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Brassinosteroids mitigate cadmium toxicity in cowpea plants

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Abstract

Anthropogenic activities and improper uses of phosphate fertilizers have led to an increase in cadmium concentrations in agricultural soils. Brassinosteroids are steroid hormones that are rapidly assimilated and metabolised with beneficial roles in physiological and biochemical processes in plants. Our aim was to ascertain whether exogenous treatment with 24-epibrassinolide (EBR) can mitigate the Cd toxicity, and whether this substance can reduce the Cd accumulation in plant tissues. Furthermore, the dose response to EBR was determined following exposure to Cd in *Vigna unguiculata*. The experiment was a completely randomised factorial design with two concentrations of Cd (0 and 500 μM) and three concentrations of EBR (0, 50, and 100 nM). Spraying plants exposed to Cd with EBR significantly reduced the concentrations of Cd and increased nutrient contents in all tissues. The EBR treatment caused significant enhancements in leaf, root, and total dry matter. Foliar application of EBR reduced the negative effects of Cd toxicity on chlorophyll fluorescence and gas exchange parameters. Pretreatment with EBR also increased contents of pigments in plants exposed to Cd, compared with the identical treatments without EBR. Cd elevated contents of oxidant compounds, inducing cell damages, while EBR significantly decreased the concentrations of these compounds. We confirmed that EBR mitigated the negative effects related to Cd toxicity, reduced the absorption and transport of Cd, and increased the contents of essential elements. In plants exposed to Cd, the most apparent dose response was found for 100 nM EBR, with beneficial repercussions on growth, gas exchange, primary photosynthetic processes, and photosynthetic pigments, which were intrinsically connected to lower production of oxidant compounds and cell damage.

Additional key words: cadmium; chlorophyll; net photosynthetic rate; quantum yield of photosystem II; *Vigna unguiculata*.

Introduction

Cadmium (Cd) is a highly toxic heavy metal that has continued to increase in the environment because of anthropogenic activities (Perfus-Barbeoch *et al.* 2002). The improper uses of phosphate fertilizers have largely contributed to the increasing contents of Cd in agricultural soils (Irfan *et al.* 2014). Cadmium often competes with the absorption of other essential minerals (Hernandez *et al.* 1996, Pinto *et al.* 2004) and is easily absorbed and transported to different parts of plants (Gallego *et al.* 2012). Approximately 70% of Cd ingested by humans is from plants (Wagner 1993).

The uptake of Cd by plants can inhibit growth and

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Abbreviations: BCF – bioconcentration factor; BRs – brassinosteroids; Car – carotenoids; Cd_{ns} – Cd concentration in nutritive solution; Cd_{pt} – Cd concentration in plant tissue; Cd_s – Cd concentration in the shoots; Cd_r – Cd concentration in roots; Chl – chlorophyll; C_i – intercellular CO_2 concentration; DAE – day of experiment; E – transpiration rate; EBR – 24-epibrassinolide; EL – electrolyte leakage; ETR – electron transport rate; ETR/P_N – ratio between the apparent electron-transport rate and net photosynthetic rate; EXC – relative energy excess at the PSII level; F_m – maximal fluorescence yield of the dark-adapted state; F_0 – minimal fluorescence yield of the dark-adapted state; F_v – variable fluorescence; F_v/F_m – maximal quantum yield of PSII photochemistry; FM – fresh mass; g_s – stomatal conductance to water vapor; LDM – leaf dry matter; MDA – malondialdehyde; NPQ – nonphotochemical quenching; P_N – net photosynthetic rate; P_N/C_i – instantaneous carboxylation efficiency; q_P – photochemical quenching; RDM – root dry matter; ROS – reactive oxygen species; STM – stem dry matter; TF – translocation factor; TDM – total dry matter; WUE – water-use efficiency; Φ_{PSII} – effective quantum yield of PSII photochemistry;.
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cause death by direct interference in physiological processes (Djebali *et al.* 2005, Wahid *et al.* 2007, Hayat *et al.* 2014). In studies, which examine the effects of Cd on plants, this metal lowers chlorophyll (Chl) biosynthesis (Jain *et al.* 2007), interferes negatively with gas exchange (Wan *et al.* 2011), alters the ultrastructure of chloroplasts (Sandalio *et al.* 2001, Djebali *et al.* 2005), damages root structure (Najeeb *et al.* 2011), leads to an imbalance in the nutrient absorption (Zhang *et al.* 2014), and reduces growth and development (Ci *et al.* 2009, Li *et al.* 2013), in addition to accumulating in seeds (Rojas-Cifuentes *et al.* 2012).

Brassinosteroids (BRs) are polyhydroxy steroidal phytohormones, which affect plant growth, originally isolated from the pollen of *Brassica napus* (Grove *et al.* 1979, Fridman and Savaldi-Goldstein 2013, Vardhini and Anjum 2015). BRs are nontoxic, rapidly assimilated and metabolised, and occur in all plant organs (Bajguz and Tretyn 2003). BRs are classified by the number of structural carbons, *i.e.*, C_{27}, C_{28} or C_{29}. More than sixty BRs have been identified (Haubrick and Assmann 2006), and three bioactive BRs frequently used at experimental scales at low concentrations are 24-epibrassinolide (EBR), 28-homobrassinolide (HBL), and brassinolide (BL) (Vardhini *et al.* 2006, Vardhini and Anjum 2015).

BRs affect a variety of physiological, biochemical, and molecular processes, including growth (Mandava *et al.* 1981), senescence (He *et al.* 1996), seed germination (Jones-Held *et al.* 1996), flowering (Pipattanawong *et al.* 1996), vascular differentiation (Clouse and Sasse 1998), gas exchange (Farooq *et al.* 2009), antioxidant enzymes (Sharma *et al.* 2010), cell division and elongation (Xie *et al.* 2011), and gene expression (Villiers *et al.* 2012, Ahammed *et al.* 2013). BRs also contribute to the mechanisms that plants use to tolerate abiotic stresses, such as drought (Yuan *et al.* 2010), high temperature (Hayat *et al.* 2010a), polycyclic aromatic hydrocarbons (Ahammed *et al.* 2012), salinity (Alyemeni *et al.* 2013), and heavy metals (Allagulova *et al.* 2015, Ramakrishna and Rao 2015). The effects of BRs in relation to Cd-induced stress have been examined in several plant species and the alleviation of Cd toxicity by these sterols was usually observed. This alleviation has been demonstrated as the reduction of the negative effects of Cd on production components in *Cicer arietinum* (Hasan *et al.* 2008), mitigation of damages on gas exchange in *Raphanus sativus* (Anuradha and Rao 2009), improvement of antioxidant systems in *Phaseolus vulgaris* (Rady 2011), increases in Chl contents of *Solanum lycopersicum* (Hayat *et al.* 2012), and improvement of growth parameters in *Triticum aestivum* (Hayat *et al.* 2014). However, the actions of different concentrations of BRs on *Vigna unguiculata* exposed to Cd toxicity have not been yet examined.

Our aim was to ascertain whether exogenous treatment with EBR can mitigate the Cd toxicity in cowpea, and whether this alleviation could be related to reduced Cd accumulation in plant tissues. Furthermore, the dose response to EBR was determined in *V. unguiculata* plants following their exposure to Cd.

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) during January of 2015. The study was conducted in a greenhouse with temperature and humidity controlled. The minimum, maximum, and median temperatures were 23, 32, and 26.5°C, respectively. The 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 3:1 proportions, respectively. Plants were cultivated in semihydroponic conditions, and the pots had one hole in the bottom covered with mesh to maintain the substrate and aerate the roots. Solution absorption was by capillarity, with the pots placed into other containers (0.15 m in height and 0.15 m in diameter) containing 500 mL of distilled water for 5 d. Modified Hoagland and Arnon (1950) solution was used as nutrients, and the ionic strength began at 50% and was modified to 100% after one day. After one day, the nutritive solution remained at the total ionic strength.

