Physiol Mol Biol Plants (January–March 2017) 23(1):59–72
DOI 10.1007/s12298-016-0410-y

RESEARCH ARTICLE

Brassinosteroids improve photosystem II efficiency, gas exchange, antioxidant enzymes and growth of cowpea plants exposed to water deficit

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Received: 8 September 2016 / Revised: 9 December 2016 / Accepted: 23 December 2016 / Published online: 4 January 2017

Abstract Water deficit is considered the main abiotic stress that limits agricultural production worldwide. Brassinosteroids (BRs) are natural substances that play roles in plant tolerance against abiotic stresses, including water deficit. This research aims to determine whether BRs can mitigate the negative effects caused by water deficiency, revealing how BRs act and their possible contribution to increased tolerance of cowpea plants to water deficit. The experiment was a factorial design with the factors completely randomised, with two water conditions (control and water deficit) and three levels of brassinosteroids (0, 50 and 100 nM 24-epibrassinolide; EBR is an active BRs). Plants sprayed with 100 nM EBR under the water deficit presented significant increases in Φ_{PSII}, q_P and ETR compared with plants subjected to the water deficit without EBR. With respect to gas exchange, P_N, E and g_s exhibited significant reductions after water deficit, but application of 100 nM EBR caused increases in these variables of 96, 24 and 33%, respectively, compared to the water deficit + 0 nM EBR treatment. To antioxidant enzymes, EBR resulted in increases in SOD, CAT, APX and POX, indicating that EBR acts on the antioxidant system, reducing cell damage. The water deficit caused significant reductions in Chl *a*, Chl *b* and total Chl, while plants sprayed with 100 nM EBR showed significant increases of 26, 58 and 33% in Chl *a*, Chl *b* and total Chl, respectively. This study revealed that EBR improves photosystem II efficiency, inducing increases in Φ_{PSII}, q_P and ETR. This substance also mitigated the negative effects on gas exchange and growth induced by the water deficit. Increases in SOD, CAT, APX and POX of plants treated with EBR indicate that this steroid clearly increased the tolerance to the water deficit, reducing reactive oxygen species, cell damage, and maintaining the photosynthetic pigments. Additionally, 100 nM EBR resulted in a better dose–response of cowpea plants exposed to the water deficit.

Keywords Antioxidant system · Brassinosteroids · Net photosynthetic rate · Quantum yield of photosystem II · *Vigna unguiculata* · Water deficiency

Abbreviations

APX	Ascorbate peroxidase
BRs	Brassinosteroids
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
EL	Electrolyte leakage
ETR	Electron transport rate
ETR/P_N	Ratio between the 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

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F_v/F_m	Maximal quantum yield of PSII photochemistry
g_s	Stomatal conductance
H_2O_2	Hydrogen peroxide
LDM	Leaf dry matter
MDA	Malondialdehyde
NPQ	Nonphotochemical quenching
O_2	Superoxide
P_N	Net photosynthetic rate
P_N/C_i	Instantaneous carboxylation efficiency
POX	Peroxidase
PSII	Photosystem II
q_P	Photochemical quenching coefficient
RDM	Root dry matter
ROS	Reactive oxygen species
RUBISCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SDM	Stem dry matter
SOD	Superoxide dismutase
TDM	Total dry matter
Total Chl	Total chrolophyllchlorophyll
WUE	Water-use efficiency
Φ_{PSII}	Effective quantum yield of PSII photochemistry
Ψ_w	Leaf water potential

Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.] is one of the most important legume species used in human food and animal feed and is largely cultivated in semi-arid regions due to its broad adaptability and low water and nutrient requirements (Agele et al. 2006; Manivannan et al. 2007; Barbosa et al. 2013). The grains represent the focus of this culture, with socioeconomic importance due to their high content of proteins, carbohydrates, vitamins, and minerals, such as phosphorus and potassium, compared with other legumes (Phillips et al. 2003; Iqbal et al. 2006; Frota et al. 2008).

Water deficit is considered the main abiotic stress that limits agricultural production worldwide (Inman-Bamber and Smith 2005). This stress often causes molecular, biochemical and physiological modifications (Marinho et al. 2016; Boughalleb et al. 2016; Pereira et al. 2016) that negatively affect metabolism (Perlikowski et al. 2016), reducing the growth and development (Mansori et al. 2015), as well as the crop yield (Luo et al. 2016). Water limitations reduce the water potential (Fernandes-Silva et al. 2016), lower the photosynthetic activity (Bertolli et al. 2012), affect stomatal closing (Spinelli et al. 2016), affect the accumulation of reactive oxygen species (Yi et al. 2016), cause cell damages (Toscano et al. 2016) and depending on the exposure time and intensity, can cause plant death (Chaves et al. 2003; Shao et al. 2008).

Brassinosteroids (BRs), compounds characterized as polyhydroxy steroids, occur in several plant organs, such as the leaf, root, flower and seed (Sasse 2003; Kagale et al. 2007; Bajguz and Hayat 2009). BRs, a class of phytohormones characterized as natural substances essential to plant growth and development (Khripach et al. 2000; Li and Feng 2011), play a role in the regulation of metabolic processes, such as respiration (Derevyanchuk et al. 2015).

BRs play roles in plant tolerance under abiotic stresses, such as salinity in *Brassica juncea* (Alyemeni et al. 2013), metal toxicity in *Raphanus sativus* (Ramakrishna and Rao 2015), high temperature in *Vigna radiata* (Hayat et al. 2010), and low light intensity in *Lycopersicon esculentum* (Cui et al. 2016). With respect to water deficit in particular, BRs have been shown to mitigate the negative effects on gas exchange of *Brassica juncea* plants, increasing the photosynthetic rate, stomatal conductance and water-use efficiency (Fariduddin et al. 2009). In addition, this substance also can reduce the oxidative damages due to increases in antioxidant enzyme activities in *Lycopersicon esculentum* plants (Yuan et al. 2010).

Our hypothesis was based on problems caused by water deficit and considered the possible beneficial role played by 24-epibrassinolide (EBR; an active BRs) in metabolism. Therefore, this research aims to determine whether EBR can mitigate the negative effects caused by water deficiency, revealing how EBR acts and its possible contribution to increasing the tolerance of cowpea plants to water deficit.

