01 μM and 0.1 nM), contributing to the removal of ROS in *O. sativa* seedlings stressed with Cr (0.5 mM). Additionally, Hayat et al. (2012) reported that EBR and HBL applications (10^{-8}M) promote increases in CAT, SOD and POX activities, alleviating the oxidative stress in *S. lycopersicum*.

Plants exposed to SAR and EBR had reductions in oxidizing compounds (O_2^-, H_2O_2, MDA and EL), and these results were connected to the antioxidant system. Pretreatment with the steroid stimulated the activities of antioxidant enzymes previously detected in this research, inducing decrease in the membrane permeability, affecting O_2^- and H_2O_2 concentrations in cell level, with consequent reduction in lipid peroxidation products, such as MDA and EL (Sharma et al. 2016). According to Dong et al. (2019), plants under stress conditions can suffer oxidative damages, resulting in lipid peroxidation of membranes, estimated by MDA. ROS (O_2^- and H_2O_2) are part of the normal metabolism of plant chloroplasts, mitochondria and peroxisomes (Berni et al. 2019) functioning as signal transduction molecules that regulate plant acclimatization processes to different environmental conditions (Choudhury et al. 2017).

However, high levels ROS can lead to biomolecule oxidation, membrane decomposition, enzyme inactivation and alterations in the gene expressions (Singh et al. 2019), in which the H_2O_2 is considered the ROS most stable and toxic (Liang and Wang 2013). Ma et al. (2019) reported that SAR spray (H_2SO_4 and HNO_3, ratio 5:1) under different pH values (4.5, 3.5 and 2.5) on *Cinnamomum camphora* plants increased the content of ROS, specifically O_2^- and H_2O_2, causing increases in antioxidant enzyme activities to minimize oxidative damages. Ahammed et al. (2013c) working with *Lycopersicon esculentum* plants treated with 100 nM EBR and exposed to polychlorinated biphenyl organic pollutants (PCBs) detected reductions in O_2^-, H_2O_2 and MDA, reinforcing that EBR improves ROS homeostasis. Fariduddin et al. (2015) detected reduction of EL EBR-mediated (10^{-8} M) in *Brassica juncea* under Mn toxicity (0, 3, 6, or 9 mM), in which exogenous EBR application decreased EL by 25 % under 9 mM Mn, maintaining membrane integrity and stability due to antioxidant system action. In addition, Ahammed et al. (2015) found that neutralization of ROS EBR-induced protects the chloroplast structures.

EBR mitigated the declines SAR-induced on growth parameters (TDM and RDM), reflecting directly on SDM, being attributed the direct and/or indirect interferences of this steroid on chlorophyll fluorescence (F_v/F_m and Φ_{PSII}), gas exchange (P_N e g_s), antioxidant defense system (SOD, CAT, APX and POX) and photosynthetic pigments (Chl *a*, Chl *b* and Car). The benefits promoted by EBR detected in RDM of this study, had a positive impact on the biomass of plants exposed to SAR. Liang and Zhang (2018) working with *Glycine max* plants under SAR stress SAR (H_2SO_4 and HNO_3 3:1), verified that under pH 4.5 and 3.0 the SAR decreased root biomass. While Sharma et al. (2019) investigating the EBR role (0.01 μM) in *O. sativa* under alkaline stress induced by $NaHCO_3$ (25 mM) found that the EBR application reduced the negative interferences on growth components, more specifically the total dry weight, as well as root and coleoptile lengths.

Conclusion

This research revealed that EBR attenuated the deleterious effects of SAR on rice plants, confirmed by improvements in chlorophyll fluorescence and gas exchange. These benefits are related to increases in electron transport, assimilation and distribution linked to CO_2. Additionally, this organic steroid improved the antioxidant system, confirmed by the increases in activities of the enzymes superoxide dismutase, catalase, ascorbate peroxidase and peroxidase, which favored the elimination of reactive oxygen species, reducing the damages on chloroplast structures. Finally, EBR actions mitigated SAR by positively modulating the leaf epidermis, increasing the trichomes, epicuticular wax and aerenchyma area. Trichomes and wax contribute to the formation of a protective barrier to damages caused by SAR, while increases in aerenchyma area higher CO_2 reserve available during photosynthetic process. These results provide clear evidences that EBR is efficient to protect rice plants exposed to SAR, modulating physiological and anatomical processes.

