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EXOGENOUS 24-EPIBRASSINOLIDE STIMULATES ROOT PROTECTION, AND LEAF ANTIOXIDANT ENZYMES IN RICE PLANTS LEAD STRESSED: CENTRAL ROLES TO MINIMIZE Pb CONTENT AND OXIDATIVE STRESS

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LIST OF 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

RESUMO

O chumbo (Pb) é um poluente ambiental que afeta negativamente as plantas de arroz, causando danos ao sistema radicular e às estruturas do cloroplasto, além de reduzir o crescimento. O 24-Epibrassinolídeo (EBR) é um regulador de crescimento vegetal com alta capacidade de modular o metabolismo antioxidante. O objetivo desta pesquisa foi investigar se a aplicação exógena de EBR pode mitigar danos oxidativos em plantas de arroz estressadas com Pb exógeno, medir estruturas anatômicas e avaliar respostas fisiológicas e bioquímicas ligadas ao metabolismo redox. O experimento foi randomizado com quatro tratamentos, incluindo dois tratamentos com chumbo (0 e 200mM $PbCl_2$, descritos como - Pb e + Pb, respectivamente) e dois tratamentos com brassinoesteróide (0 e 100 nM EBR, descritos como - EBR e + EBR, respectivamente). Os resultados revelaram que as plantas expostas ao Pb apresentaram alterações significativas, mas o EBR amenizou as interferências negativas, conforme confirmado pelas melhorias nas estruturas radiculares e no sistema antioxidante. Esse esteróide estimulou as estruturas radiculares, aumentando a espessura da epiderme (26%) e a área do aerênquima (50%), resultando em maior proteção desse tecido contra os íons Pb^{2+}. Além disso, o EBR promoveu aumentos significativos nas enzimas superóxido dismutase (26%), catalase (24%), ascorbato peroxidase (54%) e peroxidase (63%), reduzindo o estresse oxidativo na maquinaria fotossintética em plantas com estresse de Pb. Esta pesquisa provou que o EBR atenua os efeitos tóxicos gerados pelo Pb nas plantas de arroz.

PALAVRAS-CHAVE: Brassinoesteróide. Poluente ambiental. *Oryza sativa* L. Redox status. Estrutura da raiz.

ABSTRACT

Lead (Pb) is an environmental pollutant that negatively affects rice plants, causing damage to the root system and chloroplast structures, as well as reducing growth. 24-Epibrasinolide (EBR) is a plant growth regulator with a high capacity to modulate antioxidant metabolism. The objective of this research was to investigate whether exogenous EBR application can mitigate oxidative damage in Pb-stressed rice plants, measure anatomical structures and evaluate physiological and biochemical responses connected with redox metabolism. The experiment was randomized with four treatments, including two lead treatments (0 and 200mM $PbCl_2$, described as - Pb and + Pb, respectively) and two treatments with brassinosteroid (0 and 100 nM EBR, described as - EBR and + EBR, respectively). The results revealed that plants exposed to Pb showed significant changes, but the EBR alleviated the negative interferences, as confirmed by the improvements in the root structures and antioxidant system. This steroid stimulated the root structures, increasing the epidermis thickness (26%) and aerenchyma area (50%), resulting in higher protection of this tissue against Pb^{2+} ions. Additionally, EBR promoted significant increases in superoxide dismutase (26%), catalase (24%), ascorbate peroxidase (54%) and peroxidase (63%) enzymes, reducing oxidative stress on the photosynthetic machinery in Pb-stressed plants. This research proved that EBR mitigates the toxic effects generated by Pb in rice plants

KEYWORDS: Brassinosteroids. Environmental pollutant. *Oryza sativa* L. Redox status. Root structures.

SUMMARY

1 CONTEXTUALIZATION

Rice is one of the most important basic crops, it is consumed by more than half of the world population (about more than 3 billion people), and it plays an important role in the entry of mineral nutrients into the food chain (LIU *et al.*, 2018; ZEIGLER; BARCLAY, 2008; SHANTA KARKI *et al.*, 2013). Rice is grown worldwide with a production of around 755 billion tons and among the ten largest producers, nine are from Asian countries, with the exception of Brazil, which occupies the ninth place (FAOSTAT, 2019). In Brazil, with an annual production, based on husks, between 11 and 13 million tons of rice in the last harvests, participates with 78% of Mercosur production (on average from 2009/10 to 2017/18), followed by Uruguay, Argentina and, finally, Paraguay, which in the 2017/18 harvest represented around 6.00% of the total produced by the bloc (SOSBAI, 2018).

The rice cultivation system traditionally occurs in flooded areas that can harbor heavy metals (such as Pb) (MURUGAIYAN et al., 2019), and enter the food chain. This commonly happens in Asian countries that use water from rivers with high levels of Pb (ASHRAF *et al.*, 2018). In Brazil, the entry of Pb in the rice culture can occur in a punctual way, in others words, it happens when there is an irregular dumping of toxic residues, coming from mining, in soils or rivers close to the rice plantation (ANDRADE *et al.*, 2018; MORAES; ANDRADE, 2013; MILANEZ *et al.*, 2013).

Lead (Pb) is a non-essential trace element and has become an environmental contaminant that can be added to the soil by means of phosphate fertilizers, limestone, industrial waste and irregular mining activity, by atmospheric deposition and agrochemical applications, by organic compounds from urban waste recycling and sewage sludge (CAMPOS e*t al.*, 2003; PIERANGELI *et al.*, 2001a). Although there is great concern about the concentration of Pb in agricultural soils, it is not fully known how far it has reached the global rice supply chain (NORTON *et al.*, 2014). In humans, acute exposure to Pb can cause gastrointestinal disorders, kidney damage and neurological effects that can lead to seizures and death (WHO, 2010).

In plants, Pb absorption occurs mainly by the roots and its availability is impacted by several factors including soil pH, redox conditions, cation exchange capacity, soil composition (organic and inorganic ligands levels), biological and microbiological factors (PEREIRA *et al.*, 2020). Pb toxicity in plants causes limited root growth, reduced photosynthesis, overproduction of reactive oxygen species (ROS) that disturb essential cellular structures, alters the activities of antioxidant enzymes and, consequently, affects plant growth and development (ASHRAF; TANG, 2017; RAO *et al.*, 2018).

Brassinosteroids (BR) are a group of plant steroid hormones that were first isolated from Brassica pollen about 40 years ago, since then, about 60 related compounds have been identified, however, brassinolide, 24-epibrassinolide and 28 - homobrassinolide are the most bioactive BR (ANWAR *et al.*, 2018). The occurrence of BRs has been demonstrated in almost all parts of plants, such as pollen, flower buds, fruits, seeds, vascular exchange, leaves, shoots and roots (BAJGUZ; HAYAT, 2009). This class of steroids are used for responses and protection of plants against abiotic stresses such as: salinity (ALI *et al.*, 2008), osmotic stress, heavy metals (BAJGUZ, 2011; KANWAR *et al.*, 2013; ZHOU *et al.*, 2018) drought stress (KHAMSUK *et al.*, 2018), water flooding (OTIE *et al.*, 2019) and thermal stress (HU *et al.*, 2010; ZHANG *et al.*, 2013).

24-Epibrassinolide (EBR) is one of the most active and used forms of BRs and its exogenous application can reduce the accumulation of heavy metals in plants, improve growth, increase chlorophyll content, photosynthetic efficiency, improve antioxidant activity , to increase fresh and dry biomass and yield (ZHOU *et al.*, 2018). Therefore, our hypothesis was based on the damage caused by excess Pb and the possible benefits of EBR on plant metabolism. The objective of this research was to investigate the anatomical, biochemical and physiological responses of the application of EBR in rice plants under Pb stress.

2 LITERATURE REVIEW

2.1 Rice

2.1.1 *General aspects*

The cultivated rice (*Oryza sativa* L.) it is an annual and herbaceous species of the class Liliopsida (Monocotiledônea), included in the order Poales, of the family Poaceae, with the genus Oryza (AWAN *et al.*, 2017). It is classified in the group of plants C_3, and due to the presence of aerenchyma (in the stem and roots) adapts to the aquatic environment, thus allowing the passage of oxygen from the air to the rhizosphere (SOSBAI, 2016). Rice has its development cycle divided into three main phases: the seedling, the vegetative and the reproductive phase, being that the duration of each phase depends basically on some factors such as cultivar, sowing time, region of cultivation and soil fertility conditions. In general, the cycle varies from 100 to 140 days for most cultivars grown in flooded systems, while upland rice cultivars have a cycle lasting between 110 and 155 days (AWAN *et al.*, 2017).

The species *O. sativa* has two main subspecies (*japonica* and *indica*) that are directly related to wild varieties and different from each other, with two distinct domestication regions: *japonica* in China and *indica* in Índia (GROSS; ZHAO, 2014). Thus, the origin of rice was in the Asian continent, in the Assam-Meghalaya area in India and in the river valley region of southeastern China (PRASAD *et al.*, 2017).

Rice has the capacity to emit new roots until the fruiting stage, increasing the nutrient absorption capacity, which allows cultivation even in poor soils (ZHANG *et al.*, 2019). The roots, in turn, emit a large number of stems formed by a series of knots and internodes, in which a leaf and a yolk are inserted. Clumps range from three to fifty stems and each stalk ends with an inflorescence, giving rise to an open panicle, which is erect in flowering and decumbent in maturation. The flowers are hermaphroditic and the androceu has six stamens that come together in two whorls of three stamens each, while the gynecium has a single pistil, the ovary contains a single egg and the stigma, formed by three small wolves, is sessile (SOSBAI, 2018).

2.1.2 *Rice production and consumption*

Rice is one of the main cereal crops grown worldwide and one of the most important foods for human nutrition, thus making up the diet of more than three billion people (SHANTA KARKI, 2013; HAMID *et al.*, 2019). The average world consumption of rice is 70 kg / person / year, however in Asian countries (where 91% of this grain is produced) the averages are higher with 84.4 kg / person / year. In Latin America, an average of 30kg / person / year is consumed, with Brazil standing out as a major consumer with 45kg / person / year (SOSBAI, 2016).

In 2019, world rice production reached around 755 million tons, highlighting nine Asian countries that make up 90.6% of world production and, in ninth place, Brazil with around 11 million tons (FAOSTAT, 2019). Rice production in Brazil consists of two types of cultivation techniques: irrigated and rainfed (also called upland). Even with a high cost (in land preparation and inputs), irrigated rice is responsible for 88.2% of the national production and is generally produced in the southern region (mainly in Rio Grande do Sul) (SOSBAI, 2018). Its cultivation cycle has the presence of a water slide. On the other hand, upland rice has low production costs, supports soil acidity well and has been used to recover degraded pastures and crop rotation (MOURA NETO *et al.*, 2002). This type of cultivation makes up 11.8% of the national production and is produced mainly in the northern region of the country (EMBRAPA ARROZ E FEIJÃO, 2016).