Materials and methods

Location and growth conditions

The experiment was performed at the Campus of Paragominas of the Universidade Federal Rural da Amazônia, Paragominas, Brazil (2°55′ S, 47°34′ W). The study was conducted in a greenhouse under controlled temperature and humidity conditions; the minimum, maximum, and median temperatures were 23, 32 and 26.5 °C, respectively. The air relative humidity during the experimental period varied between 60 and 80%.

Plants, containers and acclimation

Seeds of *Vigna unguiculata* L. cv. BR3-Tracuateua were germinated and grown in 1.2-L pots (0.15 m in height and 0.10 m in diameter) filled with a mixed substrate of sand and vermiculite in a 3:1 ratio. Plants were cultivated under semi-hydroponic conditions, and the pots had one hole at

the bottom, which was covered with mesh to maintain the substrate and aerate the roots. Solution absorption occurred by capillarity; these pots were placed into other containers (0.15 m in height and 0.15 m in diameter) containing 500 mL of distilled water for five d. Modified Hoagland and Arnon's (1950) solution was used as a source of nutrients; the ionic strength started at 50% and was modified to 100% after 1 day. Subsequently, the nutrient solution remained at total ionic strength.

Experimental design

The experiment was a factorial design with the factors completely randomised, with two water conditions (control and water deficit) and three levels of brassinosteroids (0, 50 and 100 nM EBR). With five replicates for each of six treatments, a total of 30 experimental units were used in the experiment, with one plant in each unit. The brassinosteroids concentrations (0, 50 and 100 nM EBR) used in our research were defined in concordance with study of Amzallag and Vaisman (2006), while the application interval (six days) was determined by the responses obtained in previous studies with *Vigna unguiculata* plants.

24-epibrassinolide (EBR) preparation and application

6-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) at 6-day intervals until day 18. The 0, 50 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). On day 18 after the experiment was initiated, the plants in the water deficit treatment were subjected to water restriction.

Plant conduction and water deficit treatment

One plant per pot was used to examine the plant parameters. The plants received the following macro- and micronutrients contained in the nutrient solution: 8.75 mM KNO_3, 7.5 mM $Ca(NO_3)_2{\cdot}4H_2O$, 3.25 mM $NH_4H_2PO_4$, 1.5 mM $MgSO_4{\cdot}7\ H_2O$, 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 $CuSO_4{\cdot}5H_2O$, 0.63 µM $NaMoO_4{\cdot}5H_2O$, and 250.0 µM $NaEDTAFe{\cdot}3H_2O$. To simulate the water deficit, the solution was removed completely, the root system was placed in similar pots without water/solution, and the water deficit was applied within 2 day (days 18–20 after the start of the experiment). During the study, the nutrient solutions were changed at 07:00 h at 3-day intervals, with the pH adjusted to 5.5 using HCl or NaOH. On day 20 of the experiment, physiological and morphological parameters were measured for all plants, and leaf tissues were harvested for nutritional and biochemical analyses.

Measurement of chlorophyll fluorescence

The minimal fluorescence yield of the dark-adapted state (F_0), maximal fluorescence yield of the dark-adapted state (F_m), variable fluorescence (F_v), maximal quantum yield of PSII photochemistry (F_v/F_m), effective quantum yield of PSII photochemistry (Φ_{PSII}), photochemical quenching coefficient (q_P), nonphotochemical quenching (NPQ), electron transport rate (ETR), relative energy excess at the PSII level (EXC) and the ratio between electron transport rate and net photosynthetic rate (ETR/P_N) were determined using an modulated chlorophyll fluorometer (model OS5p; Opti-Sciences). Chlorophyll fluorescence was measured using fully expanded leaves under light conditions. 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; consequently, the third acropetal leaf from the middle third of the plant 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.

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 on 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, airflow rate and temperature in a chamber set 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.

Leaf water potential

The leaf water potential (Ψ_w) was measured using fully expanded leaves located in the middle region of the plant and exposed to light, during the period between 11:30 to 12:00 h, which corresponded to midday potential. To determinate the Ψ_w, one leaf per plant and five plants per

treatment were measured using an analogue plant moisture system (PMS Instrument Company, model 600). This system is based on the pressure chamber technique (Scholander et al. 1964), and the procedure outlined by Turner (1988) was followed.

Extraction of antioxidant enzymes, superoxide and soluble proteins

Antioxidant enzymes (SOD, CAT, APX and POX), superoxide and soluble proteins were extracted from the leaf tissue following the method of Badawi et al. (2004). The extraction mixture was prepared by homogenizing 500 mg of fresh plant material in 5 ml of extraction buffer, consisting 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 total soluble proteins was performed using the method described by Bradford (1976). The absorbance was measured at 595 nm, using bovine albumin as a standard.

Superoxide dismutase assay

For the SOD (EC 1.15.1.1) assay, 2.8 ml of 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).

Catalase assay

For the CAT (EC 1.11.1.6) assay, 0.2 ml of supernatant and 1.8 ml of 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).

Ascorbate peroxidase assay

For the APX (EC 1.11.1.11) assay, 1.8 ml of 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).

Peroxidase assay

For the POX (EC 1.11.1.7) assay, 1.78 ml of reaction mixture containing 50 mM phosphate buffer (pH 7.0) and 0.05% guaiacol was mixed with 0.2 ml of supernatant, followed by the addition of 20 µL of 10 mM hydrogen peroxide. The absorbance was then measured at 470 nm (Cakmak and Marschner 1992).

Determination of superoxide concentration

To determine 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, an identical volume of ethyl ether was added and centrifuged at 3000×*g* for 5 min. The absorbance was measured at 530 nm (Elstner and Heupel 1976).

Extraction of nonenzymatic compounds

Nonenzymatic compounds (H_2O_2 and MDA) were extracted as described by Wu et al. (2006). Briefly, a mixture to extract H_2O_2 and MDA was prepared by homogenising 500 mg of fresh leaf material in 5 mL of 5% (w/v) trichloroacetic acid. Then, the samples were centrifuged at 15,000×*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 1000 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, after which the reaction was 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 an extinction coefficient of 155 mM^{-1} cm^{-1} was used.

Determination of electrolyte leakage

Electrolyte leakage was measured according to the method of Gong et al. (1998), with minor modifications. Fresh leaves (200 mg) were 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) × 100.