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Conflict of interest

The authors declare that they have no competing interests.

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Figures

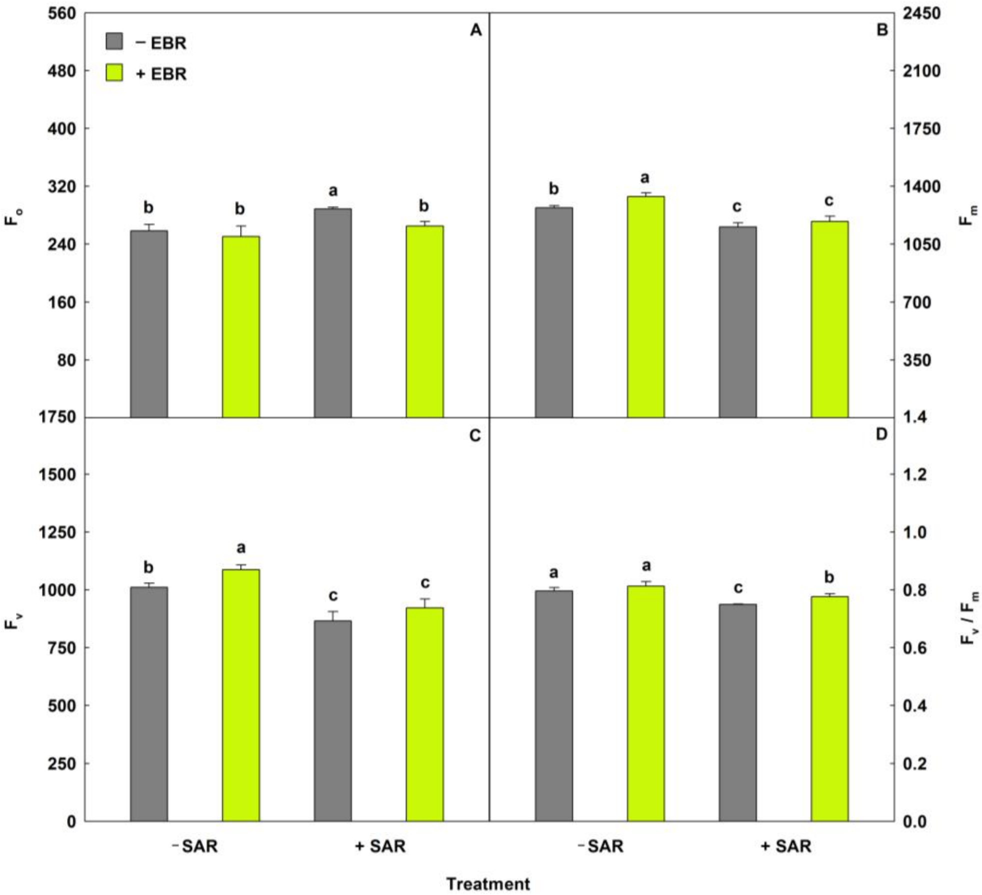

Fig. 1. A) Minimal fluorescence yield of the dark-adapted state (F0), B) maximal fluorescence yield of the dark-adapted state (Fm), C) variable fluorescence (Fv) and D) maximal quantum yield of PSII photochemistry (Fv/Fm) in rice plants treated with EBR and exposed to simulated acid rain. Bars with different letters indicate significant differences from the Scott-Knott test (P<0.05). Bars corresponding to means from five repetitions and standard deviations.