In the State of Pará, upland rice cultivation predominates with 94,508 tons of production, corresponding to 0.91% of the country's production, placing it as the tenth largest producer (IBGE, 2019). However, in 2011, irrigated rice began to be grown in the city of Cachoeira do Arari / PA (Marajó archipelago) on 3,500 hectares, generating two annual harvests (FAPESPA, 2017). In 2019, the city of Cachoeira do Arari produced 27,460 tons of rice, thus making it the first largest producer in the state with 29.06% of total production, followed by Altamira (9.95%), Santana do Araguaia (6.76 %), Ulianópolis (6.61%) and Paragominas (6.35%) (IBGE, 2019).

2.1.3. *Stress conditions*

Despite the culture of rice has undergone years of genetic improvement that has allowed a gain in productivity, still resistance to a variety of soil conditions, biotic and abiotic stress (BRESEGHELLO *et al*., 2011). Drought is one of the most significant stresses that decreases rice production, and depending on the intensity of the drought, what season it is in, and what ecosystem it is in, it is necessary to cultivate rice varieties with different levels of tolerance to drought in different areas (RAMAN *et al*., 2012). Subramanian (2012) says that increasing the tolerance of crops to water scarcity would be the most economical approach to improving productivity and minimizing agricultural use of freshwater resources.

Several elements of the soil are responsible for damage to agricultural production, such as the presence of heavy metals, such as Pb (one of the best known and most dangerous to human health) that can affect the roots and aerial parts of rice (IGALAVITHANA *et al.*, 2017). Pb also affects growth in rice seedlings, triggers oxidative stress and affects nutrient absorption (KHAN *et al.*, 2018). The limit concentration of Pb in rice is 0.2 $\mu g g^{-1}$ per grain of rice (NORTON *et al.*, 2014). Fangmin *et al*. (2006) carried out a study with more than 250 rice samples from southeastern China and found that the Pb concentration in some rice grains reached 1,136 $\mu g g^{-1}$, exceeding the acceptable limit and explains that this high concentration can be due to local anthropogenic sources. Fu *et al*. (2008) inspected Pb in rice produced in an area where electronic waste is recycled and found that the average for Pb values in rice grain was 2,042 $\mu g g^{-1}$.

Saline stress is another factor that can interfere with rice productivity and is classified as a crop vulnerable to salt in its seedling stages (LUTTS *et al*., 1995). In general, high salinity leads to severe inhibition of plant growth and development, damage to the membrane, ionic imbalances due to the accumulation of Na^+ and Cl^-, increased lipid peroxidation and increased production of ROS (KUMAR *et al*., 2013). Heat stress is one of the reasons for the reduction in agricultural production of some cereals such as wheat, rice and barley. For rice, the high temperature affects the flowering phase, leading to spikelet sterility or generates the lack of harvest caused by the inhibition of anther dehiscence, pollen sterility and failure to germinate in the stigma (ZHANG *et al*., 2018).

Another stressor is the pathogenic fungus *Magnaporthe oryzae*, responsible for causing blast disease in rice. This destructive disease affects the leaves, causing small brownish lesions that can grow and burn, causing the death of the leaf (ISHIHARA *et al*., 2014). In the nodes and stems, in addition to brown spots, it can cause total necrosis in the affected part and thus prevent the circulation of sap (SILVA-LOBO; FILIPPI, 2017).

In addition, soil fertility, which should be sufficient to supply the nutritional demand of plants throughout their vegetative and reproductive cycle (GARCÍA SUÁREZ, 2016), mainly in Brazil, in the Amazon region, where the soils are low in nutrients and acids (QUESADA *et al*., 2011). Hou et al. (2019) observed reductions in rice productivity due to less nutrient availability in irrigated rice, while Zhang et al. (2019) reported gains due to fertilization. The presence of contaminating elements in the soil can reduce the productivity of plants sensitive to these elements (ZHANG et al., 2019).

2.2 Lead (Pb)

Pb is a toxic, dense, soft, malleable metal and poor conductor of electricity and quite resistant to corrosion. It has a bluish-white color when recently cut, but it also acquires a gray color when exposed to air (LENZI *et al*., 2009). It is generally found in ores associated with Zn, Ag and Cu and occurs naturally in the environment with <50mg / kg in the earth's crust, but the anthropogenic sources of Pb are considered the most common (RAJ *et al*., 2020). Is usually used in civil construction, acid batteries, in ceramic enamels, sewage sludge, phosphate fertilizers, ammunition, protection against X-rays and gamma rays and forms part of metal alloys for the production of welds, fuses, electrical cable coatings, antifriction materials, letterpress metals and others (GAGNETEN *et al*., 2007). Andrade *et al*. (2010) studied the steel residue to use as a source of zinc in rice, and the toxicity of Pb and Cd in the residue in three types of soil and obtained the content of 14.10 mg kg-1 Pb in red yellow argisol, preventing the use in rice.

Another important anthropogenic source is atmospheric pollution by Pb. When subjected to high temperatures, Pb (which has volatile compounds) is immediately emitted into the atmosphere, and this can occur in places of Pb ore smelting, coal burning and use of petrol with Pb in automobiles (STEINNES, 2012). Tao *et al*. (2021) collected data to identify the sources of atmospheric Pb in a Pequin megacity and observed that the concentrations and isotopic composition of Pb were higher in winter (168.1 ± 32.0 ng / m3) because of the increased burning of coal. Pb affects all living organisms (including humans and wild animals), in addition to being widely distributed and resistant in the environment, which creates major global problems (MONCLÚS; SHORE; KRONE, 2020).

Worldwide, the production of lead ore / concentrate obtained 5.5 Mt of contained metal, bringing together China (2.9 Mt), Australia (720 kt) and the United States (355 kt) as the main producers, while in Brazil, the its production reaches 11 kt, representing 0.2% of world production (DNPM, 2015). All production of lead concentrate in Brazil is exported and is obtained from recycling (from automotive, industrial and telecommunications batteries), since it does not have primary production of metallic lead, but it has refining plants that are located in the Northeast (Pernambuco), South (Rio Grande do Sul and Paraná) and Southeast regions (São Paulo, Rio de Janeiro and Minas Gerais) which together have the capacity to generate 160 kt / year (DNPM, 2015). It is worth mentioning that in the periods from 1960 to 1993, Brazil owned Companhia Brasileira de Chumbo - Cobrac (in 1990 it became mining company Plumbum), a mining company responsible for the extraction of lead in the region of Santo Amaro (BA) and this was responsible for making this city known as the "most polluted in the world", due to illegal heavy metal discharges (Pb and Cd) (MORAES; ANDRADE, 2013).

In the Amazon region, mineral extractions, such as bauxite and kaolinite, from this area present trace elements (including Pb) in their geological compositions and during the extraction stages, they can expose these materials to chemistry and weathering (QUEIROZ *et al*., 2019). After industrial processing (geological material disintegrates through physical and chemical processes), trace elements can be released and pre-concentrate on waste, which generates or emits the material into atmospheric particles (BONGLAISIN *et al*., 2011). In addition, in the city of Ulianópolis- PA (4th largest rice producer), Companhia Brasileira de Bauxita - CBB creates the Environmental Liabilities Plant to give adequate final destination to toxic waste sent by 100 national and multinational companies, however the disposal it was made in the soil of the region and in the river that cuts the city, gurupizinho river, that passes along several surrounding cities, being able to cause contamination to the population around (CNPG, 2016).

2.2.1. Lead in soil

The occurrence of Pb in nature is generally associated with the presence of minerals from the sulfide group such as galena (PbS) and sphalerite (ZnS), as well as associated with other metals such as Fe, Cu, As, and small amounts of Au (USEPA, 1994). Its bioavailability, as well as other metal cations, is governed by speciation in soil solution and their interactions with the solid phase system (PIERANGELI *et al.*, 2001). In agricultural areas, concentrations of Pb can be introduced through the application of pesticides or even through the application of fertilizer sources (NACKE *et al*., 2013). In places that have high levels of toxic metal and metalloid contamination, such as in mines, these metals can migrate through the percolation of water through the soil profile to the water table (OGAWA *et al*., 2020). Frachini *et al*. (2020) declares that priority hazardous substances, such as Pb, Hg and Cd may be related to environmental mining liabilities, since soil analysis in mining areas indicated that they are seriously polluted by these metals. In their research, Buschle *et al*. (2010) evaluated the Pb release kinetics of soils in the mining and heavy metal metallurgy area in the city of Adrianópolis (PR) and obtained high total Pb levels (maximum of 24,755.6 mg kg^{-1}), which indicated intense soil contamination. However, the permanence and availability of toxic metals in soils, sediments and in aquatic environments, depend not only on the physical and chemical properties of the soil, but also on the mineralogy and biological activity of the soil, as well as on the ionic composition of the soil solution with effects on metals competition (FRACHINI *et al*., 2020).

2.2.2. Lead in plants

Pb is not an essential metal for plant metabolism, however, several plants grow in soil contaminated with Pb and accumulate this metal in different parts, however, most of it accumulates in the roots (DALYAN *et al*., 2018). In their research, Jensen *et al*., (2009) studied the growth performance and the absorption of heavy metals (Cd, Cu, Pb and Zn) in willow plants (*Salix viminalis*) and reported that in parts of plants above the ground, Pb was more concentrated in leaves than in branches. In contrast, Evangelou *et al*., (2013) found a higher Pb content in the willow stem than in the leaves. Pb negatively affects physiological and developmental processes, such as seed germination, reduced seedling growth, dry root and shoot biomass, water absorption, mineral nutrition, photosynthesis, respiration, reduction of division growth and enzymatic activities in plants (ZHONG *et al*., 2020).

When Pb enters the root, it moves mainly through apoplasts and follows water courses until it reaches the endodermis and can be immobilized by negatively charged pectins on the cell wall, precipitation of insoluble lead salts in intercellular spaces, accumulation in plasma membranes or sequestration in the vacuoles of rhizodermal and cortical cells (POURRUT *et al*., 2011).

In high concentrations of Pb it can destroy the physical barriers found in the roots and the transport of the metal to the shoot can occur via xylem, and is probably driven by transpiration. Pb interrupts several physiological and biochemical pathways, resulting in decreased growth, restricts aquaporins, inhibits the absorption of nutrients, degrades the chlorophyll molecule, increases lipid peroxidation, interferes with ionic homeostasis and increases the production of reactive oxygen species (ROS) (PIRZADAH *et al.*, 2020). Liu *et al*. (2010) reported that in 30 cultivars of *Brassica pekinensis*, the increase in Pb levels in the soil also increased the percentage of translocation to aerial parts of plants. Ashraf *et al.* (2020) evaluated the absorption of Pb in different cultivars of aromatic rice, and as a result, all 5 cultivars accumulated different concentrations of Pb (higher in the roots and lower in the grains).