Determination of photosynthetic pigments

The chlorophyll and carotenoid determinations were performed using 40 mg of leaf tissue. The samples were homogenised in the dark with 8 mL of 90% methanol (Nuclear). The homogenate was centrifuged at 6000×*g* for 10 min at 5 °C. The supernatant was removed, and the chlorophyll *a* (Chl *a*) and *b* (Chl *b*), and 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

Root, stem and leaf growth was measured based on constant dry weights (g) obtained after drying in a forced-air ventilation oven at 65 °C.

Data analysis

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

Results

Effects of water deficit and EBR on chlorophyll fluorescence

Plants subjected to the water deficit exhibited a reduction in Ψ_w. However, the application of EBR diminished the effects of the water restriction, increasing the Ψ_w measured in response to 100 nM EBR by 40% (Fig. 1a) compared with the water deficit + 0 nM EBR treatment. There was a significant difference in F_v/F_m between the water conditions. The plants sprayed with EBR had higher F_v/F_m values, mainly at a concentration of 100 nM EBR (Fig. 1b). Significant differences were observed in F_0 after the water deficit was imposed, and there was a more intense reduction (15%) in the 100 nM EBR treatment compared with the water deficit + 0 nM EBR treatment (Fig. 1c). The application of 100 nM EBR increased F_m in the control and water deficit plants by 29 and 32%, respectively. The water deficit induced a significant reduction in values compared with the respective controls (Fig. 1d).

The Φ_{PSII}, q_P and ETR values decreased in response to the water deficit; significant differences were detected relative to the control plants at the same concentration of EBR. However, plants sprayed with 100 nM EBR under the water deficit exhibited significantly higher values for these variables, i.e., 74, 112 and 72%, respectively, compared with the water deficit + 0 nM EBR treatment (Table 1). Significant increases in NPQ, EXC and ETR/P_N occurred in response to the water deficit; however, the application of 100 nM EBR resulted in decreases of 30, 19 and 12%, respectively, compared with the water deficit + 0 nM EBR treatment (Table 1).

Improvements in the gas exchange of plants subjected to the water deficit

P_N, E and g_s were significantly reduced by the water restriction. However, 100 nM EBR caused increases of 96, 24 and 33%, respectively, in these variables compared with the water deficit + 0 nM EBR treatment (Table 2). The C_i levels increased with the water deficit, but the application of 100 nM EBR resulted in a significant decrease of 18% compared with the water deficit treatment without EBR (Table 2). Plants subjected to the water deficit had lower WUE and P_N/C_i values, whereas the application of 100 nM EBR caused increases of 49 and 141%, respectively, compared with the water deficit + 0 nM EBR treatment (Table 2).

EBR increase the activities of antioxidant enzymes

The SOD activity increased as a result of the water deficit, and EBR caused a variation of 25% in the activity of this enzyme in plants subjected to the water deficit + 100 nM EBR treatment (Fig. 2a) compared with those subjected to the water deficit + 0 nM EBR treatment. The CAT activity increased significantly in response to the water deficit; there was a variation of 29% in plants exposed to the water deficit + 100 nM EBR treatment (Fig. 2b) in comparison to the water deficit + 0 nM EBR treatment. The application of EBR resulted in a 50% increase in APX in the water deficit + EBR 100 nM treatment, and the water deficit produced strong increases in APX activity compared with the control plants (Fig. 2c). There were significant differences in POX between the water deficit treatments, and the water deficit + 100 nM EBR