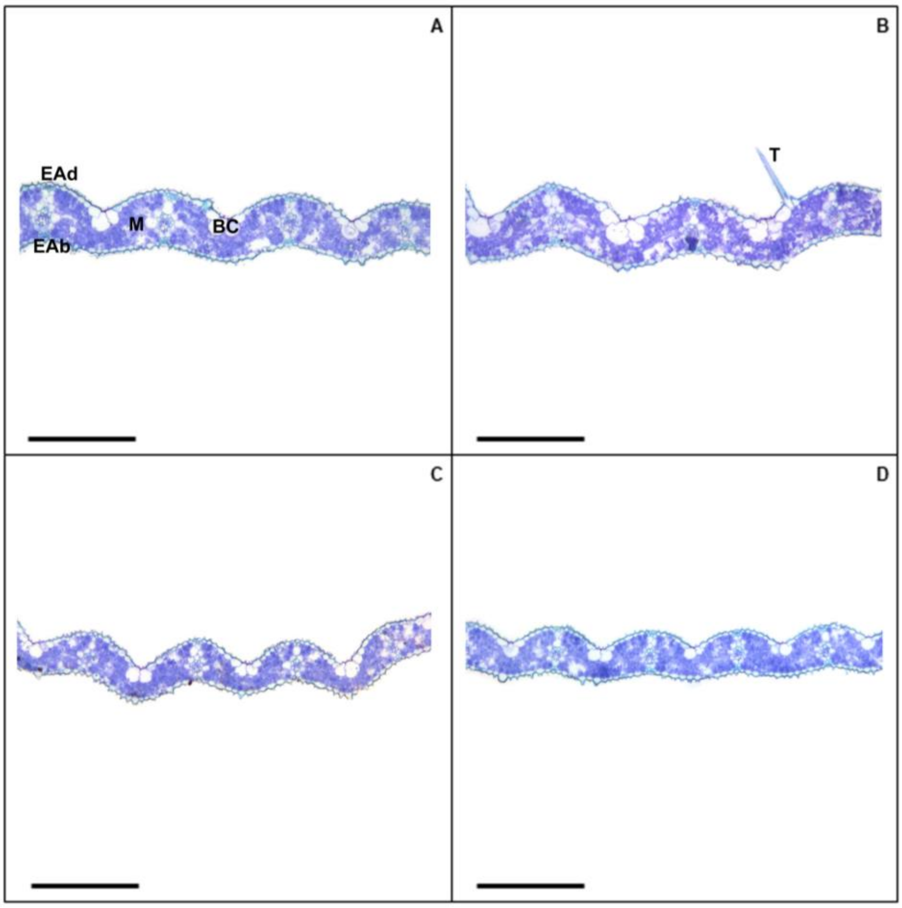

Fig. 2. Cross section of leaf mesophyll of rice plants treated with EBR and exposed to simulated acid rain. − SAR / −EBR (A), − SAR / + EBR (B), + SAR / − EBR (C) and + SAR / + EBR (D). Legends: BC = Bulliform cell; EAb = Abaxial epidermis; EAd = adaxial epidermis; M = Mesophyll; T = Trichome. Bars: 150 µm.

