



## Research article

# The putative role of endogenous nitric oxide in brassinosteroid-induced antioxidant defence system in pepper (*Capsicum annuum* L.) plants under water stress

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## ABSTRACT

Brassinosteroids (BRs) have been rarely tested for their effective roles in mitigation of deleterious effects of water stress (WS) on plants. In addition, the contribution of nitric oxide (NO) in BR-improved plant tolerance to water stress needs to be elucidated. So, a trial was carried out to uncover the contribution of NO in BR-induced tolerance of pepper plants to WS. For well-watered and water-stressed plants, soil water availability was sustained at 80% and 40% of the full water storage capacity, respectively. BR (24-epibrassinolide, EB; 1.0 μM) was sprayed to the leaves of both well-watered and water stressed-pepper plants every two days for 10 days prior to the initiation of stress treatment. After starting WS treatment, cPTIO was sprayed to plant leaves twice a week for four weeks. Water stress caused a reduced plant growth and oxidative stress, but the application of EB increased plant growth and reversed the oxidative stress. The EB treatment increased endogenous NO and reinforced antioxidant defence systems, but the cPTIO application reversed the NO levels, downregulated the antioxidant defence systems, and aggravated oxidative damages caused by WS. These results show that EB-induced NO generation and NO-mediated antioxidant defence systems might be crucial mechanisms for EB-improved tolerance of pepper plants to WS. So, both EB and NO jointly are responsible for achieving improved tolerance of pepper plants to water stress.

## 1. Introduction

Drought stress is one of the main abiotic stresses suppressing plant growth and yield, particularly in dry and semi-dry areas (Boutraa et al., 2010). During the last decades, the investigation on drought stress has gained considerable attention of plant scientists (Boutraa et al., 2010). Increasing population and climate changes will aggravate the current pressure on water resources (Gosling et al., 2016). The damaging effect of drought starts mainly with disturbance of osmotic balance, and then progressively continues with physiological and metabolic disorders in plants (Salehi-Lisar and Bakhshayeshan-Agdam, 2016). The harshness of water stress is increasing progressively and it has been projected that it will lead to markedly suppress crop production up to 30% relative to the present yields by 2025 (Siddique et al., 2016). Furthermore, drought stress can disrupt the balance between reactive oxygen species (ROS) and antioxidant defence system in plants (Sofa et al., 2005; Zhang et al., 2010). Over-generation of ROS, e.g., H<sub>2</sub>O<sub>2</sub>, and O<sub>2</sub><sup>•-</sup> due

to water stress inhibits plant growth (Cui et al., 2015) and disturbs the metabolic stability of cells in plants (Nxele et al., 2017). Moreover, increased ROS generation leads to the degradation of chlorophyll molecules and finally reduces the photosynthetic activity of plants under water stress conditions (Sharma and Zheng, 2019).

Like other vegetable crops, pepper is one of the major crops in the Mediterranean area, where water deficit is one of main issues restricting plant growth and yield (Penella et al., 2014). Pepper is classified as one of the most sensitive crops to water stress, primarily because of large leaf area and high stomatal conductance leading to high water loss (Delfine et al., 2002).

Brassinosteroids (BRs) are known to maintain plant growth, development and several physiological processes (Jiroutova et al., 2018). Since their discovery, main components of the recognized BR signalling cascade have been evaluated through a variety of genetic and biochemical tools (Yokota and Mori, 2018). In the last few decades, BR has been reported to be essential cell elongation. Several preliminary

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investigations on hypocotyl elongation have shown insight into the transcriptional responses that activate elongation (Szekeres et al., 1996). However, lately they have been found to be involved in cell division (Hu et al., 2000), thereby promoting growth and development in plants (Planas-Riverola et al., 2019). Many studies show that BRs can alleviate the detrimental effects of several stresses on plants (Ahmed et al., 2013; Çoban and Baydar, 2016; Wani et al., 2017; Jiroutova et al., 2018). Application of BR can enhance water stress tolerance in various plant species, such as peppermint (Çoban and Baydar, 2016), tomato (Li et al., 2017), and rice (Riboldi et al., 2018).