2.3 Brassinosteroids (Brs)

Currently, Brassinosteroids are known as a class of plant polyhydroxysteroids that have been recognized as a new type of phytohormone that plays an essential role in plant development (BAJGUZ, 2011). However, in 1979 the presence of steroid hormones was not confirmed in plants. That same year, American scientists published data on a new steroidal lactone called brassinolide (BL), which was isolated from pollen from *Brassica napus* collected by bees (GROVE *et al*., 1979). Three years later, castasterone, the immediate biosynthetic precursor of BL, was isolated from gall insects of *Castanea crenata* (YOKOTA *et al*., 1982). Since the discovery, 68 BRs have been isolated (besides several conjugates) from 64 plant species, including 53 angiosperms (12 monocotyledons and 41 dicotyledons), six gymnosperms, one pteridophyte (*Equisetum arvense* L.), one bryophyte (*Marchantia polymorpha* L.) and three algae (*Chlorella vulgaris* Beijerinck, *Cystoseira myrica* (SGGmelin) C. Agardh and *Hydrodictyon reticulatum* (Linnaeus) Bory) (FUJIOKA *et al*., 1998; BAJGUZ E TRETYN, 2003).

In the first researches on the action of BRs on plants it was based on exogenous application and followed by the recording of the observable response (CLOUSE, 2011), in the same way, the biosynthesis routes, as well as their precursors, were little known. Currently, to identify BR biosynthesis and signal transduction pathways, utilized is the isolation and characterization of mutants deficient in biosynthesis or response BRs (genes involved *dwf1*, *dwf2*, *dwf3*, *dwf4*, *dwf5*, *dwf6*, *dwf7*, *dwf8*, *dwf11*, *dwf12*, *dwf(d)*, *bas*, *brd1*, *sax*, *bak1-1D*, *bim1*, *bee1,2,3*, *bes1*, *bin2*, *brs*, *bru1*, *bsu1*, *bzr1* e *tch4*) (PEREIRA-NETTO, 2007). Yokota *et al.* (1991) researching BR biosynthesis, predicted that campesterol, a plant sterol, would be converted to BL via teasterone, tifasterol and castasterone. Other BRs are also derived from common plant sterols with an appropriate side chain structure, such as sitosterol, isofucosterol, 24-methylene cholesterol and 24-epicampesterol (YAKOTA, 1997). Clause (2011) explains that the BL biosynthesis starts in sterol campesterol and is converted to campestanol via DET2, then converted to castasterone through the Early and Late routes in the oxidation branches at C-6, and thus converted to BL. These two routes coexist in pea and rice crops and in *Arabidopsis*.

BRs act in multiple processes throughout the life cycle of plants, including germination, elongation of roots and stems, photomorphogenesis of seedlings, vascular differentiation, male fertility, senescence and flowering time and resistance to biotic and abiotic stresses (CLOUSE E SASSE, 1998). In rice, BRs affect many agricultural traits that influence grain yield, including plant height, leaf angle, grain size, and tiller number (ZHANG et al., 2014).

24-Epibrassinolide (EBR) is one of the most biologically active growth regulators in the BR group that specifically modulate plant responses to biotic and abiotic stress (KHALID; AFTAB, 2016). Fonseca *et al.* (2020) proved that the steroid spray improved the antioxidant system in rice plants stressed by acid rain, according to the increases in the activities of the SOD, CAT, APX and POX enzymes, resulting in the elimination of ROS and reducing the damages in the structure of the chloroplasts. EBR demonstrated effects in increasing plant growth under low and high air temperatures, as well as in the presence of salt and heavy metal stresses (ZHONG *et al.*, 2020). Feijuan *et al.* (2018) treated brown rice with exogenous EBR and managed to reduce the cd content, decreasing the absorption and transport of this metal from the root to the aerial part. Li *et al.* (2015) studied the effects of EBR on pepper under induced cooling, and obtained improvement in photoinhibition, increase in Fv / Fm, in photochemical efficiency and in the activity of antioxidant enzymes (SOD, POX, CAT e APX).

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PAPER

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Exogenous 24-Epibrassinolide stimulates root protection, and leaf antioxidant enzymes in lead stressed rice plants: Central roles to minimize Pb content and oxidative stress☆

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Lead (Pb) is an environmental pollutant that negatively affects rice plants, causing damage to the root system and chloroplast structures, as well as reducing growth. 24-Epibrasnolide (EBR) is a plant growth regulator with a high capacity to modulate antioxidant metabolism. The objective of this research was to investigate whether exogenous EBR application can mitigate oxidative damage in Pb-stressed rice plants, measure anatomical structures and evaluate physiological and biochemical responses connected with redox metabolism. The experiment was randomized with four treatments, including two lead treatments (0 and 200 μM $PbCl_2$, described as - Pb and + Pb, respectively) and two treatments with brassinosteroid (0 and 100 nM EBR, described as - EBR and + EBR, respectively). The results revealed that plants exposed to Pb suffered significant disturbances, but the EBR alleviated the negative interferences, as confirmed by the improvements in the root structures and antioxidant system. This steroid stimulated the root structures, increasing the epidermis thickness (26%) and aerenchyma area (50%), resulting in higher protection of this tissue against Pb^{2+} ions. Additionally, EBR promoted significant increases in superoxide dismutase (26%), catalase (24%), ascorbate peroxidase (54%) and peroxidase (63%) enzymes, reducing oxidative stress on the photosynthetic machinery in Pb-stressed plants. This research proved that EBR mitigates the toxic effects generated by Pb in rice plants.

1. Introduction

Rice is one of the most produced crops and serves as a basic food for most of the world's population. Rice is an important source of carbohydrates in human nutrition and has an effective increase in the yield of grain (Zeng et al., 2019), mainly due to the understanding of plant metabolism and the adoption of cultivation technologies (Zhang et al., 2019). It is a model plant and was the first plant among cereal crops that had its genomic map sequenced (Hong et al., 2019). Rice cultivation can be carried out in uplands and lowlands, with tolerance mechanisms essential to development in these environments (Gao et al., 2008). In general, this species presents a high risk of accumulating heavy metals in plant tissues and grains due to environmental contamination, affecting human and animal health (Hamid et al., 2019).

Lead (Pb) is a heavy metal that is highly toxic and is becoming a major environmental pollutant with significant risk to the agro-ecosystem (Rao et al., 2018). It can be inserted into the environment through anthropogenic activities and industrial waste, such as chemical fertilizers, herbicides, pesticides and contaminated irrigation water (Khan et al., 2018). In plants, Pb can accumulate in roots and/or be transported to shoots (Dalyan et al., 2018), negatively impacting metabolic processes (Sukhmeen Kaur Kohli et al., 2018), including leading to overproduction of reactive oxygen species (ROS), limitations on photosynthetic activity and oxidative

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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
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
Pb	Lead
$PbCl_2$	Lead chloride
PDS	Polar diameter of the stomata
pH	Hydrogen potential
P_N	Net photosynthetic rate
P_N/C_i	Instantaneous carboxylation efficiency
POX	Peroxidase
PSII	Photosystem II
q_P	Photochemical quenching
RDM	Root dry matter
RET	Root epidermis thickness
RXT	Root exodermis thickness
RDT	Root endodermis thickness
RCT	Root cortex thickness
ROS	Reactive oxygen species
RAA	Root aerenchyma area
RUBISCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SD	Stomatal density
SDM	Shoot dry matter
SF	Stomatal functionality
SOD	Superoxide dismutase
TD	Trichome density
RMD	Root metaxylem diameter
TDM	Total dry matter
Total Chl	Total Chlorophyll
TS	Trichome size
VCD	Vascular cylinder diameter
WUE	Water-use efficiency
Zn	Zinc
Φ_{PSII}	Effective quantum yield of PSII photochemistry

stress (Kohli et al., 2017), resulting in delays in growth and germination (Ye et al., 2018).

The root is the first tissue affected during stress situations induced by heavy metals available in the substrate, often inhibiting the elongation rate and biomass (Dalyan et al., 2018), with negative impacts on the uptake, transport and accumulation of water and nutrients (Potters et al., 2007; Ribeiro et al., 2020). However, adaptive strategies in species in contaminated environments that were focused on anatomical structures suggest opportunities to improve tolerance to heavy metal stress in crops, including rice plants (Gomes et al., 2011; Gowayed and Almaghrabi, 2013). Adejumo et al. (2020) studied the root anatomical responses of native plants growing on Pb-contaminated sites and found thickened epidermal cells and large air spaces within their internal tissues in all species evaluated. Tadaiesky et al. (2020) described that root aerenchyma contributes to the formation of an oxidative barrier, reducing Fe mobilization at the root surface in rice plants under Fe toxicity.

Heavy metals frequently induce oxidative stress in plant tissues, provoking overproduction of reactive oxygen species (ROS), species such as the hydroxy radical anion (−OH), superoxide anion (O_2^-), and hydrogen peroxide (H_2O_2) (Amari et al., 2017; Pinho and Ladeiro, 2012). ROS are also triggered by Pb^{2+} ions through multiple mechanisms, including activation of NADPH oxidase, displacement of the prosthetic groups of several enzymes, and inhibition of the activity of enzymes that contain −SH groups (Singh et al., 2020). On the other hand, antioxidant enzymes play relevant roles against oxidative stress, acting as the first line of defence and maintaining redox homeostasis. In other words, superoxide dismutase (SOD) removes O_2^-, releasing H_2O_2 and O_2. Catalase (CAT) is an enzyme associated with the dismutation of H_2O_2 in H_2O and O_2, in addition to other enzymes included in this antioxidant machinery, including ascorbate peroxidase (APX) and peroxidase (POX) (Das and Roychoudhury, 2014; Gill and Tuteja, 2010).

Brassinosteroids (BRs) are plant steroids and are a class composed of more than 60 natural molecules described in the literature (Bartwal et al., 2013). These substances are considered plant growth regulators (Liu et al., 2009), and they act on cell elongation and division (Thussagunpanit et al., 2015a), reproductive development (Fridman and Savaldi-Goldstein, 2013), synthesis of nucleic acids and proteins (Kim et al., 2012) and photosynthesis (Xi et al., 2013). Exogenous BR application can enhance crop performance under stress conditions, and significant increases in gene expressions (*CHLASE*, *CHS* and *PAL*, *POD*, *CAT*, *GR* and *GST1*) stimulates tolerance mechanisms (S. K. Kohli et al., 2018a, 2018b; Kohli et al., 2017), resulting in incremental changes in biomass and yield (Rao and Dixon, 2017; Sharma et al., 2013). 24-Epibrassinolide (EBR) is one of the most active forms of BRs, with multiple roles in metabolism and practical applications in agricultural contexts (Yao et al., 2017). More specifically, EBR contributes to fundamental processes, including vascular differentiation (Krishna, 2003), modulation of gene expression (Wang et al., 2011), seed germination (Wu et al., 2015), root and stem growth (Mussig et al., 2003), fruit development, abscission and maturation (Shu et al., 2016), being often used at low concentrations and having natural and biodegradable advantages (Feijuan et al., 2018).