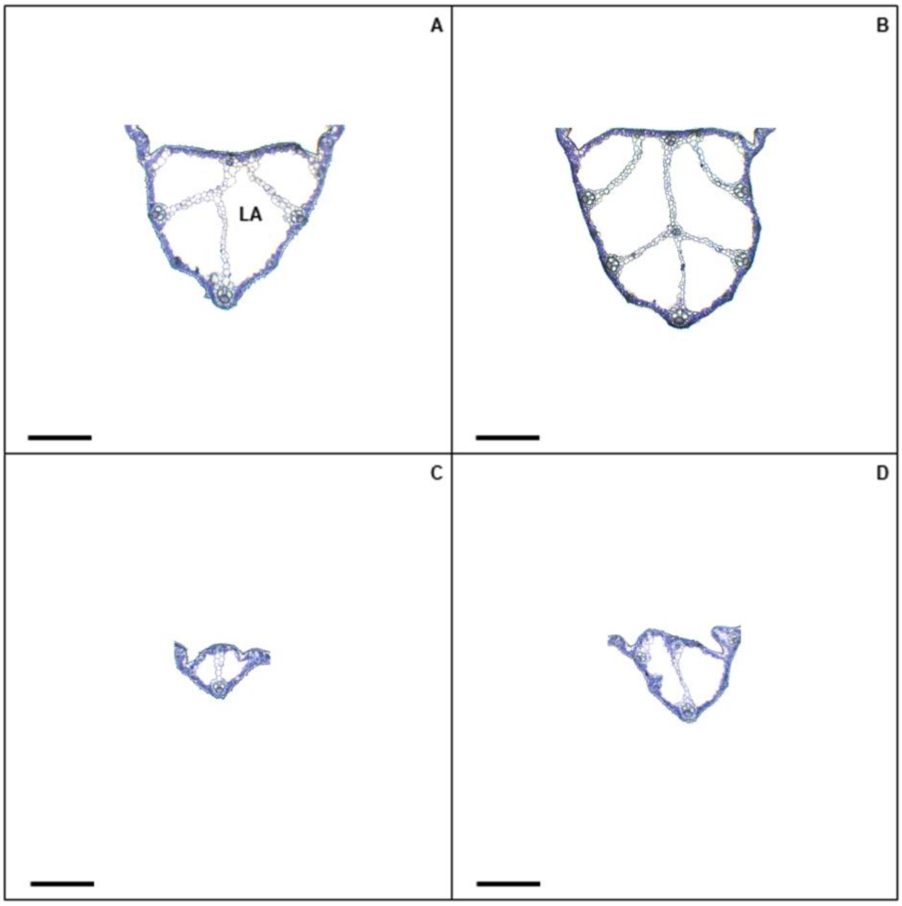

Fig. 3. Cross section of central leaf vein of rice plants treated with EBR and exposed to simulated acid rain. − SAR / − EBR (A), − SAR / + EBR (B), + SAR / − EBR (C) and + SAR / + EBR (D). Legends: LA = leaf aerenchyma; EAb = Abaxial epidermis; EAd = adaxial epidermis. Bars: 500 µm.

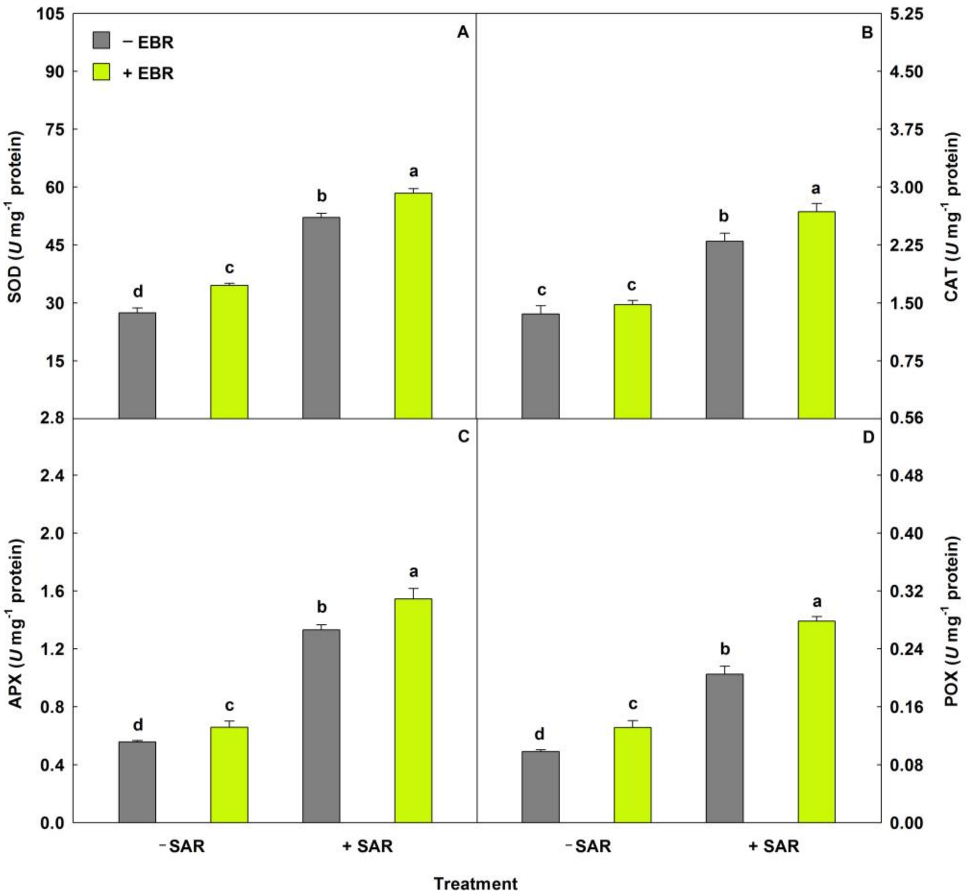

Fig. 4. A) Activities of superoxide dismutase (SOD), B) catalase (CAT), C) ascorbate peroxidase (APX) and D) peroxidase (POX) in rice plants treated with EBR and exposed to simulated acid rain. Bars with different letters indicate significant differences from the Scott-Knott test (*P*<0.05). Bars corresponding to means from five repetitions and standard deviations.