Experimental design: The experiment was a factorial design with the factors completely randomised, with two concentrations of Cd (0 and 500 μM) and three concentrations 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.

24-epibrassinolide (EBR) preparation and application: Six-day-old plants (leaves and stem) were sprayed with 10 mL per plant of EBR or Milli-Q water (as control, containing a proportion of ethanol which was equal to that used for preparation of the EBR solution) at 6-d intervals until day 24. 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.* 2013). On day 18 after the experiment implementation, the plants were exposed to 0 and 500 μM Cd.

Cadmium treatments: The plants received the following macro- and micronutrients from the nutritive solution (*Sigma-Aldrich*, USA):

KNO_3	8.75 mM
$Ca(NO_3)_2 \cdot 4H_2O$	7.50 mM
$NH_4H_2PO_4$	3.25 mM
$MgSO_4 \cdot 7\ H_2O$	1.50 mM
KCl	62.50 µM
H_3BO_3	31.25 µM
$MnSO_4 \cdot H_2O$	2.50 µM
$ZnSO_4 \cdot 7H_2O$	2.50 µM
$CuSO_4 \cdot 5H_2O$	0.63 µM
$NaMoO_4 \cdot 5H_2O$	0.63 µM
NaEDTA-Fe·$3H_2O$	250 µM

In order to simulate Cd exposure, $CdCl_2$ was used at concentrations of 0 and 500 µM and applied during 6 d (day 18–24 after the start of the experiment, DAE). During the study, the solutions were changed at 07:00h at 3-d intervals, with the pH adjusted to 5.5 using HCl or NaOH. After 24 DAE, physiological and morphological parameters were measured for all plants, and tissues (roots, stem, and leaves) were harvested for nutritional and biochemical analyses.

Chlorophyll (Chl) 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 Chl fluorometer (*OS5p*; *Opti-Sciences*, USA). The Chl fluorescence was measured in fully expanded leaves under light, between 9:00 and 10:00 h. Preliminary tests determined the location of the leaf, the part of the leaf, and the time required to obtain the greatest F_v/F_m ratio; therefore, the acropetal third of leaves, which were in the middle third of the plant and adapted to the dark for 30 min, were used for the evaluation. The intensity and duration of the saturation-light pulse were 7,500 µmol(photon) m^{-2} s^{-1} and 0.7 s, respectively.

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 (*LCPro*$^+$, *ADC BioScientific*, UK). These parameters were measured at the adaxial surface of fully expanded leaves, which 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, PAR, air-flow rate, and temperature in a chamber at 360 µmol(CO_2) mol^{-1}, 800 µmol(photon) m^{-2} s^{-1}, 300 µmol s^{-1}, and 28ºC, respectively, between 10:00 and 12:00 h.

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

Superoxide concentration: The mixture for extraction of O_2^- was prepared by homogenising 500 mg of fresh plant material in 5 ml of extraction buffer, which consisted of 50 mM phosphate buffer (pH 7.6), 1.0 mM ascorbate, and 1.0 mM EDTA. Samples were centrifuged at 14,000 × *g* for 4 min at 3°C in order to collect the supernatant. For the determinations of O_2^-, 1 ml of extract was incubated with 30 mM phosphate buffer (pH 7.6) and 0.51 mM hydroxylamine hydrochloride for 20 min at 25°C. Then, 17 mM sulphanilamide and 7 mM α-naphthylamine were added to the incubation mixture for 20 min at 25°C. After the reaction, ethyl ether was added in the identical volume and centrifuged at 3,000 × *g* for 5 min. The absorbance was measured at 530 nm (Elstner and Heupel 1976) using a spectrophotometer (*UV-M51*, *Bel Photonics*, Italy). O_2^- concentration was expressed in nmol min^{-1} g^{-1}(FM).

Extraction of nonenzymatic compounds: Nonenzymatic compounds (H_2O_2 and malondialdehyde, MDA) were extracted as described by Wu *et al.* (2006). Briefly, a mixture for extraction of 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.

Hydrogen peroxide concentration: To measure a content of H_2O_2, 200 µL of supernatant and 1,800 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) using a spectrophotometer (*UV-M51*, *Bel Photonics*, Italy. H_2O_2 concentration was expressed in µmol g^{-1}(FM).

MDA concentration: MDA was determined by mixing 500 µL of the supernatant with 1,000 µL of the reaction mixture, which contained 0.5% (w/v) thiobarbituric acid in 20% trichloroacetic acid. The mixture was incubated in boiling water at 95°C for 20 min, with the reaction terminated by placing the reaction container in an ice bath. The samples were centrifuged at 10,000 × *g* for 10 min, and the absorbance was measured at 532 nm using a spectrophotometer (*UV-M51*, *Bel Photonics*, Italy). The

nonspecific absorption at 600 nm was subtracted from the absorbance data. The MDA–TBA complex (red pigment) amount was calculated based on the method of Cakmak and Horst (1991), with minor modifications and using an extinction coefficient of 155 mM^{-1} cm^{-1}. MDA concentration was expressed in nmol g^{-1}(FM).

Electrolyte leakage was measured according to the method of Gong *et al.* (1998) with minor modifications. Fresh leaves and roots (200 mg) were cut into pieces of 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 $EL\ [\%] = (EC_1/EC_2) \times 100$.

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

Determining of Cd and nutrients: Samples with 100 mg of milled material (roots, stem, and leaves) were placed in 50-ml conical tubes (*Falcon*, *Corning*, Mexico) and predigested (48 h) with 2 ml of subdestilled HNO_3 (*DST 1000*, *Savillex*, USA). Afterwards, 8 ml of a solution containing 4 ml of H_2O_2 (30 % v/v, *Synth*, Brasil) and 4 ml of ultrapure water (*Milli-Q System*, *Millipore*, USA) were added and the mixture was transferred to a teflon digestion vessel, closed, and heated in a block digester (*EasyDigest®*, *Analab*, France) according to the following program: 100ºC during 30 min (1); 150ºC during 30 min (2); 130ºC during 10 min (3); 100ºC during 30 min and (4), left to cool (5). The volume was made up to 50 mL with ultrapure water and iridium was used as internal standard at 10 μg l^{-1}, in agreement with Batista *et al.* (2014). The determinations of Cd, Ca, Mg, Mn, Fe, Cu, and Zn were carried out by using an inductively coupled plasma mass spectrometer (*ICP-MS 7900*, *Agilent*, USA). Certified reference materials (*NIST 1570a* and *NIST 1577c*) were run in each batch for quality control purposes. All found values were in agreement with certified values.

Bioconcentration and translocation factors: The bioconcentration factor (BCF) was calculated by the equation $BCF = Cd_{pt}/Cd_{ns}$ as described by Yoon *et al.* (2006), where Cd_{pt} represents the concentration of Cd in plant tissue and Cd_{ns} represents the Cd concentration in the nutritive solution. The translocation factor (TF) was obtained by the Cd_s/Cd_r formula in agreement with Yoon *et al.* (2006), where Cd_s represents the Cd concentration in the shoot (stem + leaves) and Cd_r represents the Cd concentration in the roots.

Data analysis: The data were subjected to an analysis of variance (*ANOVA*), and significant differences between the means were determined by *Scott-Knott*'s test at a probability level of 5% (Steel *et al.* 2006). Standard deviations were calculated for each treatment. Statistical analyses were performed using *Assistat* software (Silva and Azevedo 2002).

Results

Cd concentration: Spraying the plants of *V. unguiculata* with EBR significantly reduced the concentrations of Cd in the tissues (Table 1). After 100 nM EBR + 500 μM Cd treatment, the concentrations of Cd decreased 25.2, 51.3, and 19.2% in the roots, stems, and leaves, respectively, compared with those at the 0 nM EBR + 500 μM Cd. EBR had the identical effect on the BCF, which was verified in plants of the 100 nM EBR + 500 μM Cd treatment with reductions of 25.1 (roots), 53.1 (stem), and 25% (leaves) compared with the plants of the control exposed to Cd. For TF, the effect of EBR was not significant in plants exposed to Cd toxicity.