Several studies have demonstrated that EBR can mitigate biotic and abiotic stresses, such as saline stress (Dong et al., 2017; Wu et al., 2017), herbicides and pesticides (Wang et al., 2017; Xia et al., 2011), heavy metals (Bajguz, 2011; Kanwar et al., 2012; Zhou et al., 2018), drought stress (Hu et al., 2013; Khamsuk et al., 2018), and low and high temperatures (Cui et al., 2016; Hu et al., 2010; Zhang et al., 2013). Therefore, our hypothesis was created in the absence of information available in the literature on EBR roles in rice plants under Pb excess, with a focus on anatomical responses. The objective of this research was to investigate whether exogenous EBR application can mitigate oxidative damage in Pb-stressed rice plants, measure anatomical structures and evaluate physiological and biochemical responses connected with redox metabolism.

2. Materials and methods

2.1. Location and growth conditions

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

2.2. Plants, containers and acclimation

Five-day-old seedlings of *Oryza sativa* L. cv. Puitá INTA CL™ were selected (similar aspects and sizes) and placed in 0.5-L pots (10 cm in height and 8 cm in diameter). All plants were cultivated under hydroponic conditions. Nutritive solution was used for nutrients (Fonseca et al., 2020), with the ionic strength beginning at 50% (8th day) and later modified to 100% after two days (10th day). After this period, the nutritive solution remained at the total ionic strength.

2.3. Experimental design

The experiment was randomized with four treatments, with two lead concentrations (0 and 200 μM Pb, described as − Pb and + Pb, respectively) and two concentrations of 24-Epibrassinolide (0 and 100 nM EBR, described as − EBR and + EBR, respectively). Twelve replicates for each of the four treatments were conducted, yielding 48 experimental units in the experiment, with three plants in each unit.

2.4. 24-Epibrasnolide (EBR) preparation and application

Ten-day-old seedlings were sprayed with 24-epibrassinolide (EBR) or Milli-Q water (containing a proportion of ethanol that was equally used in preparation of the EBR solution) for 20 days (days 10–30 after the start of the experiment), and this steroid was applied at intervals of 5 days. The 0 and 100 nM EBR (Sigma-Aldrich, USA) solutions were prepared by dissolving the solute in ethanol followed by dilution with Milli-Q water [ethanol:water (v/v) = 1:10,000] (Ahammed et al., 2013). The EBR concentration used in this research was defined in concordance with Maia et al. (2018).

2.5. Plant conduction and Pb treatment

Plants received the following macro- and micronutrients contained in the nutrient solution described by Fonseca et al. (2020). To simulate Pb^{2+} exposure, $PbCl_2$ was used at concentrations of 0 and 200 μM Pb, being defined this Pb concentration in agreement with Khan et al. (2018) and preliminary experiments, which was applied over 5 days (days 25–30 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 6.5 using HCl or NaOH. On day 30 of the experiment, the physiological and morphological parameters were measured for all plants, and the leaf tissues were collected for nutritional, anatomical and biochemical analyses.

2.6. Determining of Pb and nutrients

Milled samples (100 mg) of root, stem and leaf tissues were pre-digested using conical tubes (50 ml) with 2 ml of sub boiled HNO_3. Subsequently, 8 ml of a solution containing 4 ml of H_2O_2 (30% v/v) and 4 ml of ultra-pure water were added, and transferred to a Teflon digestion vessel in agreement with Paniz et al. (2018). The determination of lead (Pb), potassium (K), calcium (Ca), magnesium (Mg) , manganese (Mn), copper (Cu) and zinc (Zn) was performed using an inductively coupled plasma mass spectrometer (model ICP-MS 7900; Agilent).

2.7. Measurement of chlorophyll fluorescence and gas exchange

Chlorophyll fluorescence was measured in fully expanded leaves under light using a modulated chlorophyll fluorometer (model OS5p; Opti-Sciences). 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 the leaves, which was the middle third of the plant and was adapted to the dark for 30 min, was used in the evaluation. The intensity and duration of the saturation light pulse were 7500 μmol m^{-2} s^{-1} and 0.7 s, respectively. Gas exchange was evaluated in all plants and measured in the expanded leaves in the middle region of the plant using an infrared gas analyser (model LCPro$^+$; ADC BioScientific) in a chamber under constant CO_2, photosynthetically active radiation, air-flow rate and temperature conditions 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. 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 that was described by Aragão et al. (2012).

2.8. Measurements of root and leaf anatomical variables

Samples were collected from the middle region of the leaf limb of fully expanded leaves and roots 5 cm from the root apex. Subsequently, all collected botanical material was fixed in FAA 70 for 24 h, dehydrated in ethanol and embedded in historesin Leica™ (Leica, Nussloch, Germany). Transverse sections with a thickness of 5 μm were obtained with a rotating microtome (model Leica RM 2245, Leica Biosystems) and stained with toluidine blue (O'Brien et al., 1964). For stomatal characterization, the epidermal impression method was used according to Segatto et al. (2004). The slides were observed and photomicrographed under an optical microscope (Motic BA 310, Motic Group Co. LTD.) coupled to a digital camera (Motic 2500, Motic Group Co., LTD.). The images were analysed with Motic plus 2.0 previously calibrated with a micrometre slide from the manufacturer.

2.9. Determination of the 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 total soluble proteins were quantified using the methodology described by Bradford (1976). The SOD assay was measured at 560 nm (Giannopolitis and Ries, 1977), and the SOD activity was expressed in mg^{-1} protein. The CAT assay was detected at 240 nm (Havir and McHale, 1987), and the CAT activity was expressed in μmol H_2O_2 mg^{-1} protein min^{-1}. The APX assay was measured at 290 nm (Nakano and Asada, 1981), and the APX activity was expressed in μmol AsA mg^{-1} protein min^{-1}. The POX assay was detected at 470 nm (Cakmak and Marschner, 1992), and the activity was expressed in μmol tetraguaiacol mg^{-1} protein min^{-1}. O_2^- was measured at 530 nm (Elstner and Heupel, 1976).

2.10. Quantification of hydrogen peroxide, malondialdehyde and electrolyte leakage

Stress indicators (H_2O_2 and MDA) were extracted using the methodology described by Wu et al. (2006). H_2O_2 was measured using the procedures described by Velikova et al. (2000). MDA was determined by the method of Cakmak and Horst (1991) using an extinction coefficient of 155 mM^{-1} cm^{-1}. EL was measured according to Gong et al. (1998) and calculated by the formula EL (%) = $(EC_1/EC_2) \times 100$.

2.11. Determination of photosynthetic pigments and biomass

Chlorophyll and carotenoid determinations were performed using a spectrophotometer (model UV-M51; Bel Photonics) according to the methodology of Lichtenthaler and Buschmann (2001). The biomass of roots and shoots was measured based on constant dry weights (g) after drying in a forced-air ventilation oven at 65 °C.

2.12. Data analysis

Normality of residuals was tested using Shapiro-Wilk test and data subjected to one-way ANOVA. Significant differences between means were determined by the Scott–Knott test at a probability level of 5% (Steel et al., 2006). Standard errors were calculated for each treatment. The statistical analyses were performed with Assistat 7.7 software.

3. Results

3.1. EBR minimized the Pb contents in plants exposed to toxicity

The Pb contents in the roots and shoots of rice plants subjected to Pb exposure increased significantly, mainly in the roots (Table 1). However, plants submitted to the treatment of 200 μM Pb and 100 nM EBR had significant reductions in this metal by 20% and 13% in the root and shoot, respectively, when compared to treatment with Pb alone.

3.2. Pretreatment with EBR induced higher protection against Pb in roots and leaves

Plants exposed to Pb exposure had decreases in the values of RET, RDT, RCT, RAA and VCD (Table 2 and Fig. 1). Plants treated with EBR and exposed to Pb had increases of 26%, 19% and 50% in RET, RCT and RAA, respectively, compared to the same treatment without EBR. For leaf structures, plants treated with 200 μM Pb suffered reductions in ETAd, ETAb, MT, LAA and BCD (Table 2 and Fig. 1). However, plants exposed to Pb excess with EBR spray showed increases of 15% and 42% in ETAd and LAA, respectively, compared to equal treatment without steroids.

3.3. Steroids positively regulate nutrient contents and metal homeostasis

Pb excess caused negative changes in the nutrient contents in the roots and shoots (Table 3). However, plants treated with Pb and EBR exhibited increases in the levels of K, Ca, Mg, Mn, Cu and Zn in roots of 10%, 13%, 18%, 8%, 9% and 7%, respectively, while in shoots, the increases were 9%, 14%, 10% and 4%, respectively, when compared to treatment without EBR. At ionic rates, plants submitted to Pb and EBR showed increases in the Ca^{+2}/Pb^{+2} and Mg^{+2}/Pb^{+2} ratios at the root of 100% and 50% (Table 1), respectively, with increases in the shoot of 35% and 23%, respectively, compared to Pb treatment without EBR.

3.4. EBR attenuated the Pb impact on pigments, light capture and gas exchange

Photosynthetic pigments (Chl *a*, Chl *b* and Total Chl) in plants treated with Pb were reduced (Table 4). However, plants subjected to Pb and EBR treatment had significant increases of 15%, 23% and 17% for Chl *a*, Chl *b* and Total Chl, respectively. In relation to light capture, exposure to Pb increased F_0 and decreased F_m, F_v and F_v/F_m (Fig. 2). However, the combined Pb and EBR treatment decreased F_0 by 6% and increased F_m, F_v and F_v/F_m by 2%, 4% and 3%, respectively, compared to treatment without EBR. Furthermore, plants subjected to Pb treatment suffered reductions in Φ_{PSII}, q_P and ETR and had increased NPQ, ETR and EXC/P_N values (Table 4). Plants subjected to 100 nM EBR and exposed to 200 μM Pb had significant increases of 33%, 19% and 34% in Φ_{PSII}, q_P and ETR, respectively, and a reduction in NPQ (23%) and EXC (6%) compared to treatment without EBR. Regarding gas exchange, plants subjected to Pb excess suffered significant reductions in P_N, WUE and P_N/C_i and increases in E and C_i (Table 4). For the Pb and EBR treatments, there were significant increases in P_N (39%), WUE (46%) and P_N/C_i (50%) and decreases of 5% and 9% in E and C_i, respectively, when compared to the treatment without EBR.