Nitric oxide (NO) is a diffusible molecule through cellular membranes because of its lipophilic nature. It plays a role in numerous metabolic and biochemical events in plants (Simontacchi et al., 2015; Pucciariello and Perata, 2017). Over the last decades, some studies have reported that NO can be triggered promptly, and it acts as a powerful bio-stimulator in stressed plants (Fancy et al., 2017). There is a strong evidence that NO plays a significant role in plant response to a range of stresses, e.g., iron deficiency (Shanmugam et al., 2015), salinity stress (Ahmad et al., 2016), and water stress (Cechin et al., 2015). On the other hand, the integrative role of endogenous NO in EB-mediated oxidative defence mechanisms needs to be elucidated. Hence, the main aim of conducting this trial was to assess that up to what extent NO synthesis contributes to EB-induced tolerance to water stress in pepper plants.

## 2. Material and methods

### 2.1. Cultivation of plant and treatments

A trial was set up using pepper (*Capsicum annum* L.) cv. “Semerkand” under glasshouse conditions. Day and night temperatures in the entire course of the experimentation remained at  $25 \pm 2^\circ\text{C}$  and  $15 \pm 2^\circ\text{C}$ , respectively by using a heating system. The photoperiod during the whole experimentation period was 11 h/d. Seeds of pepper were sterilised with sodium hypochlorite (NaOCl; 1% v/v) solution and after rinsing with deionised water, they were sown in a tray containing glass beads. Water was supplied to the tray every day. After the second leaf was completely expanded, the seedlings were used for experimentation. Three seedlings were transplanted into each pot containing 8 kg of air-dried soil. The soil was clay loam with pH of 7.8,  $\text{CaCO}_3$  25.2%, organic matter content 1.2%, available P 4.4 mg/kg [0.5 M  $\text{NaHCO}_3$  extractable  $\text{P}_2\text{O}_5$  (Olsen et al., 1954)], plant-available S  $3.1 \mu\text{g g}^{-1}$  [0.01 M  $\text{CaCl}_2$  extractable  $\text{SO}_4^{2-}\text{-S}$  (Bardsley and Lancaster, 1960)] and electrical conductivity of saturation extract  $1.1 \text{ dS m}^{-1}$ . The exchangeable nutrient contents of  $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Na}^+$  were 13.5, 255, 122 and  $6.7 \text{ mmol kg}^{-1}$ , respectively. Electrical conductivity of the water used was  $0.52 \text{ dS m}^{-1}$  and its pH 7.2. Nitrogen,  $\text{P}_2\text{O}_5$  and  $\text{K}_2\text{O}$  were applied to the soil as granular urea (100 mg/kg), triple superphosphate (50 mg/kg) and potassium sulphate (120 mg/kg), respectively.

For well-watered and water-stressed plants, soil water availability was maintained at 80 and 40% of the full water storage capacity, respectively. Prior to initiating water stress treatment, half of the seedlings were sprayed with  $1.0 \mu\text{M}$  24-epibrassinolide 85% (EB, Sigma-Aldrich) solution prepared in 0.01% tween-20, every two days for 10 days to the foliage of plants grown under water stress, but to the rest of the plants only 0.01% tween-20 solution was sprayed. Water stress treatment was initiated after acclimatizing the plants for two weeks under optimum growth conditions. After the initiation of water stress treatment, 0.1 mM cPTIO, a NO scavenger, was dissolved in ethanol as described in the product information sheet and then was sprayed to the foliage of plants under different treatments twice a week for four weeks. The same amount of ethanol used to dissolve cPTIO was also added to the deionised water for spraying the control seedlings.

To initiate water stress treatment, the full soil water retention capacity was measured by testing three pots with holes in their bottoms.

Each pot was saturated with distilled water, covered with a plastic film and allowed to drain excess water for 24 h. Thereafter, the pots were reweighed and the maximum water storing capacity of the soil was calculated following Bonfim-Silva et al. (2015). The evaporation was measured by weighing each pot twice a day with an electronic scale and the water lost was re-added based on the gravimetric procedure and omitted increase in plant weight (da Silva Leite et al., 2019).

Each treatment had 3 replications and there were 3 pots (3 plants per pot) in each replicate, i.e., 9 pots per treatment. For determining shoot fresh and dry weights, three plants per replication (9 plants per treatment) were harvested. After measuring their fresh weight, they were oven-dried at  $75^\circ\text{C}$  for three days and then their dry weights measured. Fully expanded youngest leaves of the remaining 6 plants per replicate were collected to record data of the following parameters:

### 2.2. Chlorophyll contents

A 1.0 g of leaf tissue was homogenised in an acetone solution (90%). After filtration, homogenates were centrifuged at  $5000 \times g$  and then the absorbance values recorded on a Shimadzu UV-Visible spectrophotometer (UV-1201, Japan) for measurement of chlorophyll contents of all leaf samples according to the equation proposed by Strain and Svec (1966).