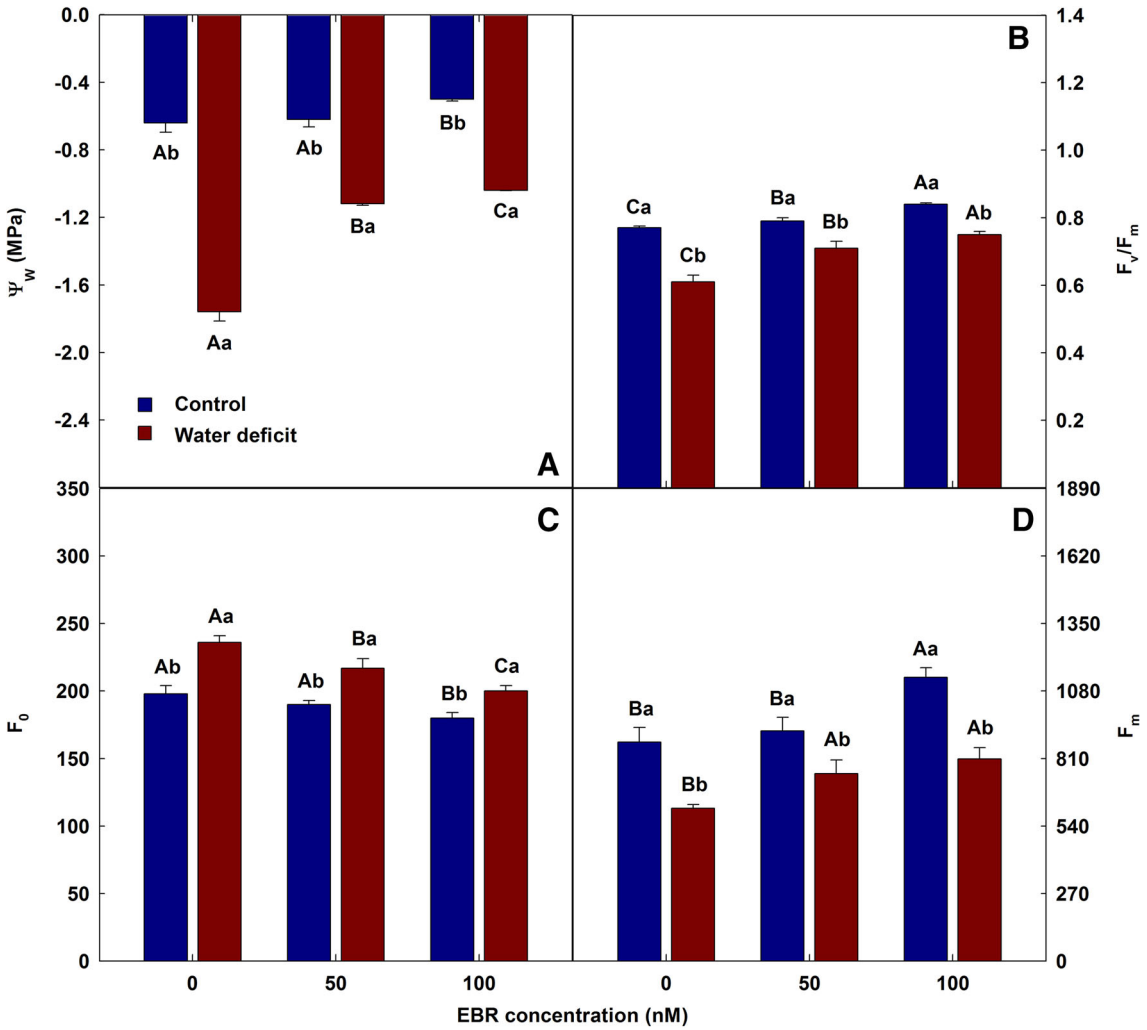

Fig. 1 Leaf water potential (**a**), maximal quantum yield of PSII photochemistry (**b**), minimal fluorescence yield of the dark-adapted state (**c**) and maximal fluorescence yield of the dark-adapted state (**d**) in *Vigna unguiculata* plants splayed with EBR and exposed to water deficit. Different *uppercase letters* between EBR levels (0, 50 and 100 nM EBR under equal water condition) and *lowercase letters* between water conditions (control and water deficit under equal EBR concentration) indicate significant differences from the Scott-Knott test ($P < 0.05$). Columns described corresponding to means from five repetitions and standard deviations

Table 1 Chlorophyll fluorescence in *Vigna unguiculata* plants splayed with EBR and exposed to water deficit

Water condition	EBR (nM)	Φ_{PSII}	Q_P	NPQ	ETR (μmol m^{-2} s^{-1})	EXC (μmol m^{-2} s^{-1})	ETR/P_N
Control	0	0.40 ± 0.03Ba	0.79 ± 0.06Ba	0.82 ± 0.07Ab	59.0 ± 5.1Ba	0.47 ± 0.03Ab	3.81 ± 0.29Ab
Control	50	0.42 ± 0.01Ba	0.86 ± 0.05Ba	0.79 ± 0.07Ab	62.3 ± 3.4Ba	0.46 ± 0.03Ab	3.65 ± 0.16Ab
Control	100	0.46 ± 0.02Aa	0.97 ± 0.04Aa	0.78 ± 0.06Ab	68.7 ± 2.2Aa	0.44 ± 0.02Ab	3.57 ± 0.14Ab
Water deficit	0	0.19 ± 0.01Cb	0.25 ± 0.04Cb	1.51 ± 0.11Aa	28.5 ± 0.8Cb	0.68 ± 0.01Aa	5.45 ± 0.18Aa
Water deficit	50	0.27 ± 0.01Bb	0.37 ± 0.06Bb	1.09 ± 0.05Ba	39.9 ± 1.6Bb	0.61 ± 0.03Ba	4.98 ± 0.21Ba
Water deficit	100	0.33 ± 0.02Ab	0.53 ± 0.06Ab	1.06 ± 0.04Ba	49.0 ± 3.0Ab	0.55 ± 0.03Ca	4.81 ± 0.24Ba

Φ_{PSII} Effective quantum yield of PSII photochemistry; q_P Photochemical quenching coefficient; *NPQ* Nonphotochemical quenching; *ETR* Electron transport rate; *EXC* Relative energy excess at the PSII level; ETR/P_N Ratio between the electron transport rate and net photosynthetic rate. Columns with different uppercase letters between EBR levels (0, 50 and 100 nM EBR under equal water condition) and lowercase letters between water conditions (control and water deficit under equal EBR concentration) indicate significant differences from the Scott–Knott test ($P < 0.05$). Values described corresponding to means from five repetitions and standard deviations

Table 2 Gas exchange in *Vigna unguiculata* plants splayed with EBR and exposed to water deficit

Water condition	EBR (nM)	P_N (μmol m^{-2} s^{-1})	E (mmol m^{-2} s^{-1})	G_s (mol m^{-2} s^{-1})	C_i (μmol mol^{-1})	WUE (μmol $mmol^{-1}$)	P_N/C_i (μmol m^{-2} s^{-1} Pa^{-1})
Control	0	15.6 ± 0.5Ca	3.38 ± 0.02Aa	0.37 ± 0.02Aa	247 ± 15Ab	4.62 ± 0.12Ca	0.063 ± 0.005Ba
Control	50	17.0 ± 0.7Ba	3.38 ± 0.05Aa	0.36 ± 0.01Aa	247 ± 20Ab	5.03 ± 0.15Ba	0.065 ± 0.004Ba
Control	100	19.2 ± 0.4Aa	3.46 ± 0.07Aa	0.38 ± 0.02Aa	247 ± 17Aa	5.55 ± 0.16Aa	0.078 ± 0.006Aa
Water deficit	0	5.2 ± 0.2Cb	1.42 ± 0.09Bb	0.09 ± 0.01Bb	303 ± 10Aa	3.88 ± 0.18Cb	0.017 ± 0.001Cb
Water deficit	50	8.0 ± 0.4Bb	1.67 ± 0.09Ab	0.12 ± 0.01Ab	272 ± 15Ba	4.81 ± 0.22Ba	0.029 ± 0.003Bb
Water deficit	100	10.2 ± 0.5Ab	1.76 ± 0.08Ab	0.12 ± 0.01Ab	249 ± 22Ba	5.80 ± 0.31Aa	0.041 ± 0.003Ab

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 uppercase letters between EBR levels (0, 50 and 100 nM EBR under equal water condition) and lowercase letters between water conditions (control and water deficit under equal EBR concentration) indicate significant differences from the Scott–Knott test ($P < 0.05$). Values described corresponding to means from five repetitions and standard deviations