3.5. Stomata and trichomes are modulated by EBR

Application of 200 μM Pb caused increases in PDS and EDS (abaxial face) and decreases in TD and TS present in the adaxial face (Table 5). However, treatment with 200 μM Pb and 100 nM EBR presented significant increases of 18% and 10% in TD and TS (adaxial face), in this order, and decreases in the values of PDS (8%) and EDS (11%) on the abaxial face, when compared to treatment without EBR.

3.6. Benefits on antioxidant system and lower ROS concentrations induced by the EBR

Antioxidant enzymes (SOD, CAT, APX and POX) had significant increases in their activities when subjected to Pb excess (Fig. 3). EBR spray on plants exposed to Pb exposure promoted increases of 26%, 24%, 54% and 63% for SOD, CAT, APX and POX, respectively, in relation with equal treatment without EBR. Regarding stress indicators, plants under Pb stress had significant production of oxidative compounds (Fig. 3). However, plants exposed to 200 μM Pb and 100 nM EBR had reductions of 10%, 15%, 16% and 23% in O_2^-, H_2O_2, MDA and EL, respectively, compared to treatment without EBR.

3.7. EBR reduced the deleterious effects caused by Pb on biomass

The growth was slightly affected in plants treated with 200 μM Pb (Fig. 4). Control plants sprayed with EBR had increases in

Table 1
Pb contents and ionic ratios in rice plants sprayed with EBR and exposed to Pb toxicity.

Pb contents			
Pb	EBR	Pb in root (μg g DM^{-1})	Pb in shoot (μg g DM^{-1})
–	–	0.47 ± 0.02c	0.44 ± 0.04c
–	+	0.22 ± 0.01 d	0.19 ± 0.01 d
+	–	194.69 ± 3.65a	15.90 ± 0.57a
+	+	156.39 ± 4.04 b	13.84 ± 0.67 b
Ionic ratios			
Pb	EBR	Ca^{2+}/Pb^{2+} ratio in root	Ca^{2+}/Pb^{2+} ratio in shoot
–	–	5.67 ± 0.36 b	13.97 ± 1.19 b
–	+	14.32 ± 1.27a	33.71 ± 0.97a
+	–	0.01 ± 0.00 d	0.26 ± 0.01 d
+	+	0.02 ± 0.00c	0.35 ± 0.02c
Pb	EBR	Mg^{2+}/Pb^{2+} ratio in root	Mg^{2+}/Pb^{2+} ratio in shoot
–	–	9.81 ± 0.95 b	9.51 ± 0.88 b
–	+	21.37 ± 1.77a	24.22 ± 0.62a
+	–	0.02 ± 0.00 d	0.22 ± 0.01 d
+	+	0.03 ± 0.00c	0.27 ± 0.01c

Pb = Lead; Ca^{2+}/Pb^{2+} = Calcium and lead ratio; Mg^{2+}/Pb^{2+} = Magnesium and lead ratio. 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 and leaf structures in rice plants sprayed with EBR and exposed to Pb toxicity.

Root structures								
Pb	EBR	RET (μm)	RXT (μm)	RDT (μm)	RCT (μm)	RAA (mm^2)	VCD (μm)	RMD (μm)
–	–	17.11 ± 0.25 b	18.21 ± 0.65 b	313.16 ± 4.99 b	13.63 ± 0.95a	0.08 ± 0.01 b	203.65 ± 3.17a	42.92 ± 1.44a
–	+	18.12 ± 0.39a	20.64 ± 1.39a	349.38 ± 8.10a	14.64 ± 1.11a	0.11 ± 0.00a	205.91 ± 3.94a	43.25 ± 1.58a
+	–	12.49 ± 0.15 d	16.32 ± 1.24 b	295.28 ± 3.17c	10.08 ± 0.80c	0.04 ± 0.01 d	194.67 ± 1.97 b	41.30 ± 1.45a
+	+	15.73 ± 0.24c	17.01 ± 0.82 b	300.92 ± 4.42c	11.95 ± 0.65 b	0.06 ± 0.00c	197.02 ± 1.32 b	42.71 ± 1.28a
Leaf structures								
Pb	EBR	ETAd (μm)	ETAb (μm)	MT (μm)	LAA (mm^2)	BCD (μm)		
–	–	9.84 ± 0.84a	9.48 ± 0.50a	57.52 ± 1.24 b	0.40 ± 0.03 b	27.60 ± 1.49 b		
–	+	9.98 ± 0.68a	9.77 ± 0.71a	62.49 ± 1.89a	0.59 ± 0.03a	31.31 ± 1.70a		
+	–	7.32 ± 0.39c	7.62 ± 0.69 b	47.95 ± 1.90c	0.26 ± 0.02c	22.19 ± 1.36c		
+	+	8.42 ± 0.52 b	8.01 ± 0.32 b	52.65 ± 2.89c	0.37 ± 0.00 b	24.09 ± 1.17c		

SDM and RDM of 7% and 16%, respectively. In plants treated with EBR + Pb excess, increases of 9% (SDM) and 8% (RDM) were verified. Additionally, in both treatments (control + EBR and Pb + EBR), the increases in TDM were 9%.

4. Discussion

Pb values found in the roots and shoots of rice plants confirm the toxic effect caused by the application of 200 μM Pb. However, plants subjected to 100 nM EBR had reductions in Pb contents, probably due to stimulation of phytochelatin (PC) biosynthesis caused by EBR application, in which these substances can exercise protective roles, reducing metal toxicity in higher plants. Concomitantly, EBR caused increases in root structures, more specifically RET and RAA, which are structures that can act as barriers and play an important role in protecting against nutrient stresses, and their thickening can be a strategy to minimize the translocation of metals (Gomes et al., 2011). Bajguz (2002) showed increases in PCs of *Chlorella vulgaris* treated with EBR and exposed Pb. Plants exposed to high concentrations of Pb often tend to restrict the metal in the roots, inhibiting translocation to shoots, because leaf tissue is more sensitive to intoxication (Fahr et al., 2013; Gupta et al., 2013), corroborating our data with higher contents in roots. Talarek-Karwel et al. (2019) described the positive impacts of 1 μM EBR on *Acutodesmus obliquus* subjected to Pb stress, showing positive modulations on phytoquelatin synthesis, primary metabolites and antioxidant compounds.

EBR alleviated the toxic effects associated with Pb and increased the content of macronutrients (K, Ca and Mg) and micronutrients (Mn, Cu and Zn), which may be related to increases verified in root structures, contributing to nutrient uptake. Simultaneously, the increase in RMD promoted by EBR suggests that a higher diameter of this tissue can enhance the transport of water and nutrients from roots to shoots (Y et al., 2020). Pb excess modifies the selectivity linked to cell membranes in root tissues, affecting the uptake, transport and accumulation of essential nutrients in several plant organs, resulting in a nutritional imbalance and inadequate absorption of K, Ca, Mg, Zn and Cu by the root (Alves et al., 2014). Our results confirm the positive actions of this steroid on Ca^{+2}/Pb^{+2} and Mg^{+2}/Pb^{+2} ratios in roots and shoots, confirming the ionic homeostasis induced by EBR. Pb^{+2} can be absorbed by channels permeable to Ca^{+2} and Mg^{+2} of root cells because they have the same valence, interfering with the nutritional status of these essential elements (Kim et al., 2002). Jan et al. (2018), studied *Pisum sativum* plants subjected to 150 mg L^{-1} Cd stress and treated with 10^{-7} M EBR, which showed improvement in nutritional status, more specifically in the macronutrients Ca, Mg and K in the root and shoot tissues. Bukhari et al. (2016), working with three *Nicotiana tabaccum* genotypes (L, H and M) under exogenous EBR (0.1 μM) application and with Cr toxicity (50 μM), observed significant increases in micronutrients Mn and Zn in genotype L.

The EBR spray caused increases in Chl *a*, Chl *b* and Chl total, which were related to increments in nutrient contents, more specifically Mg. EBR increased the Mg contents and mitigated the interferences on chlorophylls caused by Pb exposure, improving the Mg supply necessary for the synthesis of these pigments, as confirmed by the increases in Mg contents and the Mg^{2+}/Pb^{2+} ratio. During Pb excess, the replacement of Mg^{2+} ions for Pb^{2+} frequently