### 2.3. Chlorophyll fluorescence measurements

Before measuring this physiological attribute, the leaves already dark adapted for 30 min from each replicate were subjected to the Walz Mini-PAM Photosynthesis Yield Analyser.

### 2.4. Leaf water potential

Measurements for leaf water potential were performed early in the morning using newly expanded leaf, and subjected to a water potential measurement system (PMS model 600, USA).

### 2.5. Leaf free proline

The ninhydrin procedure outlined by Bates et al. (1973) was followed to analyse it. A 0.5 g of fresh leaf sample was homogenised in 10 ml of aqueous sulfosalicylic acid (3%) and then the homogenised solution was filtered. A solution of 2 ml of acid ninhydrin and glacial acetic acid (each) were added to the filtrate (2 ml). All samples were placed in a water bath at  $80^\circ\text{C}$  for 1 h and then in order to terminate the reaction they were placed in an ice bath. Following the addition of toluene (4 ml), the mixtures were mixed for 15–20 s using a test tube mixer. The absorbance values were read at 520 nm. A standard curve for proline was prepared with proline solutions ranging from 0.04 to 1 mM.

### 2.6. Assay of total soluble sugars

It was quantified based on the procedure reported by Yemm and Willis (1954). A leaf tissue (100 mg) was extracted in ethanol (10 ml, 80%) and subjected to centrifugation at 2683g for 20 min. The supernatant was taken and the remaining residue was re-extracted in ethanol (10 ml, 80%) and centrifuged at 2683 g for 20 min. The supernatants so obtained were mixed up and then 0.2 ml of the supernatant was taken and evaporated to dryness in a test tube in a water bath and cooled down it to  $25^\circ\text{C}$ . Distilled water (1 ml) was added to each test tube and mixed carefully. The Anthrone reagent (4 ml) was added to each test tube and mixed softly, incubated in a water bath at  $100^\circ\text{C}$  for 10 min, cooled down quickly under running cold water and optical density was measured at 620 nm.

## 2.7. Leaf soluble proteins

The protocol reported by Bradford (1976) was followed to appraise leaf soluble proteins. A fresh leaf tissue (50 g) was ground in 0.2 mM phosphate buffer solution (pH 6.2). The ground sample was then subjected to centrifugation at 2000g for 10 min. The phosphate buffer solution was used to raise the volume of the supernatant to 10 ml. A 5 ml aliquot of the Coomassie Blue R-250 reagent was added to 1 mL of the sample solution, and then shaken in a vortex mixer for 30 s. The optical density of each sample was noted at 595 nm. Bovine serum albumin was used as a standard to determine soluble proteins.

## 2.8. Nitric oxide (NO) determination

The modified method outlined by Zhou et al. (2005) was followed to determine NO in the leaves. Fresh leaf sample (each 0.6 mg) was triturated in 3 ml of cold acetic acid buffer (50 mM, pH 3.6, consisting of 4% zinc diacetate) in a mortar and pestle and then the extraction was subjected to centrifugation at 10,000 g at 4 °C for 15 min. The supernatant was taken out, but the pellet was washed using a 1.0 ml of extraction buffer after centrifugation. Both supernatants were mixed, and then 100 mg of charcoal were added to it. After filtrating and vortexing, 1 ml of each of the mixture and the Greiss reagent were retained for 30 min at room temperature. The optical density of each treated sample was noted at 540 nm.

## 2.9. Assay of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)

The method of Velikova et al. (2000) was employed to determine leaf H<sub>2</sub>O<sub>2</sub>. A fresh leaf sample (500 mg) was macerated in TCA (3 ml, 1% w/v). Following centrifugation of each sample, an aliquot (0.75 ml) was added to potassium phosphate (K–P) buffer (0.75 ml, 10 mM) and KI (1.5 ml, 1M). The absorbance of each treated sample was recorded at 390 nm. The quantity of H<sub>2</sub>O<sub>2</sub> was determined using a standard curve prepared using solutions containing H<sub>2</sub>O<sub>2</sub> ranging 0–100 nmolar.

## 2.10. Leaf malondialdehyde (MDA)

MDA, a lipid peroxidation product, in the leaves was appraised following the procedure reported by Weisany et al. (2012). MDA content was determined based on the content of total 2-thiobarbituric acid reactive substances (TBARS).