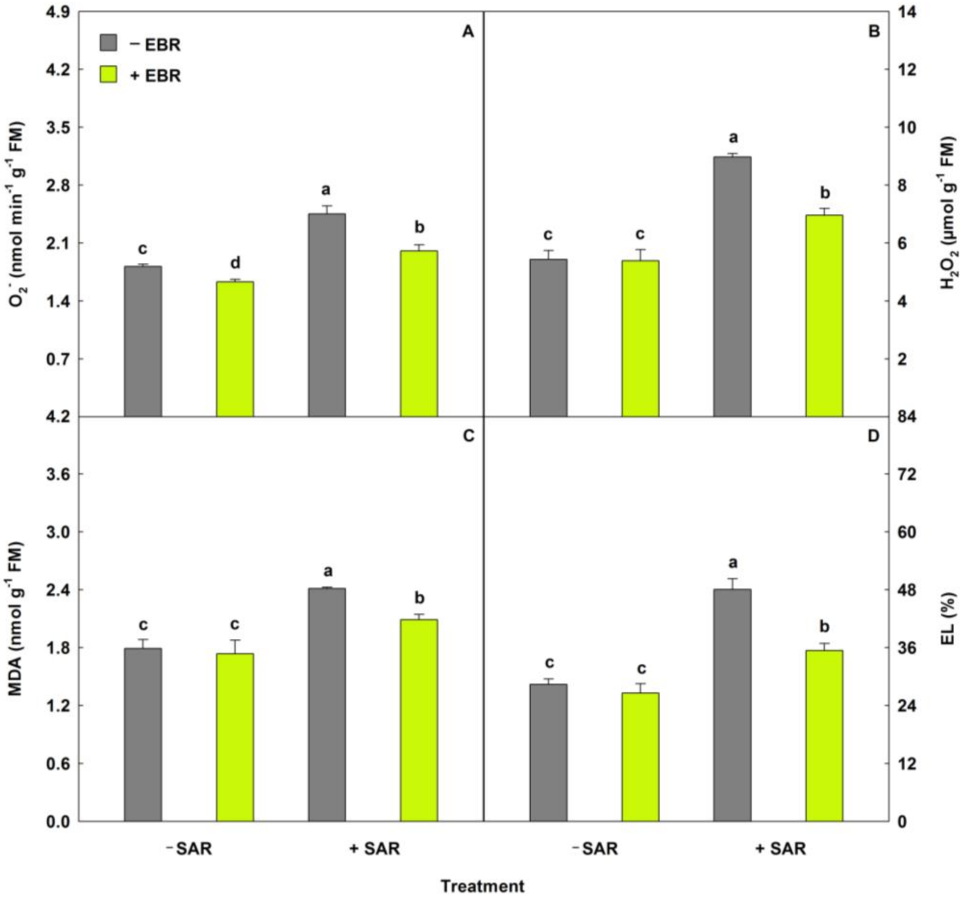

Fig. 5. A) Superoxide (O_2^-), B) hydrogen peroxide (H_2O_2), C) malondialdehyde (MDA) and D) electrolyte leakage (EL) in rice plants treated with EBR and exposed to simulated acid rain. Bars with different letters indicate significant differences from the Scott-Knott test (P<0.05). Bars corresponding to means from five repetitions and standard deviations.