Nutrient concentration: Cd toxicity modified the macronutrient concentrations in plant tissues, with significant reductions in the concentrations of Ca and Mg (Table 2). However, when the 100 nM EBR + 500 μM Cd and 0 nM EBR + 500 μM Cd treatments were compared, the concentrations of Ca and Mg increased with EBR by 27.3 and 18% (roots), 28 and 55.6% (stem), and 15 and 105% (leaves), respectively. In leaf tissue, the concentrations of all nutrients increased significantly when sprayed with 100 nM EBR compared with those after the 0 nM EBR + 0 μM Cd treatment. For micronutrients (*i.e.*, Mn, Fe, Cu, and Zn), concentrations in the roots, stems, and leaves of *V. unguiculata* decreased significantly after the treatment with 500 μM Cd (Table 2). However, when the plants of the 100 nM EBR + 500 μM Cd treatment and those of the control exposed to Cd were compared, the concentrations of Mn, Fe, Cu, and Zn increased significantly with the application of EBR, with the largest changes recorded for Mn (82.2%) and Fe (41.2%) in stems and for Cu (125%) and Zn (95.8%) in leaves.

EBR and Cd produced interference on Chl fluorescence: Values of F_0 for plants after the treatment with 100 nM EBR and 0 μM Cd increased significantly by 9.8%, when compared with the treatment with 0 nM EBR. With the addition of Cd, the effect of EBR on the treatments was positive (Fig. 1*A*). For F_m, the values were not significantly different between the treatments with different concentrations of EBR and 0 μM Cd. The application of Cd

Table 1. Cd contents and translocation in *Vigna unguiculata* plants with EBR and exposed to Cd toxicity. BCF – bioconcentration factor; TF – translocation factor. Columns with different *uppercase letters* between EBR concentrations (0, 50, and 100 nM under equal Cd concentration) and *lowercase letters* between Cd concentrations (0 and 500 μM under equal EBR concentration) indicate significant differences from the *Scott-Knott* test (P<0.05). Means ± SD, n = 5.

Cd [μM]	EBR [nM]	Cd content [mg kg^{-1}]			BCF			TF
		Root	Stem	Leaf	Root	Stem	Leaf	
0	0	0.1 ± 0.0^{Ab}	0.1 ± 0.0^{Ab}	0.0 ± 0.0^{Ab}	0.00 ± 0.00^{Ab}	0.0 ± 0.00^{Ab}	0.00 ± 0.00^{Ab}	0.99 ± 0.07^{Aa}
0	50	0.1 ± 0.0^{Ab}	0.1 ± 0.0^{Ab}	0.0 ± 0.0^{Ab}	0.00 ± 0.00^{Ab}	0.0 ± 0.00^{Ab}	0.00 ± 0.00^{Ab}	0.69 ± 0.03^{Ba}
0	100	0.1 ± 0.0^{Ab}	0.0 ± 0.0^{Ab}	0.0 ± 0.0^{Ab}	0.00 ± 0.00^{Ab}	0.0 ± 0.00^{Ab}	0.00 ± 0.00^{Ab}	0.62 ± 0.02^{Ca}
500	0	342.3 ± 19.6^{Aa}	27.5 ± 1.0^{Aa}	5.1 ± 0.5^{Aa}	6.09 ± 0.35^{Aa}	0.49 ± 0.02^{Aa}	0.28 ± 0.01^{Aa}	0.12 ± 0.01^{Ab}
500	50	266.7 ± 15.3^{Ba}	15.2 ± 0.6^{Ba}	3.9 ± 0.6^{Ba}	4.74 ± 0.27^{Ba}	0.27 ± 0.01^{Ba}	0.25 ± 0.01^{Ba}	0.11 ± 0.01^{Ab}
500	100	256.1 ± 8.7^{Ba}	13.4 ± 0.9^{Ca}	2.2 ± 0.8^{Ca}	4.56 ± 0.16^{Ba}	0.23 ± 0.02^{Ca}	0.21 ± 0.02^{Ca}	0.10 ± 0.02^{Ab}

Table 2. Nutrient contents in *Vigna unguiculata* plants with EBR and exposed to Cd toxicity. Columns with different *uppercase letters* between EBR concentrations (0, 50 and 100 nM under equal Cd concentration) and *lowercase letters* between Cd concentrations (0 and 500 μM under equal EBR concentration) indicate significant differences from the *Scott-Knott* test (P<0.05). Means ± SD, n = 5.

Cd [μM]	EBR [nM]	Ca [mg g^{-1} (DM)]	Mg [mg g^{-1} (DM)]	Mn [μg g^{-1} (DM)]	Fe [μg g^{-1} (DM)]	Cu [μg g^{-1} (DM)]	Zn [μg g^{-1} (DM)]
Root							
0	0	2.7 ± 0.1^{Ba}	18.8 ± 1.5^{Ba}	356.5 ± 13.3^{Ba}	112.4 ± 8.0^{Aa}	10.5 ± 0.7^{Ba}	41.4 ± 1.4^{Ba}
0	50	3.1 ± 0.2^{Aa}	19.8 ± 1.1^{Ba}	390.8 ± 12.3^{Aa}	113.9 ± 9.5^{Aa}	11.3 ± 0.8^{Ba}	47.2 ± 2.1^{Aa}
0	100	3.2 ± 0.1^{Aa}	23.2 ± 1.9^{Aa}	394.6 ± 9.0^{Aa}	119.9 ± 11.4^{Aa}	12.9 ± 0.5^{Aa}	51.3 ± 4.4^{Aa}
500	0	2.2 ± 0.1^{Cb}	12.2 ± 0.6^{Bb}	122.9 ± 4.8^{Cb}	66.3 ± 6.0^{Bb}	7.8 ± 0.7^{Bb}	28.3 ± 1.1^{Bb}
500	50	2.5 ± 0.1^{Bb}	12.5 ± 0.8^{Bb}	137.5 ± 6.0^{Bb}	74.4 ± 6.1^{Bb}	9.6 ± 0.9^{Ab}	33.2 ± 3.0^{Ab}
500	100	2.8 ± 0.1^{Ab}	14.4 ± 0.9^{Ab}	152.5 ± 4.3^{Ab}	88.2 ± 7.1^{Ab}	10.5 ± 0.7^{Ab}	35.2 ± 3.3^{Ab}
Stem							
0	0	3.2 ± 0.1^{Ca}	2.8 ± 0.1^{Ba}	33.7 ± 2.8^{Aa}	85.6 ± 7.0^{Ba}	1.4 ± 0.1^{Ba}	18.7 ± 0.4^{Ca}
0	50	3.7 ± 0.1^{Ba}	3.1 ± 0.1^{Aa}	35.6 ± 2.8^{Aa}	110.8 ± 6.5^{Aa}	1.4 ± 0.0^{Ba}	21.9 ± 1.9^{Ba}
0	100	4.0 ± 0.1^{Aa}	3.2 ± 0.1^{Aa}	37.4 ± 3.4^{Aa}	115.0 ± 4.4^{Aa}	2.0 ± 0.1^{Aa}	33.1 ± 3.2^{Aa}
500	0	2.5 ± 0.1^{Bb}	1.8 ± 0.1^{Cb}	15.7 ± 1.0^{Bb}	53.9 ± 4.7^{Bb}	1.0 ± 0.1^{Cb}	9.3 ± 0.4^{Bb}
500	50	3.1 ± 0.2^{Ab}	2.4 ± 0.2^{Bb}	26.2 ± 2.1^{Ab}	68.6 ± 6.5^{Ab}	1.3 ± 0.1^{Bb}	14.6 ± 0.9^{Ab}
500	100	3.2 ± 0.2^{Ab}	2.8 ± 0.1^{Ab}	28.6 ± 1.6^{Ab}	76.1 ± 5.5^{Ab}	1.6 ± 0.1^{Ab}	15.8 ± 1.2^{Ab}
Leaf							
0	0	6.8 ± 0.1^{Ba}	4.3 ± 0.1^{Ca}	66.0 ± 2.1^{Ca}	196.1 ± 10.4^{Ca}	0.7 ± 0.0^{Ca}	13.9 ± 1.1^{Ba}
0	50	7.5 ± 0.1^{Aa}	5.3 ± 0.1^{Ba}	70.0 ± 1.4^{Ba}	215.1 ± 6.8^{Ba}	0.8 ± 0.0^{Bb}	16.3 ± 1.0^{Aa}
0	100	7.4 ± 0.1^{Aa}	5.6 ± 0.1^{Aa}	77.8 ± 2.7^{Aa}	230.2 ± 9.3^{Aa}	1.1 ± 0.1^{Aa}	17.4 ± 0.6^{Aa}
500	0	6.0 ± 0.0^{Cb}	2.0 ± 0.0^{Cb}	35.7 ± 0.8^{Cb}	155.5 ± 3.0^{Cb}	0.4 ± 0.1^{Bb}	7.1 ± 0.7^{Cb}
500	50	6.6 ± 0.1^{Bb}	3.8 ± 0.1^{Bb}	40.5 ± 1.5^{Bb}	179.3 ± 10.3^{Bb}	0.9 ± 0.0^{Aa}	9.6 ± 0.6^{Bb}
500	100	6.9 ± 0.1^{Ab}	4.1 ± 0.1^{Ab}	52.4 ± 0.8^{Ab}	191.6 ± 4.9^{Ab}	0.9 ± 0.1^{Ab}	13.9 ± 1.4^{Ab}