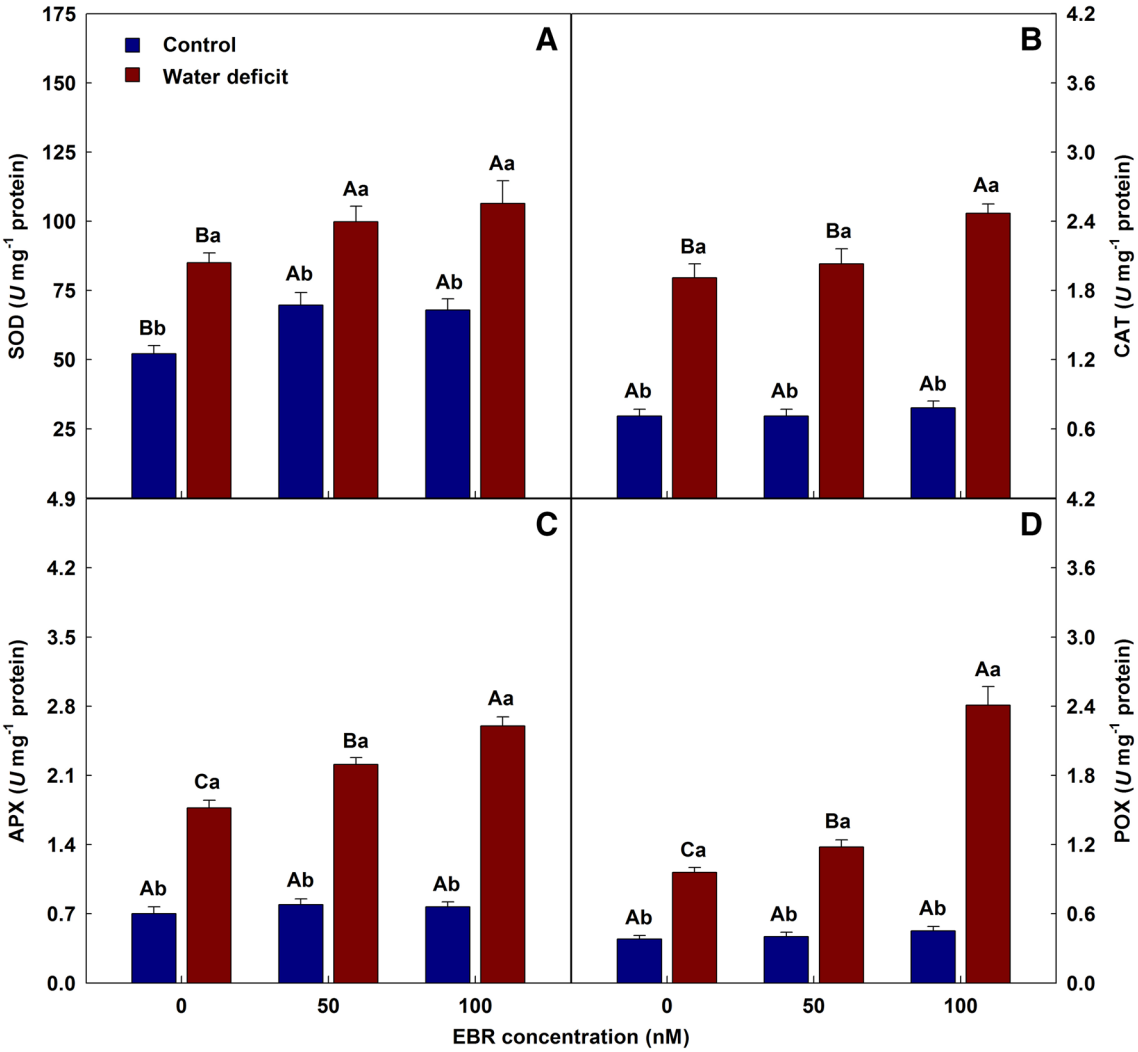

Fig. 2 Activities of superoxide dismutase (**a**), catalase (**b**), ascorbate peroxidase (**c**) and peroxidase (**d**) in *Vigna unguiculata* plants splayed with EBR and exposed to water deficit. Different *uppercase letters* between EBR levels (0, 50 and 100 nM EBR under equal water condition) and *lowercase letters* between water conditions (control and water deficit under equal EBR concentration) indicate significant differences from the Scott–Knott test ($P < 0.05$). Columns described corresponding to means from five repetitions and standard deviations