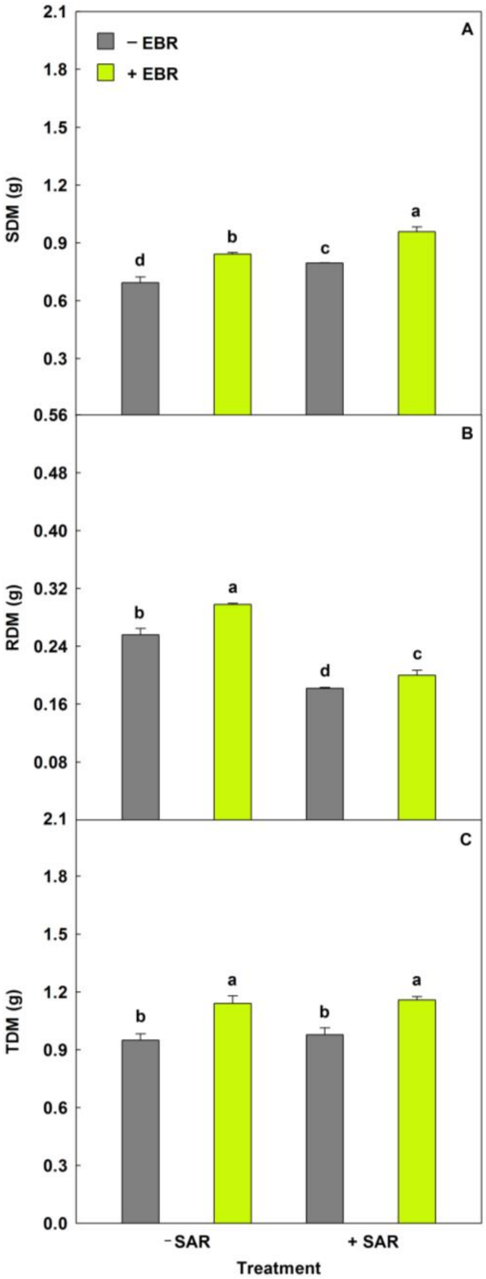

Fig. 6. A) Shoot dry matter (SDM), B) root dry matter (RDM) and C) total dry matter (TDM) in rice plants treated with EBR and exposed to simulated acid rain. Bars with different letters indicate significant differences from the Scott-Knott test ($P<0.05$). Bars corresponding to means from five repetitions and standard deviations.

Tables

Table 1. Photosynthetic pigments, chlorophyll fluorescence and gas exchange in rice plants treated with EBR and exposed to simulated acid rain.

Photosynthetic pigments							
SAR	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
−	−	8.47 ± 0.28b	3.57 ± 0.02b	12.04 ± 0.27b	0.48 ± 0.03b	2.37 ± 0.09c	25.30 ± 2.06c
−	+	9.79 ± 0.55a	4.30 ± 0.19a	14.09 ± 0.61a	0.57 ± 0.01a	2.28 ± 0.15c	24.89 ± 0.62c
+	−	7.03 ± 0.09d	1.52 ± 0.09d	8.54 ± 0.04d	0.23 ± 0.02d	4.66 ± 0.38a	36.60 ± 3.41a
+	+	7.83 ± 0.36c	2.48 ± 0.12c	10.31 ± 0.32c	0.36 ± 0.02c	3.17 ± 0.26b	28.48 ± 2.19b
Chlorophyll fluorescence							
SAR	EBR	Φ_{PSII}	q_P	NPQ	ETR (µmol m^{-2} s^{-1})	EXC (µmol m^{-2} s^{-1})	ETR/P_N
−	−	0.14 ± 0.01b	0.18 ± 0.01b	0.18 ± 0.01c	20.00 ± 1.18b	0.83 ± 0.01b	1.48 ± 0.13b
−	+	0.15 ± 0.01a	0.21 ± 0.01a	0.15 ± 0.01d	22.40 ± 1.96a	0.81 ± 0.01c	1.42 ± 0.11b
+	−	0.10 ± 0.01c	0.16 ± 0.01c	0.26 ± 0.01a	15.20 ± 0.92c	0.86 ± 0.01a	1.63 ± 0.09a
+	+	0.13 ± 0.01b	0.18 ± 0.01b	0.20 ± 0.01b	18.80 ± 1.23b	0.84 ± 0.01b	1.57 ± 0.08a
Gas exchange							
SAR	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})
−	−	13.58 ± 0.39b	2.19 ± 0.17a	0.25 ± 0.02a	254 ± 13b	6.23 ± 0.49b	0.054 ± 0.003b
−	+	15.76 ± 0.23a	2.32 ± 0.16a	0.27 ± 0.01a	247 ± 15b	6.82 ± 0.44a	0.064 ± 0.005a
+	−	9.30 ± 0.30d	2.30 ± 0.12a	0.20 ± 0.01c	282 ± 13a	4.05 ± 0.17d	0.033 ± 0.002d
+	+	12.01 ± 0.60c	2.38 ± 0.14a	0.23 ± 0.01b	264 ± 10b	5.05 ± 0.44c	0.046 ± 0.001c