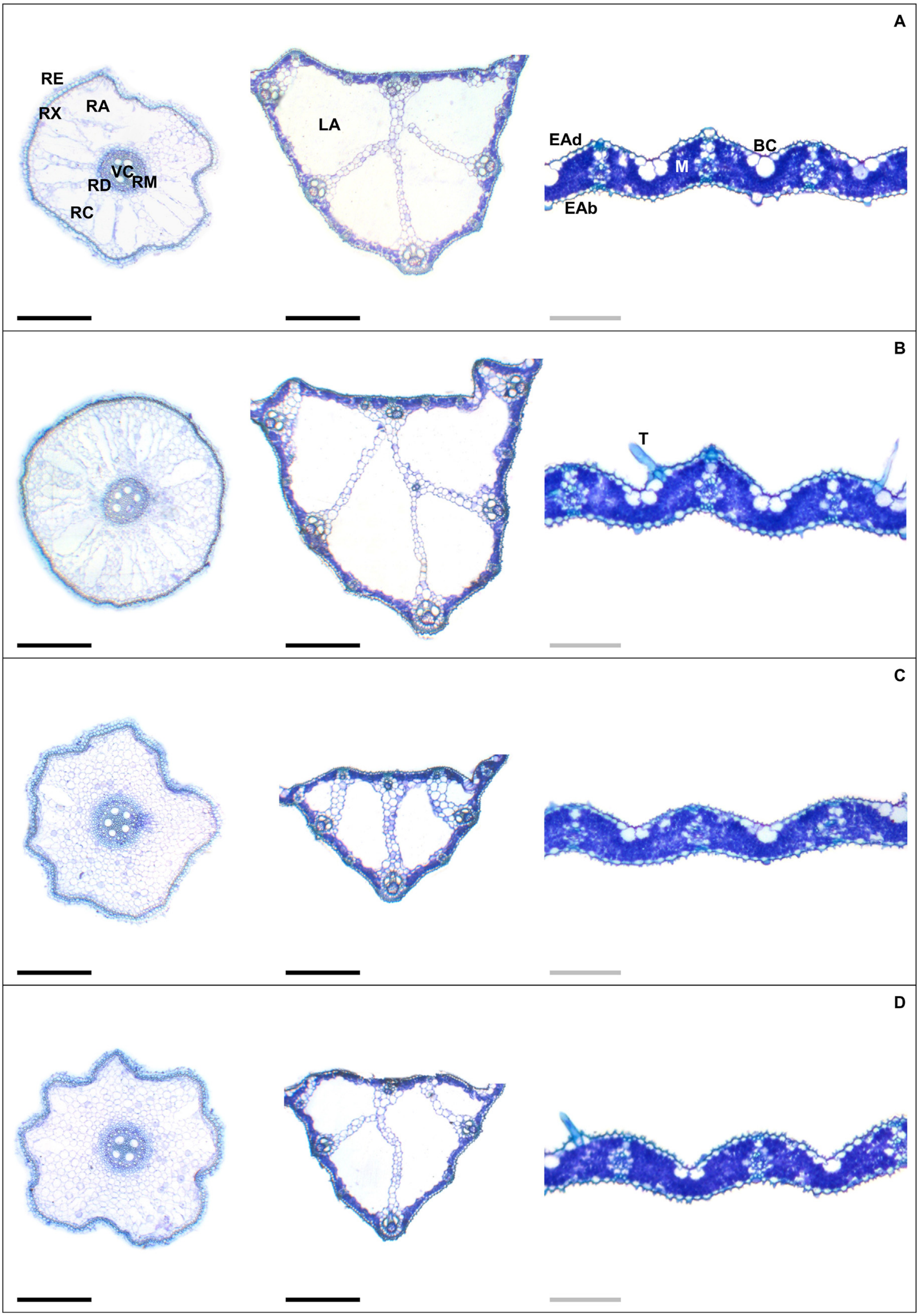

Fig. 1. Root and leaf cross sections in rice plants sprayed with EBR and exposed to Pb toxicity. Pb^{+2}/ EBR (A), Pb^{+2}/+ EBR (B), + Pb^{+2}/ EBR (C) and + Pb^{+2}/+ EBR (D). Legends: RE = Root epidermis; RX = Root exodermis; RC = Root cortex; RA = Root aerenchyma; RD = Root endodermis; VC = Vascular cylinder; RM = Root metaxylem; LA = Leaf aerenchyma; EAb = Adaxial epidermis; EAb = Abaxial epidermis; BC = Bulliform cell; M = Mesophyll; T = Trichome. Black bars: Black = 300 μm; Gray bars = 100 μm.

Table 3
Nutrient contents in rice plants sprayed with EBR and exposed to Pb toxicity.

Contents in root							
Pb	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})
_	_	53.66 ± 0.68 b	2.64 ± 0.13 b	4.55 ± 0.29a	67.22 ± 1.48 b	13.27 ± 0.59a	68.04 ± 0.57 b
_	+	55.54 ± 0.82a	3.09 ± 0.18a	4.61 ± 0.28a	74.31 ± 0.54a	13.86 ± 0.46a	70.79 ± 0.76a
+	_	43.71 ± 0.58 d	2.36 ± 0.07c	3.82 ± 0.15 b	62.07 ± 1.09c	11.87 ± 0.48 b	59.32 ± 0.49 d
+	+	48.18 ± 0.61c	2.67 ± 0.07 b	4.50 ± 0.12a	66.94 ± 0.66 b	12.97 ± 0.56a	63.58 ± 0.64c
Contents in shoot							
Pb	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})
_	_	40.48 ± 0.60 b	6.16 ± 0.05 b	4.19 ± 0.14 b	351.91 ± 9.83 b	11.46 ± 0.48a	46.12 ± 1.12 b
_	+	42.29 ± 0.27a	6.35 ± 0.06a	4.57 ± 0.07a	506.51 ± 13.80a	11.81 ± 0.68a	50.67 ± 1.30a
+	_	35.71 ± 0.74 d	4.21 ± 0.08 d	3.43 ± 0.12 d	318.34 ± 7.04c	10.72 ± 0.51a	44.05 ± 0.56c
+	+	38.78 ± 0.57c	4.82 ± 0.17c	3.77 ± 0.09c	334.18 ± 4.82c	11.08 ± 0.71a	45.90 ± 0.36 b

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
Photosynthetic pigments, chlorophyll fluorescence and gas exchange in rice plants sprayed with EBR and exposed to Pb toxicity.

Photosynthetic pigments							
Pb	EBR	Chl *a* (mg g^{-1} FM)	Chl *b* (mg g^{-1} FM)	Total Chl (mg g^{-1} FM)	Car (mg g^{-1} FM)	Ratio Chl *a*/Chl *b*	Ratio Total Chl/Car
_	_	7.48 ± 0.31a	3.39 ± 0.13 b	10.87 ± 0.26 b	0.71 ± 0.07 b	2.23 ± 0.22a	14.66 ± 0.63a
_	+	7.79 ± 0.40a	3.78 ± 0.21a	11.57 ± 0.33a	0.89 ± 0.08a	2.08 ± 0.17a	13.12 ± 0.31 b
+	_	6.30 ± 0.12 b	2.59 ± 0.10c	8.89 ± 0.17c	0.62 ± 0.04 b	2.45 ± 0.22a	15.18 ± 0.57a
+	+	7.22 ± 0.29a	3.19 ± 0.11 b	10.41 ± 0.28 b	0.70 ± 0.07 b	2.28 ± 0.22a	14.97 ± 0.40a
Chlorophyll fluorescence							
Pb	EBR	Φ_{PSII}	q_P	NPQ	ETR (µmol m^{-2} s^{-1})	EXC (µmol m^{-2} s^{-1})	ETR/P_N
_	_	0.157 ± 0.009 b	0.22 ± 0.01 b	0.16 ± 0.01c	23.13 ± 1.36 b	0.80 ± 0.01 b	1.76 ± 0.12 b
_	+	0.178 ± 0.008a	0.25 ± 0.01a	0.16 ± 0.01c	26.17 ± 1.23a	0.77 ± 0.01c	1.72 ± 0.05 b
+	_	0.116 ± 0.010c	0.16 ± 0.01 d	0.26 ± 0.02a	17.09 ± 1.50c	0.85 ± 0.01a	2.18 ± 0.13a
+	+	0.154 ± 0.005 b	0.19 ± 0.01c	0.20 ± 0.01 b	22.67 ± 0.75 b	0.80 ± 0.01 b	2.09 ± 0.06a
Gas exchange							
Pb	EBR	P_N (µmol m^{-2} s^{-1})	*E* (mmol m^{-2} s^{-1})	g_s (mol m^{-2} s^{-1})	C_i (µmol mol^{-1})	WUE (µmol $mmol^{-1}$)	P_N/C_i (µmol m^{-2} s^{-1} Pa^{-1})
_	_	13.18 ± 0.24 b	1.85 ± 0.10c	0.44 ± 0.01 b	188 ± 9c	7.14 ± 0.36 b	0.070 ± 0.004 b
_	+	15.22 ± 0.90a	1.87 ± 0.07c	0.47 ± 0.01a	164 ± 8 d	8.14 ± 0.35a	0.093 ± 0.008a
+	_	7.83 ± 0.29 d	2.85 ± 0.08a	0.29 ± 0.01c	327 ± 9a	2.75 ± 0.12 d	0.024 ± 0.001 d
+	+	10.85 ± 0.08c	2.70 ± 0.05 b	0.31 ± 0.01c	298 ± 6 b	4.02 ± 0.21c	0.036 ± 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.

occurs, with Mg being an element essential to the formation of the tetrapyrrole ring of the chlorophyll molecule (John et al., 2008). Zhong et al. (2020) described the positive effects of EBR in *Festuca arundinaceae* plants, alleviating the stress of 1000 mg kg^{-1} soil Pb and significantly increasing the Chl *a*, Chl *b* and Chl total. Kohli et al. (2018b) analysed the effects of 10^{-7} mM EBR in *Brassica juncea* submitted to 0.75 mM Pb, obtaining an increase (166%) in Chl total, suggesting an improvement in light absorption promoted by EBR. Interestingly, the same study conducted by Kohli et al. (2018b) detected higher expression of the *CHLASE* gene (originating chlorophyllase, a chlorophyll-degrading enzyme) caused by Pb stress, and after application of EBR, there was a decrease in the expression of this gene.

EBR attenuated the toxic effects of Pb in F_0, F_m, F_v and F_v/F_m, as confirmed by the decrease in F_0 and an increase in the values of F_m, F_v and F_v/F_m. The decrease in F_0 in plants treated with EBR and Pb is an interesting response, demonstrating the reduction of the closed reaction centres due to an increase in oxidized quinones, minimizing possible photoinhibition (Murchie and Lawson, 2013). The increase caused by EBR in F_v/F_m is an important result, because this variable represents a sensitive indicator of stress, often influencing Φ_{PSII} (Dao and Beardall, 2016; Siddiqui et al., 2018). Pb exposure in *Oryza sativa* decreases the efficiency of the photosynthetic machinery, damaging the chloroplast structures and lipid composition linked to the thylakoid membrane. The dissociation of polypeptides from the oxygen-evolving complex (OEC) and removal of essential elements of this complex (Ca and Mn) occurs in parallel, consequently decreasing water oxidation and limiting the transport of electrons during the photosynthetic process (Ashraf et al., 2015; Kalaji et al., 2016). Pb excess in *Oryza sativa* plants interferes negatively with the photosynthetic apparatus, damaging the structures of the chloroplast and inhibiting the photosynthetic process (Ashraf et al., 2015). On the other hand, steroids clearly modulated these effects, improving the efficiency in the reaction centre of PSII and alleviating Pb impacts during CO_2 fixation. Santos et al. (2018) described a decrease in F_0 in their study with *Vigna unguiculata* plants treated with 100 nM EBR and subjected to 500 µM Cd stress. Santos et al. (2020) verified increases of 2% in F_m in *Glycine max* plants pretreated with EBR (100 nM) and subjected to Zn stress (2000 µM). Xia et al. (2011) described that EBR alleviated the decline in F_v/F_m induced by herbicide treatment (Paraquat), inducing tolerance to photooxidative stress in treated leaves of *Cucumis sativus*.