caused a significant decrease in F_m; however, F_m values increased 26.5% after the treatment with 100 nM EBR compared with those in the 0 nM EBR + 500 μM Cd treatment (Fig. 1*B*). The ratio of F_v/F_m was changed significantly by the treatment with 100 nM EBR under both 0 and 500 μM Cd. Additionally, the ratio increased significantly by 4.5% in the treatments with 100 nM EBR + Cd compared with that in the control treatment (0 nM EBR) exposed to Cd (Fig. 1*C*).

In the treatment with 0 nM EBR and Cd, Φ_{PSII} was significantly affected (Table 3). Exposure to Cd caused a significant reduction in Φ_{PSII} compared with the identical treatment without Cd. The ratio increased 59% in the treatment with 100 nM EBR compared with that in the 0 nM EBR + 500 μM Cd treatment. For q_P and NPQ, values increased and decreased with EBR, respectively (Table 3). EBR caused a significant increase in ETR, with increases of 16.9 and 55.7% observed in the plants treated with 100 nM EBR compared with those in the treatments with 0 nM EBR and concentrations of 0 and 500 μM Cd, respectively. In the treatments with EBR, EXC values decreased significantly, particularly in the plants treated with 100 nM EBR, with a reduction of 28.6% compared with those in the 0 nM EBR and 500 μM Cd treatment. For ETR/P_N, the treatment with 100 nM EBR and 500 μM Cd presented reduction of 10.4%, compared with control plants exposed to Cd (Table 3).

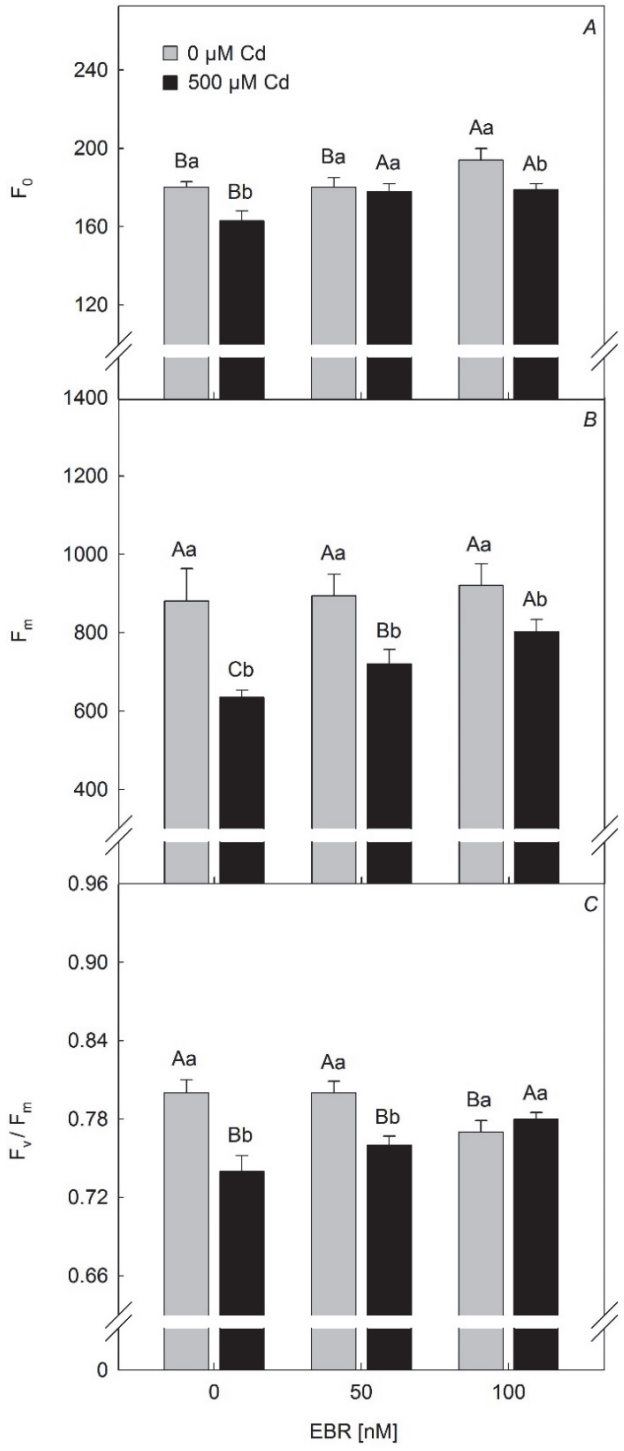

Fig. 1. Minimal fluorescence yield of the dark-adapted state (F_0; *A*), maximal fluorescence yield of the dark-adapted state (F_m; *B*), and maximal quantum yield of PSII photochemistry (F_v/F_m; *C*) in *Vigna unguiculata* plants with EBR and exposed to Cd toxicity. Different *uppercase letters* between EBR concentrations (0, 50, and 100 nM under equal Cd concentration) and *lowercase letters* between Cd concentrations (0 and 500 µM under equal EBR concentration) indicate significant differences from the *Scott-Knott* test (P<0.05). Means ± SD, n = 5.

EBR improved gas exchange in response to Cd toxicity: The P_N increased significantly with foliar application of EBR without Cd, with an increase of 24% in the plants treated with 100 nM EBR (Table 4) compared with those of the control (0 nM EBR). Although Cd lowered P_N, P_N increased significantly by 74% after the treatment with 100 nM EBR compared with that in the 0 nM EBR + 500 µM Cd treatment. In the plants with the 100 nM EBR + 0 µM Cd, E increased significantly by 6.4% compared with that in the 0 nM EBR without Cd treatment (Table 4). However, Cd negatively affected E. In the treatment with 100 nM EBR and no Cd, g_s increased significantly; however, Cd had a negative influence on g_s, which decreased. For C_i, the effect of EBR was positive, and in the the plants treated with 100 nM EBR and Cd, C_i decreased significantly by 8% compared with that in the treatment with 0 nM EBR + 500 µM Cd (Table 4). The WUE was significantly affected by EBR and an increase of 16.4% was observed after the treatment with 100 nM EBR without Cd compared with that in the treatment with 0 nM EBR + 0 µM Cd. In the plants exposed to Cd stress, significant reductions in P_N/C_i occurred; however, the ratio increased by 100% in the treatment with 100 nM EBR + 500 µM Cd compared with that in the 0 nM EBR + Cd treatment.