## 2.11. Determination of electrolyte leakage (EL)

The protocol described by Dionisio-Sese and Tobita (1998) was followed to assess EL. Detailed information on the procedure was prescribed elsewhere (Kaya and Ashraf, 2015).

## 2.12. Appraisal of antioxidant enzyme activities

A fresh leaf tissue (500 mg) was triturated in 50 mM Na–P buffer (pH 7.0) containing 1% soluble polyvinyl pyrrolidone. The leaf extract was centrifuged at 20 000 g at 4 °C for 15 min. The activities of CAT, SOD and POD in the supernatant were estimated following Kraus and Fletcher (1994), Van Rossum et al. (1997) and Chance and Maehly (1955), respectively. All enzyme activities were expressed as enzyme unit (EU) mg<sup>-1</sup> protein.

## 2.13. Assay of ascorbate (AsA)

The protocol developed by Mukherjee and Choudhuri (1983) was followed to analyse AsA content. Briefly, leaf tissues were powdered in liquid nitrogen and then they were extracted in a solution mixture consisting of 6% trichloroacetic acid (TCA) and 2% dinitrophenylhydrazine prepared in H<sub>2</sub>SO<sub>4</sub> (50%) and thiourea (10%) dissolved in

ethanol (70%). The extraction solution was boiled in a water bath for 15 min, and then its temperature was brought down to 25 °C. The solution so obtained was subjected to centrifugation at 1000 g for 10 min at 4 °C, and the resultant pellet was resuspended in H<sub>2</sub>SO<sub>4</sub> (80%). The absorbance values were read at 530 nm. Standard curves with known concentrations of AsA (ranging 0–10 nmolar) were used to determine AsA contents in plant samples.

## 2.14. Glutathione (GSH) assay

The procedure reported by Ellman (1959) was followed to analyse total GSH. Based on the procedure that when the Ellman reagent and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) are added to sulfhydryl compounds, yellow color is produced. An aliquot of 3 mL of 4% sulfosalicylic acid was added to the leaf tissue extracted (0.5 mL) in phosphate buffer. After centrifugating the mixture at 3000 g briefly, an aliquot (0.5 mL) was treated with the Ellman's reagent. The absorbance values were read at 412 nm following a lapse of 10 min.

## 2.15. Mineral nutrient analysis

The well ground leaf samples were placed in a muffle furnace at 550 °C to attain ash content, which was then used to determine K<sup>+</sup> and Ca<sup>2+</sup>. Each ash sample was treated with hot HCl (5 mL, 2M), and then distilled deionised water was added to this solution to bring the final volume of the sample to 50 mL (Chapman and Pratt, 1982). Potassium (K<sup>+</sup>) and Ca<sup>2+</sup> in the digested samples were analysed using an ICP.

## 2.16. Statistical analysis

The data were analysed following a two-way analysis of variance using CoStat program (Version v6.303). The Duncan's Multiple Range test at 5% probability was employed to appraise if the mean values differed significantly from one another.