The application of EBR increased the values of Φ_{PSII}, qp and ETR, revealing that the steroid attenuated the interferences occasioned by Pb^{2+}, stimulating the electron flux and improving the efficiency of the PSII reaction centre. Plants under Pb stress often present a decrease in photochemical activity as a consequence of disorders of

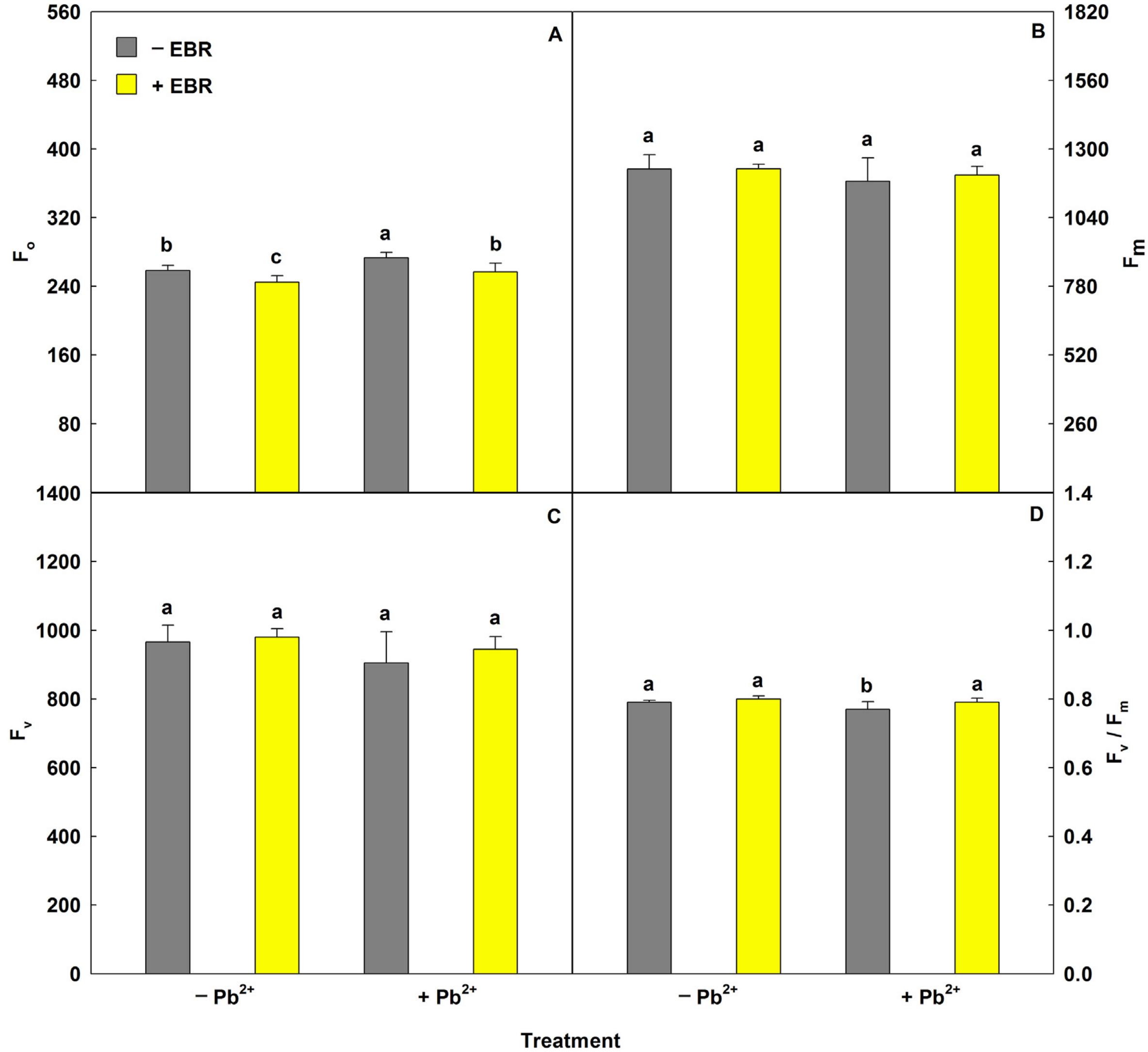

Fig. 2. Minimal fluorescence yield of the dark-adapted state (F_0; A), maximal fluorescence yield of the dark-adapted state (F_m; B), variable fluorescence (F_v; C) and maximal quantum yield of PSII photochemistry (F_v/F_m; D) in rice plants sprayed with EBR and exposed to Pb toxicity. Bars with different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

Table 5
Stomatal and trichome characteristics in rice plants sprayed with EBR and exposed to Pb toxicity.

Adaxial face							
Pb	EBR	SD (stomata per mm^2)	PDS (μm)	EDS (μm)	SF	TD	TS
−	−	400 ± 32a	6.88 ± 0.64 b	12.72 ± 0.43 b	0.55 ± 0.05a	992 ± 62 b	8.66 ± 0.43 b
−	+	421 ± 39a	6.79 ± 0.48 b	12.28 ± 0.33 b	0.56 ± 0.05a	1282 ± 93a	9.93 ± 0.52a
+	−	378 ± 34a	7.51 ± 0.29a	14.40 ± 0.52a	0.52 ± 0.04a	814 ± 42c	7.49 ± 0.28c
+	+	385 ± 36a	7.51 ± 0.49a	13.68 ± 0.24a	0.54 ± 0.05a	964 ± 69 b	8.27 ± 0.35 b
Abaxial face							
Pb	EBR	SD (stomata per mm^2)	PDS (μm)	EDS (μm)	SF	TD	TS
−	−	507 ± 18 b	7.16 ± 0.13c	12.07 ± 0.31c	0.59 ± 0.03a	–	–
−	+	555 ± 23a	6.82 ± 0.18c	11.48 ± 0.55c	0.60 ± 0.05a	–	–
+	−	478 ± 11 b	8.15 ± 0.22a	14.82 ± 1.04a	0.55 ± 0.03a	–	–
+	+	484 ± 16 b	7.52 ± 0.17 b	13.14 ± 0.48 b	0.57 ± 0.04a	–	–

SD = Stomatal density; PDS = Polar diameter of the stomata; EDS = Equatorial diameter of the stomata; SF = Stomatal functionality; TD = Trichome density; TS = Trichome size. 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.

the chloroplast ultrastructure, restriction linked to chlorophyll biosynthesis and plastoquinones, and inefficient electron transport (Sharma and Dubey, 2005). In contrast, the EBR reduced the values of NPQ, EXC and ETR/P_N, confirming that the EBR acted to reduce heat dissipation, resulting in higher energy available to the photosynthetic process intrinsically related to the PSII antenna

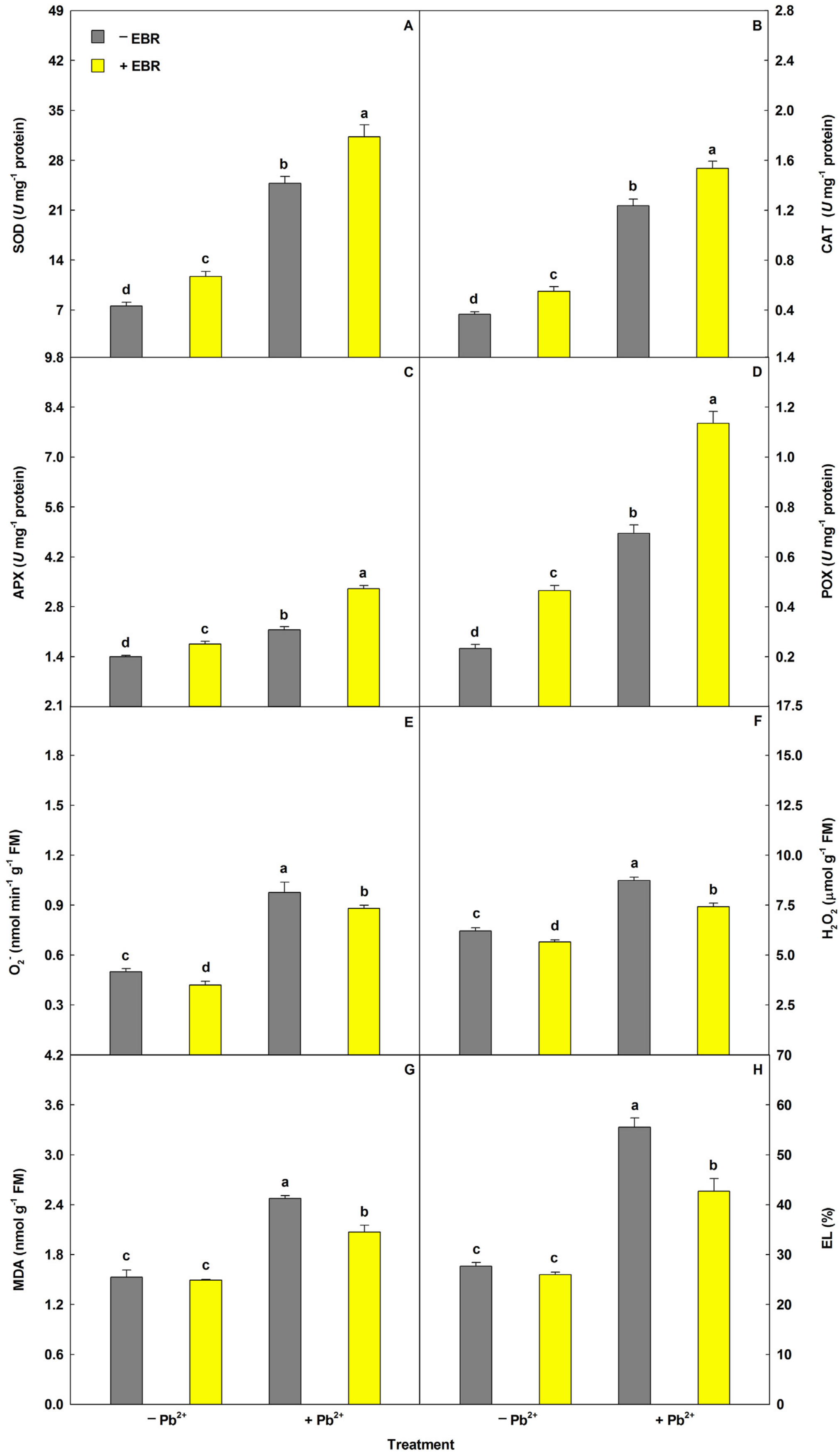

Fig. 3. Superoxide dismutase (SOD; A), catalase (CAT; B), ascorbate peroxidase (APX; C), peroxidase (POX; D), Superoxide (O_2^{-}; E), hydrogen peroxide (H_2O_2; F), malondialdehyde (MDA; G) and electrolyte leakage (EL; H) in rice plants sprayed with EBR and exposed to Pb toxicity. Bars with different letters indicate significant differences from the Scott-Knott test ($P < 0.05$). Bars corresponding to means from five repetitions and standard deviations.