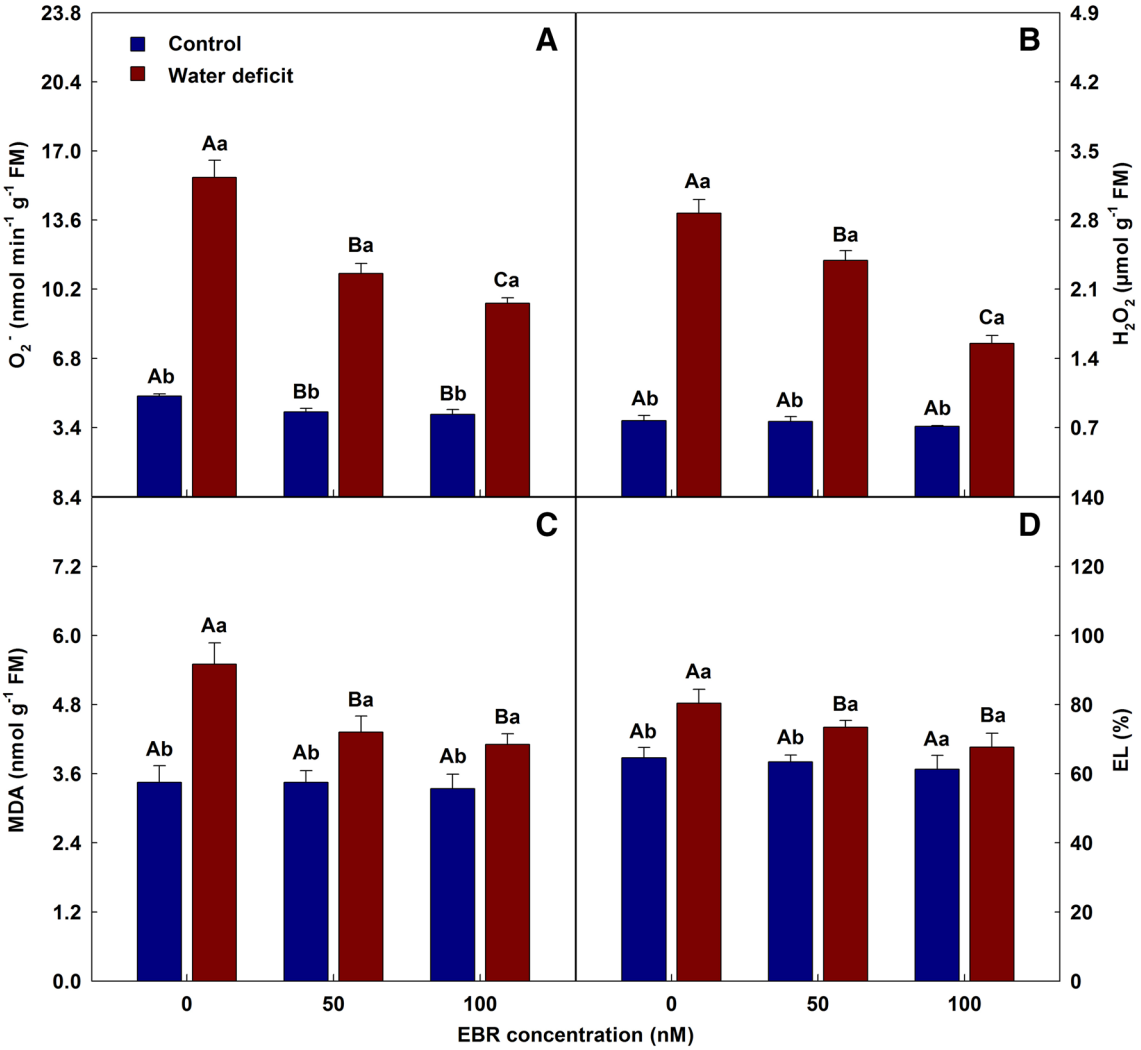

Fig. 3 Superoxide (**a**), hydrogen peroxide (**b**), malondialdehyde (**c**) and electrolyte leakage (**d**) in *Vigna unguiculata* plants splayed with EBR and exposed to water deficit. Different *uppercase letters* between EBR levels (0, 50 and 100 nM EBR under equal water condition) and *lowercase letters* between water conditions (control and water deficit under equal EBR concentration) indicate significant differences from the Scott–Knott test ($P < 0.05$). Columns described corresponding to means from five repetitions and standard deviations

treatment resulted in a significant increase in POX (149%) compared with plants in the water deficit + 0 nM EBR treatment (Fig. 2d).

Reduced production of oxidant compounds and cell damage induced by EBR

The water deficit caused an increase in the O_2^- levels of the *Vigna unguiculata* plants, but the application of EBR reduced this effect. For example, the plants that received 100 nM EBR exhibited a 39% reduction relative to the water deficit + 0 nM EBR treatment (Fig. 3a). H_2O_2 levels increased in plants subjected to the water deficit compared with the control treatment, whereas significant reductions were measured in response to the application of EBR: a decrease of 45% was measured in plants subjected to the water deficit + 100 nM EBR treatment compared with the water deficit + 0 nM EBR treatment (Fig. 3b). Plants exposed to the water deficit had significantly higher MDA contents, but the application of 100 nM EBR to plants under the water deficit caused a 25% reduction compared with those subjected to the water deficit and 0 nM EBR (Fig. 3c). In addition, the water deficit caused increases in the EL values, whereas there was a 15% decrease in these values in the water deficit + 100 nM EBR treatment compared with the water deficit + 0 nM EBR treatment (Fig. 3d).

Maintenance of pigments in plants pretreated with EBR

The water deficit caused significant reductions in Chl *a*, Chl *b* and total Chl, but these effects were attenuated by

Table 3 Photosynthetic pigments in *Vigna unguiculata* plants splayed with EBR and exposed to water deficit

Water condition	EBR (nM)	Chl *a* (mg g^{-1} FM)	Chl *b* (mg g^{-1} FM)	Total Chl (mg g^{-1} FM)	Car (mg g^{-1} FM)
Control	0	7.33 ± 0.37Ba	1.71 ± 0.05Ca	9.04 ± 0.48Ba	0.78 ± 0.06Aa
Control	50	8.49 ± 0.59Aa	1.86 ± 0.07Ba	10.22 ± 0.58Aa	0.84 ± 0.05Aa
Control	100	8.57 ± 0.50Aa	2.07 ± 0.09Aa	10.64 ± 0.52Aa	0.80 ± 0.07Aa
Water deficit	0	3.42 ± 0.28Bb	0.96 ± 0.06Cb	4.38 ± 0.34Bb	0.34 ± 0.02Bb
Water deficit	50	3.84 ± 0.20Bb	1.12 ± 0.07Bb	4.96 ± 0.28Bb	0.54 ± 0.04Ab
Water deficit	100	4.32 ± 0.18Ab	1.52 ± 0.08Ab	5.84 ± 0.39Ab	0.56 ± 0.04Ab

Chl a Chlorophyll *a*; *Chl b* Chlorophyll *b*; *Total Chl* Total chlorophyll; *Car* Carotenoids. Columns with different uppercase letters between EBR levels (0, 50 and 100 nM EBR under equal water condition) and lowercase letters between water conditions (control and water deficit under equal EBR concentration) indicate significant differences from the Scott–Knott test ($P < 0.05$). Values described corresponding to means from five repetitions and standard deviations

Table 4 Growth in *Vigna unguiculata* plants splayed with EBR and exposed to water deficit

Water condition	EBR (nM)	LDM (g)	RDM (g)	SDM (g)	TDM (g)
Control	0	1.04 ± 0.04Aa	1.54 ± 0.11Aa	0.63 ± 0.03Aa	3.21 ± 0.14Aa
Control	50	1.08 ± 0.06Aa	1.60 ± 0.14Aa	0.64 ± 0.05Aa	3.32 ± 0.21Aa
Control	100	1.08 ± 0.06Aa	1.63 ± 0.08Aa	0.64 ± 0.05Aa	3.35 ± 0.10Aa
Water deficit	0	0.96 ± 0.02Bb	1.18 ± 0.06Ab	0.59 ± 0.01Ba	2.73 ± 0.06Bb
Water deficit	50	1.02 ± 0.02Aa	1.26 ± 0.07Ab	0.64 ± 0.03Aa	2.92 ± 0.10Ab
Water deficit	100	1.07 ± 0.07Aa	1.30 ± 0.08Ab	0.63 ± 0.02Aa	3.00 ± 0.08Ab