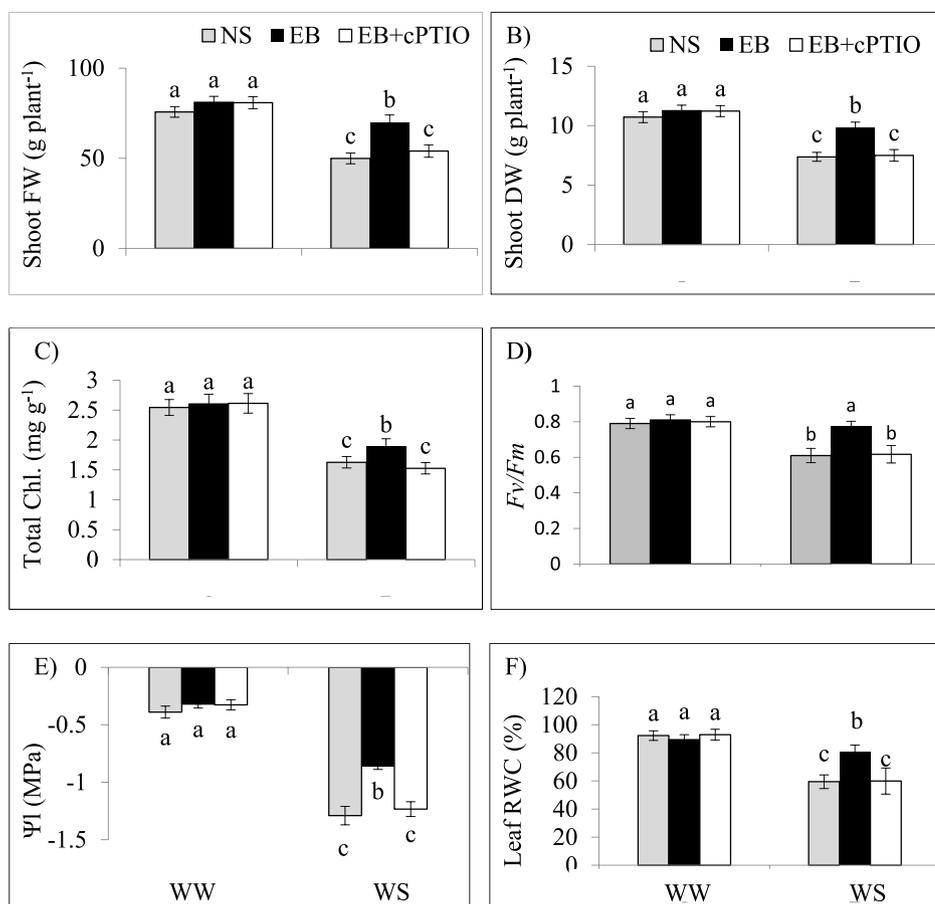
## 3. Results

### 3.1. 24-epibrassinolide (EB) enhances plant growth and photosynthetic parameters

Water stress (WS) significantly ( $P \leq 0.05$ ) decreased both shoot fresh and dry matter by 34.1 and 30.8%, respectively, total chlorophyll by 36.2% and maximum fluorescence yield ( $F_v/F_m$ ) by 22.8% of pepper plants compared to those in the well-watered plants (Fig. 1A, B, C, D). Conversely, exogenously applied 24-epibrassinolide, (EB; 1.0 μM) improved shoot fresh and dry weights by 40.5% and 33.8%, respectively, total chlorophyll by 16.04% and maximum fluorescence yield ( $F_v/F_m$ ) by 26.22% relative to those in the plants grown under WS alone. Treatment of EB was not effective in enhancing these parameters of plants grown under well-watered (WW) conditions. However, these mitigation effects of EB on the related parameters were almost totally inverted by using the scavenger of NO, cPTIO.

### 3.2. 24-epibrassinolide (EB) maintains plant water relations, total soluble sugar (TSS) content, ascorbate (AsA) and glutathione (GSH)

When plants were subjected to WS, leaf water potential ( $\Psi_l$ ) and relative water content (RWC) decreased considerably ( $P \leq 0.05$ ) by 4.31-fold and 35.36%, respectively (Fig. 1E and F), but total soluble sugar (TSS) content was increased by 4.0-fold relative to those in well-watered plants. Foliar application of EB increased  $\Psi_l$  and RWC by 33.33% and 35.65%, respectively, but it reduced the TSS content by 88%, compared to those in WS-plants alone (Fig. 2A). However, these traits did not significantly differ by the application of EB in the well-watered plants. On the other hand, application of cPTIO along with EB eliminated the ameliorative effects of EB on these parameters.



**Fig. 1.** Shoot fresh weight [FW; A)], shoot dry weight [DW; B)], chlorophyll content (C), maximum fluorescence yield [ $F_v/F_m$ ; D)], leaf water potential [ $\Psi_l$ ; E)] and leaf relative water content [RWC; F)], of pepper plants grown under well water (WW) and water stress (WS) sprayed with 1.0  $\mu\text{M}$  24-epibrassinolide (EB)/0.1 mM scavenger of NO, cPTIO or non-sprayed (NS). Data are means  $\pm$  S.E. of three replications. Mean values carrying different letters differ significantly ( $P \leq 0.05$ ) based on Duncan's multiple range test.

### 3.3. 24-epibrassinolide (EB) increases nitric oxide (NO) and proline

Water stress considerably ( $P \leq 0.05$ ) enhanced leaf NO and proline of pepper by 2.95- and 3.29-fold, respectively relative to those in the well-watered plants. Spraying EB significantly caused further elevations in leaf NO and proline by 1.89- and 1.43-fold, respectively in the water stressed plants alone (Fig. 2B and C). Conversely, when plants were sprayed with cPTIO in combination with EB treatments, the NO and proline levels were significantly reversed in the WS-plants by lowering both contents to the levels or lower than those in the WS-plants alone.