EBR alleviated oxidative stress caused by Cd: The concentrations of O_2^- increased with exposure to Cd; however, O_2^- contents decreased significantly by 51.2% in the plants of the 100 nM EBR + 500 µM Cd treatment compared with those in the control exposed to Cd (Fig. 2*A*). Similarly, the contents of H_2O_2 decreased significantly after the treatment with 100 nM EBR + 500 µM Cd by 24.6% compared with those in plants in the 0 nM EBR with Cd treatment (Fig. 2*B*). For MDA, there was no significant difference between the EBR treatments with 0 µM Cd, although the content of MDA increased with exposure to Cd. However, the content of MDA decreased by 15.8% after the treatment with 100 nM EBR compared with that in the 0 nM EBR + 500 µM Cd treatment (Fig. 2*C*). After exposure to Cd, the EL increased; however, EL decreased significantly by 17.4% in the treatment with 100 nM EBR + 500 µM Cd compared with that in the 0 nM EBR and 500 µM Cd treatment (Fig. 2*D*).

EBR promotes protection of pigments against Cd toxicity: In treatments with 0 µM Cd, Chl *a* content increased significantly by 18.2% in the plants exposed to 100 nM EBR compared with those without exposure to EBR (Table 5). For Chl *b* content, insignificant increases were observed in treatments with 50 and 100 nM EBR compared with that in the 0 nM EBR treatment (Table 5). Additionally, total Chl content increased significantly by 14.0% in plants treated with 100 nM EBR compared with that in those of the 0 nM EBR treatment. The performance of EBR in plants under Cd stress significantly affected the

Table 3. Chlorophyll fluorescence in *Vigna unguiculata* plants with EBR and exposed to Cd toxicity. 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 concentrations (0, 50, and 100 nM under equal Cd concentration) and *lowercase letters* between Cd concentrations (0 and 500 μM under equal EBR concentration) indicate significant differences from the *Scott-Knott* test (P<0.05). Means ± SD, n = 5.

Cd [μM]	EBR [nM]	Φ_{PSII}	q_P	NPQ	ETR [μmol m^{-2} s^{-1}]	EXC [μmol m^{-2} s^{-1}]	ETR/P_N
0	0	0.34 ± 0.02^{Ba}	0.72 ± 0.01^{Aa}	0.58 ± 0.05^{Aa}	50.8 ± 2.6^{Ba}	0.56 ± 0.03^{Ab}	3.63 ± 0.24^{Ab}
0	50	0.40 ± 0.02^{Aa}	0.76 ± 0.16^{Aa}	0.58 ± 0.05^{Aa}	59.2 ± 3.4^{Aa}	0.49 ± 0.03^{Bb}	3.60 ± 0.34^{Ab}
0	100	0.41 ± 0.04^{Aa}	0.77 ± 0.15^{Aa}	0.47 ± 0.04^{Bb}	61.1 ± 5.8^{Aa}	0.46 ± 0.05^{Bb}	3.55 ± 0.33^{Ab}
500	0	0.22 ± 0.02^{Cb}	0.38 ± 0.03^{Bb}	0.72 ± 0.03^{Aa}	32.5 ± 3.0^{Cb}	0.70 ± 0.02^{Aa}	6.03 ± 0.56^{Aa}
500	50	0.30 ± 0.03^{Bb}	0.67 ± 0.04^{Aa}	0.68 ± 0.02^{Aa}	44.6 ± 2.4^{Bb}	0.59 ± 0.06^{Ba}	5.73 ± 0.55^{Aa}
500	100	0.35 ± 0.01^{Ab}	0.73 ± 0.09^{Aa}	0.68 ± 0.01^{Aa}	50.6 ± 3.2^{Ab}	0.55 ± 0.03^{Ba}	5.40 ± 0.37^{Aa}

Table 4. Gas exchange in *Vigna unguiculata* plants with EBR and exposed to Cd toxicity. 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 concentrations (0, 50, and 100 nM under equal Cd concentration) and *lowercase letters* between Cd concentrations (0 and 500 μM under equal EBR concentration) indicate significant differences from the *Scott-Knott* test (P<0.05). Means ± SD, n = 5.

Cd [μM]	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}]
0	0	14.0 ± 1.1^{Ba}	3.43 ± 0.08^{Ba}	0.37 ± 0.02^{Ba}	271 ± 20.0^{Aa}	4.08 ± 0.2^{Ba}	0.052 ± 0.002^{Ba}
0	50	16.4 ± 1.0^{Aa}	3.47 ± 0.04^{Ba}	0.38 ± 0.01^{Ba}	265 ± 12.0^{Aa}	4.74 ± 0.2^{Aa}	0.062 ± 0.006^{Aa}
0	100	17.3 ± 1.7^{Aa}	3.65 ± 0.09^{Aa}	0.43 ± 0.02^{Aa}	264 ± 9.0^{Aa}	4.75 ± 0.4^{Aa}	0.066 ± 0.006^{Aa}
500	0	5.4 ± 0.3^{Cb}	2.17 ± 0.18^{Ab}	0.13 ± 0.01^{Ab}	288 ± 8.0^{Aa}	2.51 ± 0.1^{Cb}	0.018 ± 0.002^{Cb}
500	50	7.8 ± 0.5^{Bb}	2.21 ± 0.19^{Ab}	0.13 ± 0.03^{Ab}	272 ± 5.0^{Ba}	3.55 ± 0.2^{Bb}	0.028 ± 0.002^{Bb}
500	100	9.4 ± 0.8^{Ab}	2.24 ± 0.05^{Ab}	0.15 ± 0.01^{Ab}	265 ± 6.0^{Ba}	4.19 ± 0.3^{Ab}	0.036 ± 0.001^{Ab}

contents of Chl *a*, Chl *b*, and total Chl, compared with those in the 0 nM EBR with Cd treatment (Table 5). The application of EBR interferred significantly in the Car, with a significant reduction of 65.1% after the treatment with 100 nM EBR + 500 μM Cd observed if compared with that in the treatment of 0 nM EBR with Cd, respectively (Table 5). Cd caused significant reductions in contents of Chl *a*, Chl *b*, total Chl, and Car compared with those of the identical treatments without Cd (Table 5).

EBR had beneficial effects on plant growth: In the absence of Cd, LDM increased significantly by 13.2% in the 100 nM EBR treatment compared with that of the 0 nM EBR treatment. With exposure to Cd, LDM decreased significantly; however, it increased with 100 nM EBR by 26.2%, compared with that in the 0 nM EBR + 500 μM Cd treatment (Fig. 3*A*). A positive influence of EBR was also observed on RDM, primarily after the 50 nM EBR + 0 μM Cd treatment with a significant increase of 71% when compared with that in the control without Cd. The application of Cd caused a significant decrease in RDM compared with that in the 0 μM Cd treatment; however, compared with that in the 0 nM EBR + 500 μM Cd treatment, 100 nM EBR increased RDM by 20.4% (Fig. 3*B*). In the absence of Cd, SDM increased significantly in plants treated with 100 nM EBR compared with that in plants in the 0 nM EBR treatment. With the application of Cd, a significant decrease occurred in SDM (Fig. 3*C*). Treatment with EBR significantly increased TDM in the absence of Cd, and increases occurred at concentrations of 50 and 100 nM EBR compared with that in the 0 nM EBR + 0 μM Cd treatment. With exposure to Cd, a significant reduction in TDM was observed; however, TDM increased significantly by 21% in the treatment with 100 nM EBR compared with that in the 0 nM EBR treatment exposed to stress from Cd (Fig. 3*D*).