LDM Leaf dry matter; *RDM* Root dry matter; *SDM* Stem dry matter; *TDM* Total dry matter. Columns with different uppercase letters between EBR levels (0, 50 and 100 nM EBR under equal water condition) and lowercase letters between water conditions (control and water deficit under equal EBR concentration) indicate significant differences from the Scott-Knott test ($P < 0.05$). Values described corresponding to means from five repetitions and standard deviations

EBR. Plants exposed to the water deficit that received 100 nM EBR showed significant increases of 26, 58 and 33% for Chl *a*, Chl *b* and total Chl, respectively, compared with the water deficit + 0 nM EBR treatment (Table 3). The Car levels decreased as a result of the water deficit, but the application of 100 nM EBR increased the Car levels by 65% compared with the 0 nM EBR treatment in plants that were exposed to the water deficit (Table 3).

EBR mitigates the effect of the water deficit on growth

The application of EBR resulted in significant differences in the LDM and SDM of plants under the water deficit; specifically, the application of 100 nM EBR caused increases of 11 and 7%, respectively, compared with the water deficit + 0 nM EBR treatment (Table 4). The water restriction reduced the RDM values, but spraying with 100 nM EBR resulted in increases of 10% compared with the control subjected to the water deficit (Table 4). The water deficit significantly affected the TDM, and the water deficit + 100 nM EBR treatment exhibited a significant increase of 10% compared with the water deficit + 0 nM EBR treatment (Table 4).

Discussion

The application of EBR mitigated the adverse effects on the Ψ_w caused by the water deficit. This response is associated with the osmotic adjustment process, which involves the accumulation of soluble carbohydrates, such as starch and sucrose (Yu et al. 2004). Nascimento et al. (2011) studied the consequences of a water deficit on 20 genotypes of *Vigna unguiculata* and reported a 49.3% reduction in the Ψ_w of the Tracuateua-192 genotype. Zhang et al. (2008), who worked with *Glycine max* plants under two soil moisture levels, observed a positive Ψ_w response after the application of EBR.

EBR reduced the impact of the water deficit on *V. unguiculata* plants and minimized the negative effects on F_v/F_m, F_0 and F_m. This steroid resulted in a higher water retention in the tissues, shown by increases in the Ψ_w of plants treated with EBR. Increases in F_v/F_m and F_m after spraying with EBR suggest beneficial effects on the reaction centre of PSII and subsequent mitigation of the photoinhibitory process resulting from the water deficit (Maxwell and Johnson 2000; Qiu et al. 2013). Plants treated with EBR had lower F_0 values due to the increase in the flow of photons from the collector system to the

reaction centres of PSII (Baker and Rosenqvist 2004). Wang et al. (2015) observed that EBR had positive effects on F_v/F_m and F_0 in *Vitis vinifera* exposed to a water deficit. Supporting our study, Souza et al. (2004) reported similar results for *V. unguiculata* plants under water deficit and rehydration, where F_v/F_m and F_m decreased and F_0 increased as a result of water restriction for 7 days followed by rehydration for three consecutive days.

The EBR application increased the Φ_{PSII}, q_P and ETR values in plants under the water deficit and control conditions, which can be explained by positive effects of EBR on F_0 and F_m observed in this study. Plants treated with EBR exhibited increases in ETR and q_P, which are related to higher energy absorption of photons and subsequent increased flow of energy for the excitation of electrons accepted by plastoquinone (Buonasera et al. 2011). Thussagunpanit et al. (2015a) studied the action mechanisms of EBR in *Oryza sativa* plants and reported increases in Φ_{PSII} after EBR application. Research conducted by Li et al. (2015) showed that EBR increases the proportion of open PSII reaction centres, improving the efficiency of the capture of light energy for the electron transport chain. Rivas et al. (2016) observed reductions in q_P and ETR in *V. unguiculata* exposed to a water deficit due to lower activation of enzymes linked to carboxylation as well as limited extinction of fluorescence during photochemical processes.

Reductions in NPQ, EXC and ETR/P_N values in plants exposed to the water deficit + EBR are related to lower non-photochemical energy in the form of heat (Ribeiro et al. 2009). In addition, less quenching, mainly through photorespiration and secondary metabolites, such as the photoreduction of O_2 to O_2^- (Silva et al. 2011; Barbosa et al. 2014) probably occurred in plants exposed to EBR. The reduction of ETR/P_N in plants treated with EBR indicates that this steroid influences chlorophyll fluorescence and gas exchange. Guan et al. (2014) evaluated early cultivars of *Triticum aestivum* under water deficit conditions and found increases in the NPQ values, which were related to increased thermal dissipation. The use of EBR caused a reduction in EXC due to the decrease in NPQ, which can be explained by the higher efficiency in the light capture by PSII (Silva et al. 2012). Sales et al. (2013) conducted a study on the recovery of photosynthesis in *Saccharum officinarum* plants subjected to a water deficit and a low temperature substrate and reported a 25% increase in EXC. Corroborating our research, Singh and Reddy (2011), who investigated the regulation of chlorophyll fluorescence in *V. unguiculata* exposed to a water deficit, detected an increase of approximately 200% in ETR/P_N.

Plants exposed to the water deficit + EBR exhibited increases in the P_N, E and g_s values, and these results are linked to the benefits provided by the EBR, which improved the efficiency of PSII (Φ_{PSII}) and increased the water status (Ψ_w) in this study. EBR also caused an increase in E and g_s, which was induced by the increase in the Ψ_w previously described. The stomatal mechanism is dependent on the water status of the tissue and has a strong influence on gas exchange (Dias and Brüggemann 2010; Xia et al. 2014). Afzal et al. (2014) evaluated the gas exchange of *Vigna radiata* plants under a water deficit and also found reductions in P_N and g_s. Hu et al. (2013) reported that EBR application alleviates the negative effects on P_N, E and g_s in *Capsicum annuum* plants subjected to a water deficit.