### 3.4. 24-epibrassinolide increases leaf potassium and calcium

There were significant decreases ( $P \leq 0.05$ ) in leaf potassium ( $K^+$ ) and calcium ( $Ca^{2+}$ ) by 21.4% and 22.7%, respectively relative to those in the well-watered plants (Fig. 2D and E). However, significant increases were obtained by the application of EB in leaf  $K^+$  and  $Ca^{2+}$  by 43.5% and 52.9%, respectively relative to those in plants under water stress alone. The treatment of EB did not change these leaf element contents in the well-watered plants. The treatment of cPTIO in combination with EB eliminated the increase in leaf  $K^+$  and  $Ca^{2+}$  contents by the EB treatment.

### 3.5. 24-epibrassinolide induces synthesis of NO to eliminate oxidative stress

To understand that up to what extent EB-induced NO synthesis was involved in ameliorating the oxidative stress induced by WS, the levels of hydrogen peroxide ( $H_2O_2$ ), malondialdehyde (MDA) and electrolyte leakage (EL) were determined. Water stress caused a notable increase ( $P \leq 0.05$ ) in leaf  $H_2O_2$ , MDA and EL by 2.55-, 3.12- and 1.98-fold, respectively, in pepper plants relative to those in plants grown at well-watered conditions, but exogenously applied EB noticeably ( $P \leq 0.05$ )

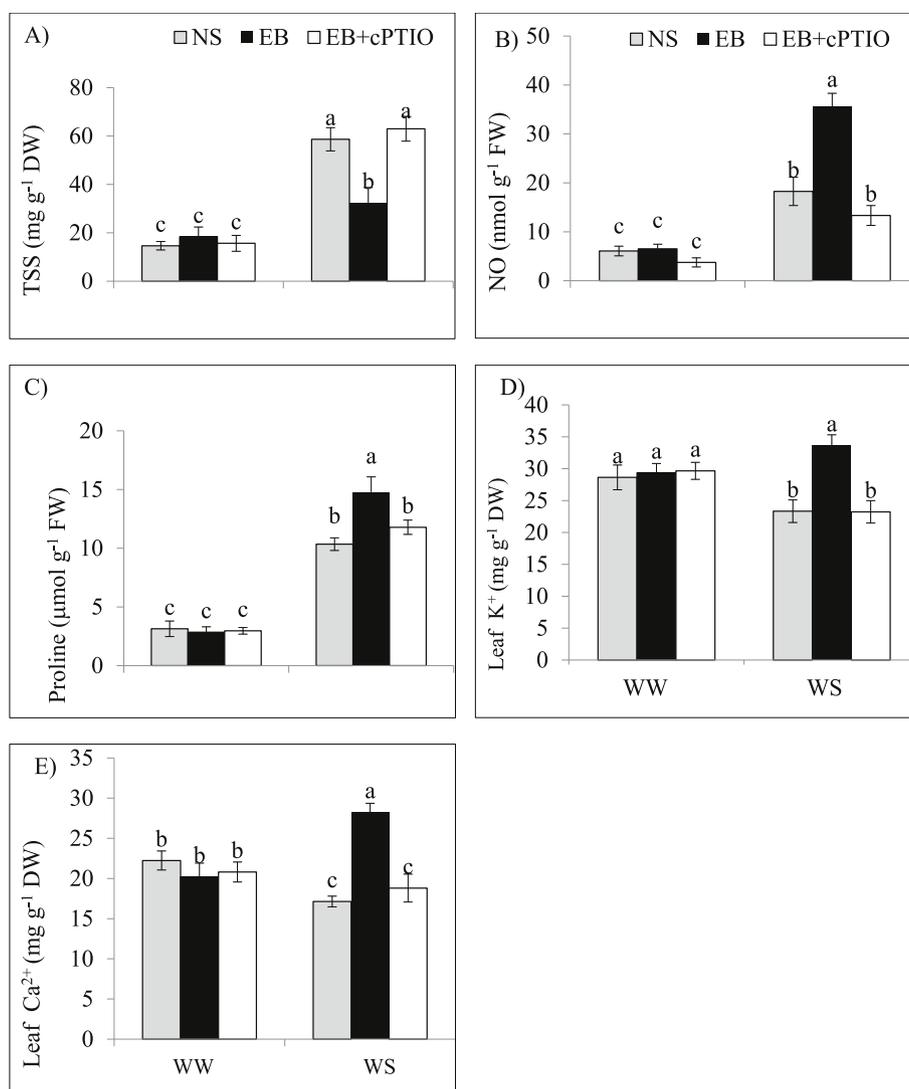
reduced,  $H_2O_2$ , MDA and EL of WS-pepper plants by 20.93%, 28.91% and 37.50%, respectively compared to those in the water-stressed plants alone. Exogenously applied EB was not significantly ( $P \leq 0.05$ ) effective in changing these oxidative parameters in the well-watered plants. However, application of cPTIO plus EB treatment totally eliminated the reduction in oxidative stress traits to the non-significant levels of those in WS-plants (Fig. 3A, B, C).