Discussion

In general, the exposure of cowpea plants to high concentrations of Cd produced negative interferences on metabolism, resulting in minor growth. On other hand, the EBR mitigated the toxic effects due to reduction of mainly the Cd and ROS (reactive oxygen species) accumulation. The Cd contents decreased in all tissues after the EBR treatment because EBR most likely increased production of phytochelatins in cells of stressed tissues. The

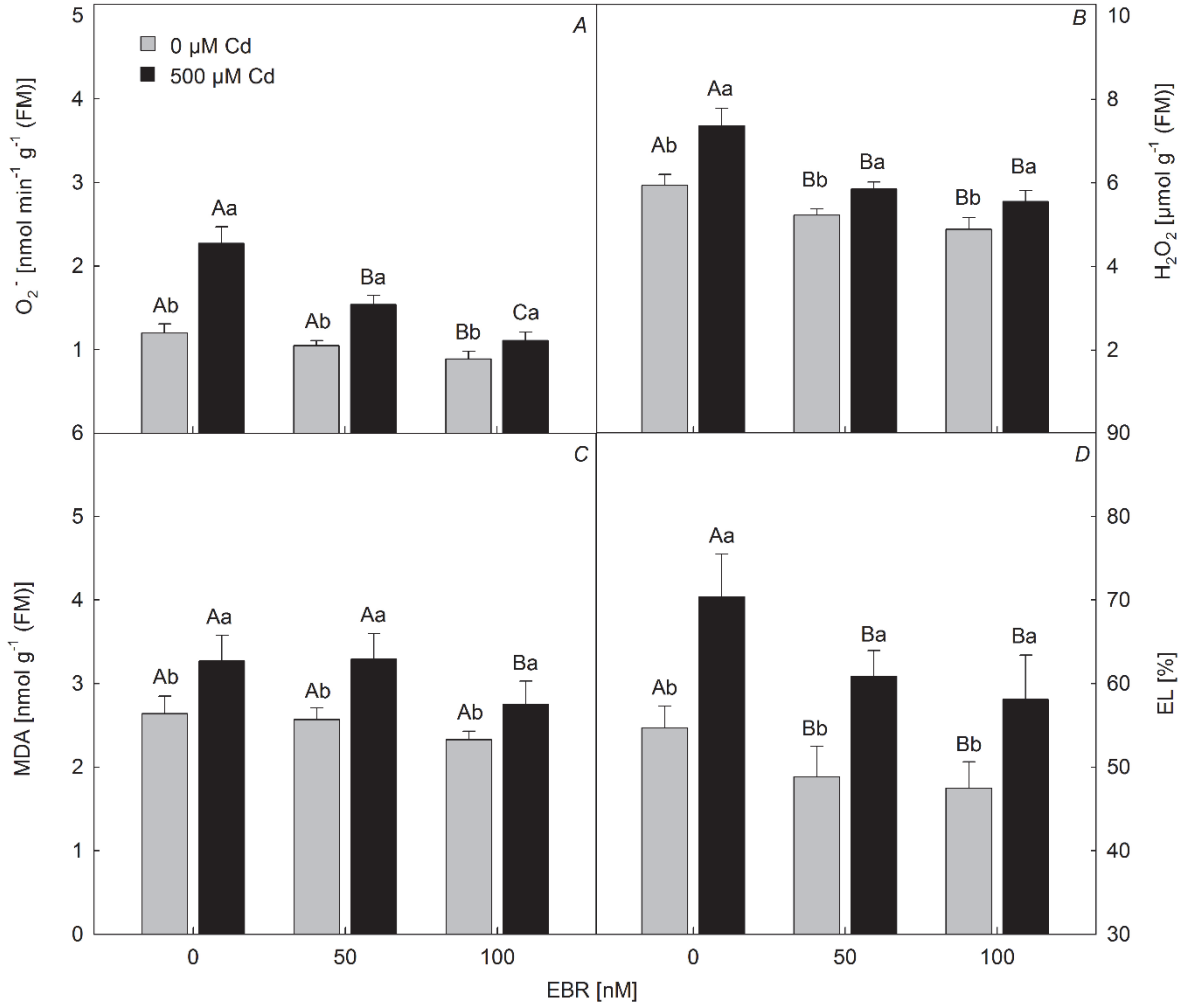

Fig. 2. Superoxide (O_2^{-}; *A*), hydrogen peroxide (H_2O_2; *B*), malondialdehyde (MDA; *C*), and electrolyte leakage (EL; *D*) in *Vigna unguiculata* plants with EBR and exposed to Cd toxicity. Different *uppercase letters* between EBR concentrations (0, 50, and 100 nM under equal Cd concentration) and *lowercase letters* between Cd concentrations (0 and 500 µM under equal EBR concentration) indicate significant differences from the *Scott-Knott* test (P<0.05). Means ± SD, n = 5.

Table 5. Photosynthetic pigments in *Vigna unguiculata* plants with EBR and exposed to Cd toxicity. Chl – chlorophyll; Car – carotenoids. Columns with different *uppercase letters* between EBR concentrations (0, 50, and 100 nM under equal Cd concentration) and *lowercase letters* between Cd concentrations (0 and 500 µM under equal EBR concentration) indicate significant differences from the *Scott-Knott* test (P<0.05). Means ± SD, n = 5.

Cd [µM]	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)]
0	0	7.51 ± 0.49^{Ba}	4.15 ± 0.31^{Aa}	11.67 ± 0.50^{Ba}	1.63 ± 0.06^{Aa}
0	50	7.83 ± 0.76^{Ba}	4.41 ± 0.31^{Aa}	12.24 ± 0.69^{Ba}	1.39 ± 0.14^{Ba}
0	100	8.88 ± 0.20^{Aa}	4.43 ± 0.35^{Aa}	13.31 ± 0.24^{Aa}	1.17 ± 0.06^{Ca}
500	0	2.22 ± 0.17^{Bb}	2.60 ± 0.26^{Bb}	4.82 ± 0.36^{Bb}	0.82 ± 0.12^{Ab}
500	50	3.17 ± 0.16^{Ab}	3.96 ± 0.31^{Ab}	7.13 ± 0.34^{Ab}	0.38 ± 0.05^{Bb}
500	100	3.57 ± 0.25^{Ab}	3.75 ± 0.32^{Ab}	7.32 ± 0.35^{Ab}	0.28 ± 0.06^{Bb}

phytochelatins provided a mechanism for heavy-metal detoxification in plants (Rajewska *et al.* 2016), and these binding peptides chelate and immobilize metal ions in cell walls, primarily in those of root tissues (Bajguz and Hayat 2009, Bajguz 2010). This action of EBR directly influenced the decrease in values of BCF and FT because minor translocation rates of Cd to stems and leaves. In *Chlorella vulgaris*, Bajguz (2011) reported that EBR increases the synthesis of phytochelatins in cells exposed to heavy metals, including Cd. Rady (2011) evaluated