The application of EBR caused reductions in the C_i values of plants exposed to the water deficit, and this response is related to the increase in P_N, suggesting that EBR increased the activity of RUBISCO, the enzyme responsible for intercellular CO_2 assimilation (Yu et al. 2004). Anyia and Herzog (2004) evaluated the gas exchange of *Vigna unguiculata* plants and observed increased C_i in plants subjected to a water deficit. EBR mitigated the negative effects caused by the water deficit and increased the WUE values; these effects resulted from the increases in P_N and E, which were caused by the beneficial actions of EBR. Anjum et al. (2011) reported a 30.4% increase in WUE after the application of EBR to *Zea mays* plants subjected to a water deficit. The P_N/C_i values also increased in plants exposed to the water deficit + EBR, which is directly linked to an increase in P_N and a reduction in C_i; these results have already been described in the current study. Corroborating our research, Farooq et al. (2009) observed increases in WUE and P_N/C_i after the application of EBR to *Oryza sativa* plants under a water deficit.

Plants under the water deficit that were treated with EBR presented increases in SOD, CAT, APX and POX, indicating that EBR alleviated the damage caused to PSII and reduced the photoinhibition. These results are corroborated by the increases in F_v/F_m and ETR and the reduction in NPQ. EBR increases the activity of antioxidant enzymes to mitigate oxidative stress by reducing ROS accumulation (Abedi and Pakniyat 2010; Ramakrishna and Rao 2015). Yuan et al. (2010) and Behnamnia et al. (2009a) observed increases in antioxidant enzyme activities (SOD, CAT, APX and POX) after the application of 0.01 and 1 μM of EBR to *Lycopersicon esculentum* plants subjected to 3 and 5 days, respectively, of a water deficit.

EBR caused decreases in the O_2^- and H_2O_2 concentrations of *Vigna unguiculata* plants subjected to the water deficit. This response is intrinsically related to increases in the activities of antioxidant enzymes that are positively induced by EBR application, aiming to neutralize the accumulation of ROS (Ahammed et al. 2013b).

Additionally, the decrease in EXC reveals lower photoreduction of O_2 to O_2^-. The O_2 reduction occurred due to the increase in SOD, which was activated by the application of EBR. SOD is the first enzyme in plant defence and catalyses the conversion of the O_2^- anion to H_2O_2 (Yusuf et al. 2011). The H_2O_2 concentrations also decreased in the water deficit + EBR treatment, but this response was associated with increases in the activities of CAT, APX and POX, which were related to the positive action of EBR. This reduction occurred through the neutralization of H_2O_2, which was converted into H_2O and O_2, a reaction mediated by CAT, APX and POX (Asada 2006; Hasan et al. 2011). Behnamnia et al. (2009b) studied the effects of two EBR concentrations and reported the benefits of applying 1 µM of EBR, which reduced H_2O_2 in *Lycopersicon esculentum* plants exposed to a 5-day water deficit.

Plants exposed to the water deficit + EBR exhibited reductions in MDA and EL, which can be explained by increases in enzyme activities (SOD, CAT, APX and POX) and reduced levels of ROS (O_2^- and H_2O_2), resulting from the exogenous application of EBR. The reduction in EL was related to the decrease in MDA caused by the beneficial action of EBR, indicating minor damages caused to the cell membrane. ROS accumulation induces lipid peroxidation due to a loss of cell membrane integrity, which negatively affects photosynthetic activity (Ye et al. 2016) and maximizes electrolyte leakage in response to stress (Demidchik et al. 2014). Li et al. (2012) observed a reduction in MDA after the application of EBR to *Chorispora bungeana* plants subjected to a water deficit. Research conducted by Mousavi et al. (2009) also showed beneficial effects of EBR, which caused a reduction in EL of *Brassica napus* subjected to a 4-day water deficit.

The foliar application of EBR to *Vigna unguiculata* plants exposed to a water deficit resulted in increases in the photosynthetic pigments (Chl *a*, Chl *b*, total Chl and Car), indicating that EBR attenuated the damage caused to the chloroplast membranes (by MDA and EL) and mitigated the accumulation of ROS (O_2^- and H_2O_2). The application of EBR maintains the photosynthetic pigments, improves photochemical activity, and also balances the distribution of excitation between the photosystems (Zhang et al. 2013). Rajasekar et al. (2016) observed a reduction in the photosynthetic pigments of *Zea mays* plants after a water deficit. Corroborating our study, Thussagunpanit et al. (2015b) observed increases in Chl *a*, Chl *b*, total chl and Car after the application of EBR to *Oryza sativa* plants subjected to heat stress (47 °C) for 7 days.

Exogenous use of EBR resulted in increases in the leaf, root, stem and total dry matter of *Vigna unguiculata* plants exposed to the water deficit. This response is directly linked to the beneficial effects of EBR on chlorophyll fluorescence and gas exchange detected through increases in Φ_{PSII} and P_N. In parallel, EBR improved the antioxidant system, mitigating the accumulation of ROS, in addition to reducing the damage caused to the membranes and photosynthetic pigments. Plants treated with EBR presented greater accumulation of biomass as a result of the increased photosynthetic rates triggered by the efficient energy absorption and proper stomatal regulation (Arora et al. 2008; Shahbaz et al. 2008). Barbosa et al. (2015) reported a reduction in the biomass of *Saccharum* spp. plants subjected to a water deficit. Corroborating our research, Zheng et al. (2016) described positive results of EBR on increases in the leaf, root, stem and total dry matter of *Lycopersicon esculentum* plants under salt stress.

This study revealed that EBR improved photosystem II efficiency, inducing increases in Φ_{PSII}, q_P and ETR. This substance also mitigated the negative effects of the water deficit on gas exchange and growth. Increases in SOD, CAT, APX and POX of plants treated with EBR indicate that this steroid clearly increased the tolerance to the water deficit, reducing reactive oxygen species, cell damage, and maintaining the photosynthetic pigments. Additionally, 100 nM EBR resulted in a better dose–response of cowpea plants exposed to the water deficit.

Acknowledgements This research was financially supported by Fundação Amazônia de Amparo a Estudos e Pesquisas (FAPESPA/Brazil), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brazil) and Universidade Federal Rural da Amazônia (UFRA/Brazil), awarded to A. K. S. Lobato. While J. V. Lima was supported by a graduate scholarship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES/Brazil).

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