### 3.6. 24-epibrassinolide induces synthesis of NO to enhance antioxidant defence system

In order to get an insight into how far NO synthesis induced by EB could play a role in enhancing antioxidant defence system impaired by WS, the activities of SOD, CAT and POD were determined. Water stress caused substantial elevations ( $P \leq 0.05$ ) in SOD, CAT and POD activities by 1.48-, 2.27- and 2.33-fold, respectively in pepper plants relative to those in plants under well-watered conditions. Exogenous application of EB caused further substantial increases ( $P \leq 0.05$ ) in the activities of SOD, CAT and POD in the leaves of pepper plants subjected to water stress by 24.79%, 34.14% and 71.92%, respectively compared to those in the WS plants alone. Exogenous application of EB did not significantly ( $P \leq 0.05$ ) alter these antioxidant defence system parameters in the well-watered plants. However, EB + cPTIO treatment totally eliminated the elevations in antioxidant defence system parameters to the non-significant levels of those in WS-plants (Fig. 3D, E, F).

### 3.7. 24-epibrassinolide (EB) maintains ascorbate (AsA) and glutathione (GSH)

When plants were subjected to WS, ascorbate (AsA) and glutathione (GSH) increased by 1.8- and 1.9-fold, respectively relative to those in well-watered plants. Foliar application of EB reduced AsA and GSH by



**Fig. 2.** Total soluble sugar [TSS; A)] content on dry weight (DW) basis, nitric oxide content [NO; (B)], proline content (C) on fresh weight (FW) basis, potassium [K<sup>+</sup>; (D)] and calcium [Ca<sup>2+</sup>; (E)] on dry weight (DW) basis in the leaves of pepper plants grown under well water (WW) and water stress (WS) sprayed with 1.0 μM 24-epibrassinolide (EB)/0.1 mM scavenger of NO, cPTIO or non-sprayed (NS). Data are means ± S.E of three replications. Mean values carrying different letters differ significantly ( $P \leq 0.05$ ) based on Duncan's multiple range test.

38% and 46% compared to those in WS-plants alone (Fig. 4A and B). However, these traits did not significantly differ by the application of EB in the well-watered plants. On the other hand, application of cPTIO along with EB eliminated the ameliorative effects of EB on these parameters.