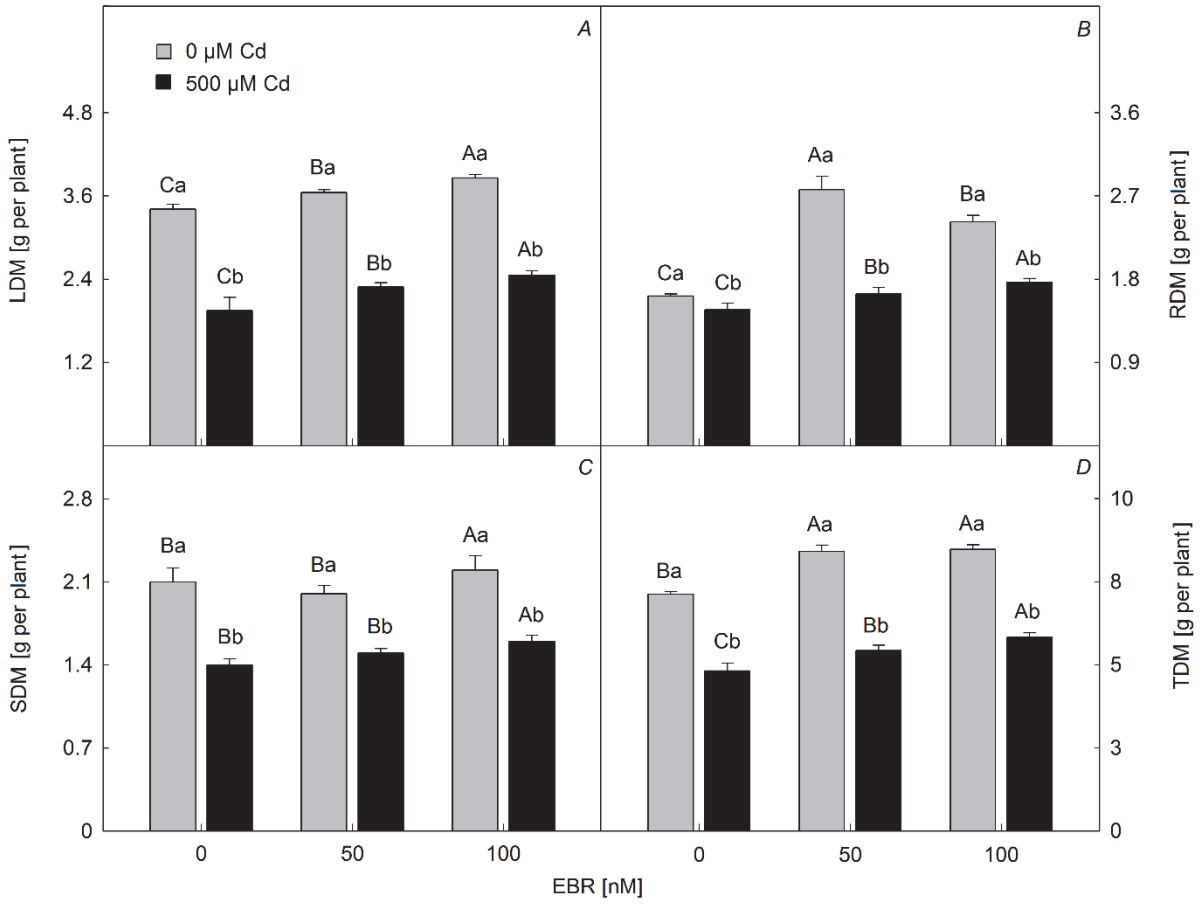

Fig. 3. Leaf dry matter (LDM; *A*), root dry matter (RDM; *B*), stem dry matter (SDM; *C*), and total dry matter (TDM; *D*) in *Vigna unguiculata* plants splayed with EBR and exposed to Cd toxicity. Different *uppercase letters* between EBR concentrations (0, 50, and 100 nM under equal Cd concentration) and *lowercase letters* between Cd concentrations (0 and 500 µM under equal EBR concentration) indicate significant differences from the *Scott-Knott* test (P<0.05). Means ± SD, n = 5.

the effects of EBR in *P. vulgaris* under salinity and Cd stress and also found a reduction in Cd concentrations. Our study corroborates the results found by Kroutil *et al.* (2010) on a decrease in Cd contents in *T. aestivum* after treatments with three different types of BRs.

EBR reduced the negative effects of Cd toxicity on Ca, Mg, Fe, Mn, Cu, and Zn concentrations in roots, stems, and leaves, which suggested that EBR helped to reduce the disturbance in ionic homeostasis of these mineral elements in tissues caused by Cd stress (Saidi *et al.* 2013). The decrease in Ca concentration following exposure to toxic Cd is correlated with damage to the intracellular defence system caused by intracellular binding proteins that frequently compromise membrane integrity (Jiang *et al.* 2004, Rodríguez-Serrano *et al.* 2009, Lu *et al.* 2010). Cd also caused a decrease in concentrations of Ca in roots, stem, and leaves of *Allium fistulosum*, as reported by Li *et al.* (2016). Reductions in the concentrations of Mg in leaves, stems, and roots exposed to Cd were found previously for *Helianthus annuus* and *Trifolium repens* (Lopes Júnior *et al.* 2014, Liu *et al.* 2015a).

The toxic effects of Cd were reduced in plants sprayed with EBR. As suggested by the green color in *V. unguiculata* leaves, the concentrations of Fe and Mn, both essential to activate the precursors linked to Chl biosynthesis, were not affected (Hermans *et al.* 2011, Lopes Júnior *et al.* 2014). Similar reduction in the concentrations of Fe and Mn in roots and leaves were reported previously after exposure to Cd (Guo *et al.* 2013, Zhang *et al.* 2014, Basa *et al.* 2014, Ali *et al.* 2015). In this study, as the Cd stress increased in tissues, the concentrations of Cu and Zn decreased significantly, consistent with results previously described in the literature (Wang *et al.* 2007, López-Millán *et al.* 2009, Tang *et al.* 2013).

In this investigation, EBR mitigated Cd toxicity in *V. unguiculata* plants by minimizing the negative effects on F_0, F_m, and F_v/F_m obtained in this study. Moradi and Ehsanzadeh (2015) described similar results for *Carthamus tinctorius* genotypes for which F_o values decreased significantly with exposure to Cd [*i.e.*, 24.1% at 4.5 mg($CdCl_2$) L^{-1}]. Janeczko *et al.* (2005) detected an increase in the F_m values during an investigation of EBR effects on the PSII of plants exposed to Cd stress. A reduction in F_m values of *Zea mays* was observed at the

highest concentration of Cd, according to Wang *et al.* (2009). Corroborating the results of our study, Hayat *et al.* (2014) found that EBR reduced the Cd phytotoxicity *T. aestivum* plants. Wael *et al.* (2015) also observed a decrease in F_v/F_m values during an evaluation of the toxic effects of Cd in *Phaseolus vulgaris*.

The application of EBR led to increases in Φ_{PSII} and q_P values, which indicated that the EBR alleviated the inhibitory effects of Cd, with probable increases in absorbed photons for use during photochemical reactions. Thus, EBR improved the capacity of the plant to absorb the excitation energy into reaction centres from PSII to transport electrons. In *Beta vulgaris*, Basa *et al.* (2014) also found that Φ_{PSII} values were strongly reduced under Cd toxicity, and decreases in q_P values were also found by Filek *et al.* (2010) in *B. napus* plants exposed to Cd toxicity.

The EBR attenuated the increases in NPQ and EXC of plants exposed to Cd, which indicated that EBR reduced the thermal dissipation linked to excitation energy. These parameters are an indication of an overexcitation of the PSII complex caused by the high availability of light energy, with this excess energy removed by quenching processes to prevent photochemical damages in PSII (Silva *et al.* 2012). In a study on Cd toxicity in *Arabidopsis thaliana*, Sobrino-Plata *et al.* (2014) also found increases in NPQ values, corroborating the values found in this research to control treatments (0.47–0.58), when compared to plants exposed to Cd (0.68–0.72).

As indicated by the increases in F_0 and F_m values in *V. unguiculata* leaves, the EBR mitigation of the effects of Cd on ETR can be explained by the increase in efficiency of the plastoquinone in oxidation-reduction reactions. Silveira *et al.* (2015) also reported a decrease in ETR of *Elephantopus mollis* plants exposed to 10, 50, and 100 μM Cd.

The decrease in ETR/P_N values of plants exposed to EBR + Cd suggested that less photochemical energy was used in other metabolic processes, such as photorespiration, the Mehler reaction, and CO_2 assimilation (Silva *et al.* 2010, Fang *et al.* 2011, Miralles-Crespo *et al.* 2011, Barbosa *et al.* 2014a). The ratio ETR/P_N is used to estimate the activity of alternative drains of electrons (da Silva *et al.* 2010) and to evaluate photorespiration as a mechanism of protection (Palliotti *et al.* 2015).