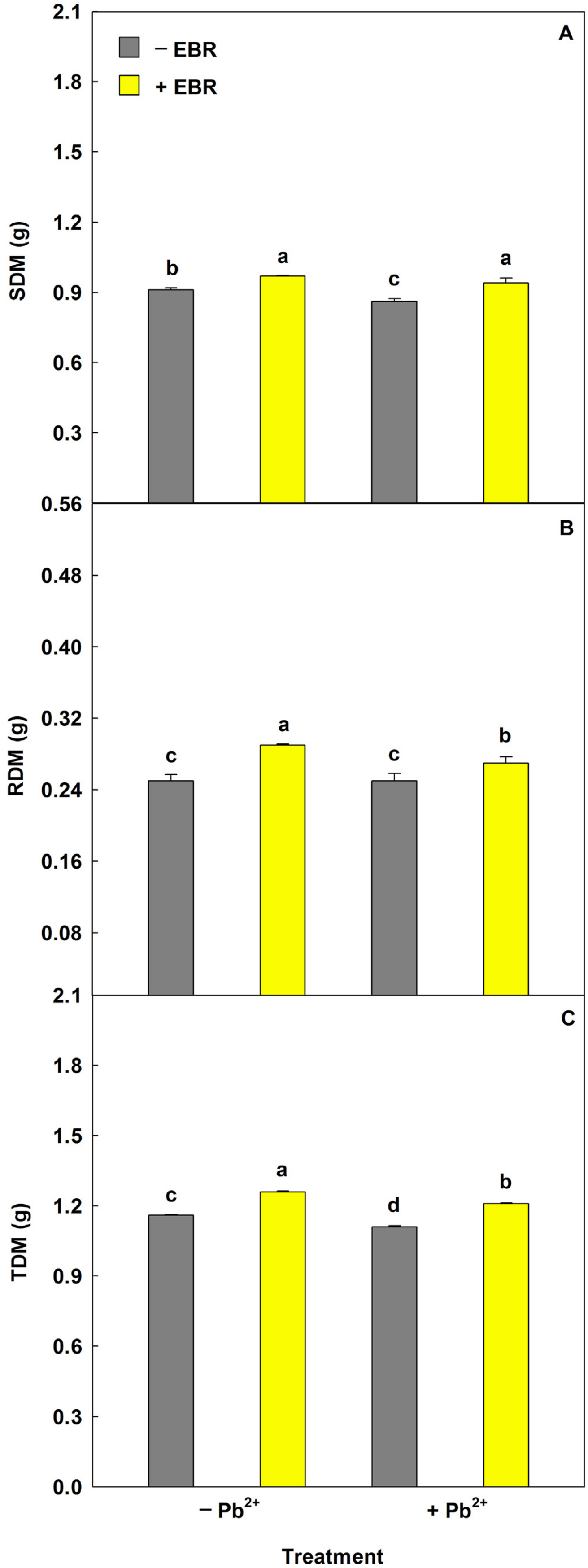

Fig. 4. Shoot dry matter (SDM; A), root dry matter (RDM; B) and total dry matter (TDM; C) in rice plants sprayed with EBR and exposed to Pb toxicity. Bars with different letters indicate significant differences from the Scott-Knott test ($P < 0.05$). Bars corresponding to means from five repetitions and standard deviations.

complex (Thussagunpanit et al., 2013). Ribeiro et al. (2020) evaluated young *Eucalyptus urophylla* plants pretreated with 100 nM EBR and exposed to 600 µM Ni and obtained increments of 44%, 4% and 42% in Φ_{PSII}, qp and ETR, respectively. Thussagunpanit et al. (2015b) studied the effects of EBR in *Oryza sativa* under high temperatures with beneficial results in chlorophyll fluorescence, more specifically increases of 5% and 102% in F_v/F_m and qp, respectively, which will improve the electron flux in the reaction centre, generating higher efficiency in Φ_{PSII} (increase of 111%) and ETR.

Plants treated with EBR and exposed to Pb^{2+} excess had increases in P_N, WUE and P_N/C_i. These results are connected with the improvements induced by the steroid on the Φ_{PSII} and ETR variables, indicating that EBR increased the photochemical efficiency, positively influencing the reactions involved in the photosynthesis process, water utilization and RuBisCO activity, and maximizing CO_2 fixation in the Calvin cycle (Yu, 2004). The decrease in C_i caused by the EBR can be associated with the increase in MT described in this study. MT is a complex structure with a large amount of chloroplasts, facilitating CO_2 consumption and reducing the CO_2 supply available in the substomatal cavity (Flexas et al., 2012), as detected in this study. CO_2 diffusion depends on the anatomical characteristics in leaf tissue, including cell forms and stomatal position (Evans et al., 2009). The toxic effects of Pb provoked increases in *E*, possibly related to oxidative damage to the mitochondria and consequent gas imbalance during the respiration process (Sharma and Dubey, 2005). Santos et al. (2020) studied the gas exchange and nutritional status in *Glycine max* plants sprayed with EBR and exposed to Zn stress and found increments in P_N, WUE and P_N/C_i of 20%, 10% and 59%, respectively. Rodrigues et al. (2020) evaluated the effects of EBR on the antioxidant system and repercussions on CO_2 fixation in *Glycine max* plants subjected to a low and high Mn supplies and reported increases in P_N, WUE and P_N/C_i and a reduction in C_i.

EBR spray in plants undergoing Pb^{2+} exposure occasioned increases in leaf anatomy (ETAd, ETAb, MT, LAA and BCD). These benefits on ETAd, ETAb and BCD are connected with the improvements previously detected on WUE because epidermal and bulliform cells contribute to plant defence against abiotic stresses, reducing the water losses connected to the leaf surface (Hameed et al., 2009; Javelle et al., 2011). High concentrations of Pb often reduce leaf area, which is explained by restrictions in division and cell expansion (Hatamian et al., 2020). Maia et al. (2018) studied the EBR (100 nM) actions on leaf and root structures in two tomato genotypes contrasting dwarfism (BR-deficient and BR-efficient), obtaining increments of 10% and 33% in ETAd and ETAb, respectively, in the BR-deficient genotype. Research conducted by Fonseca et al. (2020) with *Oryza sativa* plants pretreated with 100 nM EBR and subjected to acid rain stress (0.5 M H_2SO_4) showed benefits on leaf structures, specifically increases in MT, LAA and BCD of 14%, 100% and 12%, respectively.

Steroid alleviated the toxic effects provoked by Pb on stomatal characteristics (PDS, EDS, TD and TS). Increases in TD and TS after EBR treatment may contribute to decreasing the resistance of the boundary layer to the transport of water and CO_2, facilitating gas exchange (Bickford, 2016). Interestingly, EBR decreases the PDS and EDS values, indicating an influence of the steroid on the stomatal shape, making it more elliptical and improving its functionality (Martins et al., 2015). Fonseca et al. (2020) also obtained decreases in PDS (4%) and EDS (10%) of *Oryza sativa* plants treated with EBR and stressed by acid rain, which the authors suggested lower acid entry through the ostiole.

Increments verified in SOD, CAT, APX and POX activities in plants treated with EBR and subjected to Pb exposure indicate benefits associated with the antioxidant system linked to pretreatment with steroids. This substance enhanced antioxidant enzymes that detoxify and reduce the ROS accumulation generated by heavy metal stress, including Pb (Vázquez et al., 2013). Our study found a reduction in H_2O_2 and an increase in ETR after treatment with EBR. ROS overproduction is a common occurrence during metabolic stress, causing several negative modifications, including proteins, lipids, carbohydrates and DNA, resulting in oxidative damage (Lee and Park, 2012). Corroborating this study, Bajguz (2010) working with *C. vulgaris* exposed to several concentrations of heavy metals, including Pb (1 μM and 100 μM), obtained peroxidation of the chloroplast membranes caused by the increase in ROS production. After the application of 0.01 μM EBR, the antioxidant activity was increased, suggesting that the antioxidant enzymes contribute to the tolerance mechanism to stress. Ramakrishna and Rao (2015) evaluated antioxidant metabolism in *Raphanus sativus* plants stressed with 5 mM Zn and sprayed with three EBR concentrations (0.5, 1.0 and 2.0 μM) and described increases in the activities of antioxidant enzymes, including SOD, CAT, APX and POX.

EBR application decreased O_2^- and H_2O_2 levels in plants stressed with Pb, being effective in relation to the antioxidant system, triggered by the increases in SOD, CAT, APX and POX activities, resulting in reductions in MDA and EL. SOD acts as an O_2^- scavenger, generating H_2O_2, which CAT, APX and POX enzymes can remove (Ramakrishna and Rao, 2015). Pb stress promotes the overproduction of ROS, causing oxidative damage to membrane lipids, proteins, chloroplast pigments, enzymes and nucleic acids (Srivastava et al., 2014). Kohli et al. (2019) studied the stress indicators and osmoprotectants in *Brassica Juncea* plants and reported that EBR (0.1 mM) mitigated the toxic effects on O_2^-, H_2O_2 and MDA provoked by three Pb concentrations, with verified reductions of 43%, 36% and 53%, respectively. Zhou et al. (2018) evaluated the effect of pretreatment with EBR (0.10 mg L^{-1}) on oxidative damage and antioxidant metabolism in *Vitis vinifera* cuttings under 120 μM Cu stress, obtaining significant decreases in O_2^-, H_2O_2 and MDA in roots and leaves.

The biomass of rice plants was stimulated by the EBR spray, mitigating the deleterious effects connected to Pb on SDM, RDM and TDM. These results are clearly linked to multiple roles unraveled by steroids in pigments, chlorophyll fluorescence (Φ_{PSII}, q_p and ETR), gas exchange (P_N and P_N/C_i), nutritional status and reductions in oxidative damage. In general, Pb partially accumulates in root cells and is blocked by Caspary strips in the endoderm, but when transported to shoots, it adheres mainly to vascular bundles (Alves et al., 2008; Kumar et al., 2012). Nazir et al. (2019) obtained increases in growth (SDM and RDM) of *Solanum lycopersicum* plants treated with 10^{-8} M EBR and exposed to Ni (10 mg kg^{-1} soil). Sharma and Bhardwaj (2007) found similar results on the biomass of shoots and roots in *Brassica juncea* treated with 10^{-9} M EBR and submitted to four Co concentrations (0, 25, 50 and 100 mg kg^{-1} soil).

5. Conclusions

Our results revealed that EBR alleviated the negative interferences caused by Pb excess, which was confirmed by the improvements in the root structures and antioxidant system. This steroid modulated root anatomy, mainly the epidermis and aerenchyma; these structures act as barriers, protecting the root and minimizing Pb translocation to the shoot. Synergistically, EBR promoted significant increases in all antioxidant enzymes (SOD, CAT, APX and POX), reducing the oxidative compounds (MDA and EL), clearly stimulating the redox mechanism, and consequently decreasing the oxidative damage to the photosynthetic machinery, as confirmed by incremental changes in chlorophyll content and CO_2 fixation obtained in this research. Therefore, our results demonstrated that exogenous EBR treatment attenuates the impact provoked by Pb stress in rice plants.

Author statement

AKSL was the advisor of this project, planning all phases of the research and critically revised the manuscript. FRCMG, CFM, BRSS and BLB conducted the experiment and performed physiological, biochemical, anatomical, nutritional and morphological determinations, as well as wrote and edited the manuscript. MNA and PA critically revised the manuscript. All authors read and approved final version of manuscript.

Data availability statement

Data are available upon request to the corresponding author.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.116992.

RET = Root epidermis thickness; RXT = Root exodermis thickness; RDT = Root endodermis thickness; RCT = Root cortex thickness; RAA = Root aerenchyma area; VCD = Vascular cylinder diameter; RMD = Root metaxylem diameter; ETAd = Epidermis thickness from adaxial leaf side; ETAb = Epidermis thickness from abaxial leaf side; MT = Mesophyll thickness; LAA = Leaf arenchyma 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.

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