Chl *a* = Chlorophyll *a*; Chl *b* = Chlorophyll *b*; Total chl = Total chlorophyll; Car = Carotenoids; Φ_{PSII} = Effective quantum yield of PSII photochemistry; q_P = Photochemical quenching coefficient; NPQ = Nonphotochemical quenching; ETR = Electron transport rate; EXC = Relative energy excess at the PSII level; ETR/P_N = Ratio between the electron transport rate and net photosynthetic rate; P_N = Net photosynthetic rate; *E* = Transpiration rate; g_s = Stomatal conductance; C_i = Intercellular CO_2 concentration; WUE = Water-use efficiency; P_N/C_i = Carboxylation instantaneous efficiency. Columns with different letters indicate significant differences from the Scott-Knott test ($P<0.05$).Values described corresponding to means from five repetitions and standard deviations.

Table 2. Stomatal characteristics in rice plants treated with EBR and exposed to simulated acid rain.

SAR	EBR	SD (stomata per mm^2)	TD (trichome per mm^2)	PDS (μm)	EDS (μm)	SF
Adaxial face						
−	−	387.50 ± 20.72a	87.50 ± 2.89b	6.68 ± 0.41b	11.81 ± 0.51c	0.57 ± 0.05b
−	+	393.75 ± 23.49a	143.75 ± 8.19a	6.47 ± 0.34b	10.63 ± 0.35d	0.61 ± 0.03a
+	−	313.50 ± 20.08b	75.00 ± 4.08d	7.06 ± 0.32a	14.43 ± 0.69a	0.49 ± 0.04c
+	+	316.25 ± 24.27b	81.25 ± 5.87c	6.75 ± 0.26b	13.05 ± 0.71b	0.52 ± 0.02c
Abaxial face						
−	−	356.25 ± 23.10b	*	6.89 ± 0.33c	12.44 ± 0.25c	0.55 ± 0.02b
−	+	381.25 ± 20.66a	*	6.76 ± 0.38c	11.21 ± 0.42d	0.60 ± 0.04a
+	−	312.50 ± 24.55c	*	8.04 ± 0.44a	15.67 ± 0.24a	0.51 ± 0.03c
+	+	318.75 ± 24.56c	*	7.25 ± 0.33b	13.71 ± 0.32b	0.53 ± 0.03c

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 3. Leaf anatomy in rice plants treated with EBR and exposed to simulated acid rain.

SAR	EBR	ETAd (μm)	ETAb (μm)	EWL (mg cm^{-2})	MT (μm)	LAA (mm^2)	BCD (μm)
−	−	9.36 ± 0.44a	10.35 ± 0.49a	13.60 ± 0.19c	56.59 ± 1.61b	0.19 ± 0.01b	28.63 ± 0.21b
−	+	9.92 ± 0.36a	10.87 ± 0.67a	17.24 ± 0.48a	59.12 ± 1.05a	0.23 ± 0.01a	32.41 ± 1.72a
+	−	8.44 ± 0.36b	8.86 ± 0.39c	10.18 ± 0.42d	45.37 ± 1.92d	0.02 ± 0.00d	23.30 ± 0.71d
+	+	8.88 ± 0.64b	9.70 ± 0.54b	15.08 ± 0.36b	51.61 ± 0.59c	0.04 ± 0.00c	26.19 ± 0.69c

ETAd = Epidermis thickness from adaxial leaf side; ETAb = Epidermis thickness from abaxial leaf side; EWL = Epicuticular wax load; 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.