When exposed to Cd, which interfered with the CO_2 influx used in the photosynthetic apparatus, the interference in P_N was alleviated by EBR in leaves of *V. unguiculata*, which was associated with improved stomatal performance, as confirmed by the increase in g_s values (Gonzalez-Mendoza *et al.* 2007, Zhang *et al.* 2014). However, alterations in the ultrastructure of chloroplasts also occur with exposure to Cd (Sandalio *et al.* 2001, Djebali *et al.* 2005). Hayat *et al*. (2010b) studying two BRs forms applied as shotgun approach also detected that BRs mitigated the effects on P_N in *Lycopersicon esculentum* cultivars exposed to Cd toxicity. When *Brassica campestris* and *B. juncea* were exposed to Cd, Chen *et al.* (2011) also found decreases in P_N values, with rates that oscillated between 9 and 14 μmol m^{-2} s^{-1}.

EBR increased the E values of plants affected by Cd toxicity, with positive effects on water absorption and root elongation, as indicated by the increase in root dry matter. Cd inhibits the transport of water into the cells (Irfan *et al.* 2014) because of an increase in the oxidation of sulfhydryl groups of proteins and enzymes in cell membranes (Lösch 2004). These structural changes in proteins and the inhibition of the activity of H^+-ATPase enzymes affect the rate of cell division (Zhang *et al.* 2009, Janicka-Russak *et al.* 2012). Moreover, Cd reduces the activity of aquaporins, which are integral membrane proteins with a regulatory role in the water transport system (Przedpelska-Wasowicz and Wierzbicka 2011). In a study with *T. aestivum*, Hayat *et al.* (2014) also observed a reduction in E values when plants were exposed to the toxic effects of Cd.

The decrease in g_s values of plants exposed to Cd was likely because of the closing of the stomata caused by Cd interference with the guard cells. Cd most likely causes changes in the regulation of Ca^+ channels, which interferes with the regulation of Cl^- and K^+ ions in the cytosol of guard cells, thereby reducing g_s values (Perfus-Barbeoch *et al.* 2002). Low g_s frequently inhibits the absorption of CO_2 (de Sousa Paula *et al.* 2015). Consistent with the results of this study, Wan *et al.* (2011) observed significant decreases in g_s values in two cultivars of *B. napus* exposed to Cd.

Correlated with a decrease in P_N/C_i values, the increase in C_i values in plants stressed by Cd indicated a decrease in the fixation and consequent absorption of CO_2 by the plant. The Rubisco enzyme is responsible for carboxylation during photosynthesis (Hasan *et al.* 2011) and reduced efficiency of this enzyme causes an increase in C_i (Barbosa *et al.* 2014b). Significant increases in C_i values were also observed in studies with two varieties of *Vigna radiata* (Wahid *et al.* 2008) and *B. napus* (Ali *et al.* 2015) exposed to Cd stress.

Compared to exposure to Cd, the P_N/C_i values increased in *V. unguiculata* plants treated with EBR because of the increase in P_N and the simultaneous decrease in C_i. The increase in the P_N/C_i ratio suggested that EBR interfered with Rubisco activity and increased CO_2 fixation during photosynthesis (Wahid *et al.* 2008, Krantev *et al.* 2008). Nwugo and Huerta (2011) observed a similar decrease in P_N/C_i values in *Oryza sativa* exposed to Cd stress.

In this study, the increase in WUE in plants exposed to EBR was directly associated with the increase in P_N and E values detected in this experiment. WUE is a physiological parameter that quantitatively expresses the instantaneous behaviour of gas exchange in a leaf (Pinzón-Torres and Schiavinato 2008) for which the observed values are correlated with the amount of carbon fixed by the plant per unit of water lost (Boutraa *et al.* 2010, de Souza Ferraz *et al.* 2012). In studies with *Pisum sativum* and *Hordeum vulgare*, Januškaitienė (2010) reported a reduction and an

increase of 26.5 and 14.7%, respectively, in WUE of plants exposed to Cd stress.

The EBR treatments of plants under Cd stress decreased contents of O_2^- and H_2O_2; therefore, EBR inhibited the production of these ROS and consequently, reduced the oxidative stress caused by Cd toxicity (Hasan *et al.* 2008). In *Oryza sativa* exposed to Cd stress, Srivastava *et al.* (2014) reported a similar increase in O_2^- contents. Ahmad *et al.* (2011), in a study of Cd effects on *Brassica juncea*, described increases in H_2O_2 concentrations of 48.7 and 61.1% at 100 and 200 mg L^{-1} Cd, respectively.

Treatment with EBR reduced the content of MDA and EL, with decreases in lipid peroxidation and escape of electrolytes occurring in plants exposed to Cd stress. The rupture of membranes caused by lipid peroxidation occurs frequently under conditions of stress; therefore, these parameters are potential indicators of cell damages (Kumari *et al.* 2010, Gallego *et al.* 2012). Anuradha and Rao (2007) evaluated the effects of 24-epibrassinolide and 28-homobrassinolide on *R. sativus* and detected a reduction in contents of MDA. Moreover, Xu *et al.* (2013) observed an increase in the MDA content in *Solanum tuberosum* plants exposed to Cd, consistent with the results of this study. Allagulova *et al.* (2015) working with *T. aestivum* pretreated with EBR also reported reductions in levels of EL. Moreover, EL values increased in *Cucumis sativus* seedlings exposed to a high concentration of Cd in a study by Gonçalves *et al.* (2007).

At the highest concentration of EBR (100 nM), the contents of Chl *a*, Chl *b*, and total Chl increased in *V. unguiculata*. Simultaneously, EBR helped to mitigate the negative effects caused by Cd toxicity on photosynthetic pigments. The reductions in contents of pigments caused by Cd toxicity are associated with the substitution of Mg by Cd in the tetrapyrrole centres of both Chl types, which induces molecule breakdown and irreversible modifications of the LHCII (Gillet *et al.* 2006, Dhir *et al.* 2009). EBR application also inhibited the toxic effect of Cd on Chl *b* in *P. vulgaris* (Rady 2011), and in an evaluation of *Brassica juncea* exposed to EBR and Cd, Hayat *et al.* (2007) reported that EBR alleviated the effect of Cd on total Chl. In a study evaluating *Solanum melongena* conducted by Singh and Prasad *et al.* (2014), the contents of Chl *a* and Chl *b* were reduced significantly after exposure to two concentrations of Cd, and a decrease in total Chl content was described for the toxic effect of Cd in genotypes of *Cucumis melo* by Zhang *et al.* (2015).

The Cd interference in plant growth decreased after treatment with EBR. The EBR inhibited the toxic effects of Cd on nutrient concentrations, Chl fluorescence, gas exchange, oxidant compounds, and pigments. The Cd toxicity reduces the growth of plants because of damages to the photosynthetic apparatus and metabolic activity. The suppression of growth in plants by Cd is strongly correlated with reductions in photosynthetic pigments (Ali *et al.* 2015), damages to the ultrastructure of chloroplasts (Feng *et al.* 2010), reductions in root development (Guo *et al.* 2009, Najeeb *et al.* 2011), and the subsequent inhibition of photosynthetic capacity (Li *et al.* 2013). Our results were consistent with the positive responses obtained by Ahammed *et al.* (2013) for the role of EBR in *S. lycopersicum* plants exposed to Cd stress. Jia *et al.* (2015) also observed reduced growth (roots, stem, and leaves) of *Lonicera japonica* plants exposed to Cd toxicity.

Conclusion: Our research clearly showed that EBR can play multiple and beneficial roles in cowpea plants exposed to both control and Cd-toxicity conditions. Our results confirmed that EBR reduced the absorption and transport of Cd, besides increasing the essential element contents. The beneficial repercussions of EBR on growth, maintenance of photosynthetic pigments, primary photosynthetic processes, and gas exchange were intrinsically connected to lower production of oxidant compounds and cell damages. EBR application to plants at 50 and 100 nM concentrations improved the performances related to nutritional, physiological, biochemical, and morphological parameters, with 100 nM EBR as an optimal dose. This can be recommended for the practical utilization for plants under Cd toxicity.

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