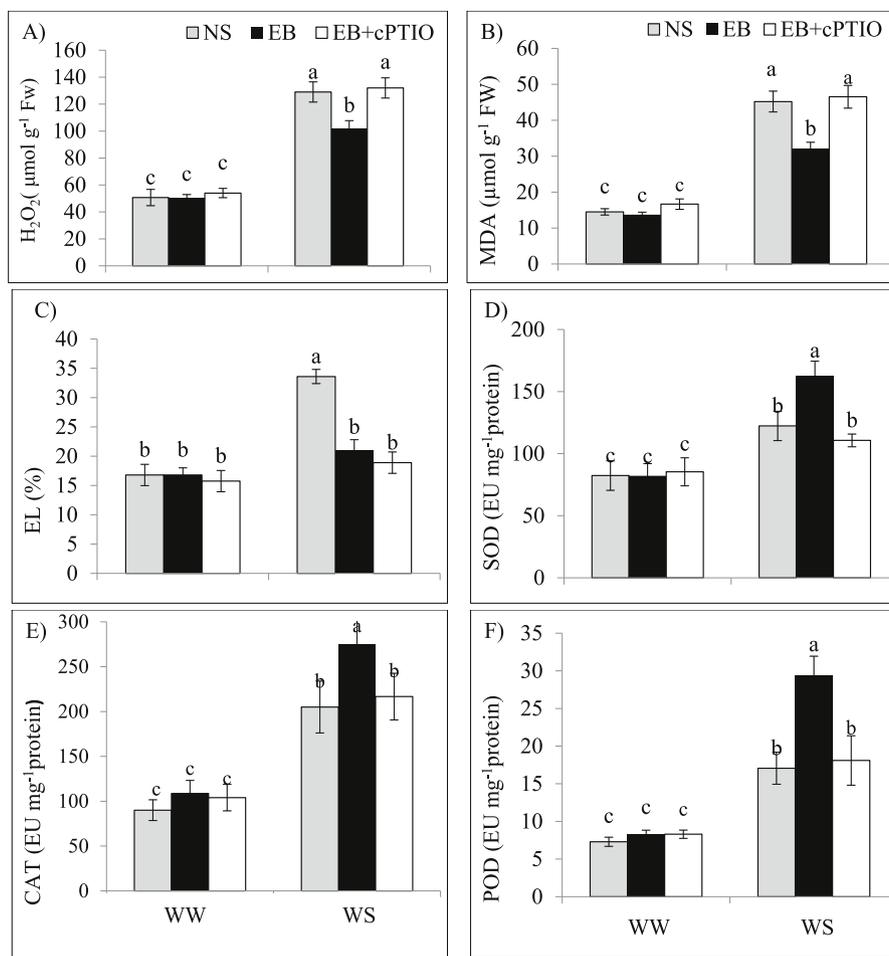
#### 4. Discussion

##### 4.1. The role of NO in EB-induced improvement in plant growth under water stress

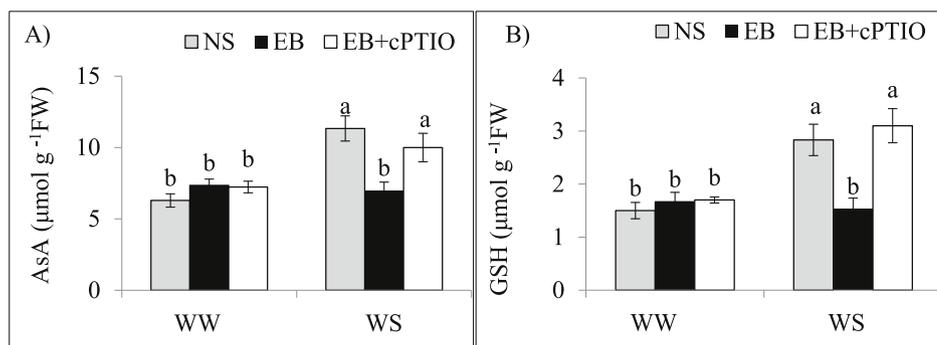
It is evident from the results that water stress reduced plant growth in terms of reduced shoot fresh and dry matter of pepper plants (Fig. 1 A, B). It has been well documented that water stress reduces plant growth in various plant species including barley (Pazirandeh et al., 2015), cherry tomato (Al Hassan et al., 2015), cucumber (Naz et al., 2016), and maize (Gao and Lynch, 2016), etc. An obvious decrease in plant growth might be because of the detrimental effect of water stress on plant metabolic events and interruption in acquisition of nutrient elements possibly due to low transport rate of nutrient elements to plant

roots by water (Selvakumar et al., 2012). The present results also reveal that the decreased plant dry matter might be linked to reduced leaf K<sup>+</sup> and Ca<sup>2+</sup> of pepper plants subjected to water stress (Fig. 2D and E).

To investigate whether or not 24-epibrassinolide (EB), a synthetic form of brassinosteroids (BRs), can mitigate the reduction in the growth of WS-plants, EB was supplied as foliar spray to the leaves of WS-plants. Our findings showed that exogenously applied EB significantly relieved the reduction in plant growth due to WS. So, this shows that EB is an effective substance in the response to water stress in pepper plants, as has been earlier reported by Damghan (2009) and Wang et al. (2015) in tomato and grape seedlings, respectively. Therefore, application of EB might be a very likely approach to mitigate the damaging effects of environmental stresses on different plants. The positive effect of EB has previously been showed on some of plant species under different stresses, e.g., cold stress (Divi and Krishna, 2010), cadmium toxicity (Jiroutova et al., 2018), zinc toxicity (Ramakrishna and Rao, 2015), heavy metal stress (Sharma et al., 2016), and saline stress (Marakli and Gozukirmizi, 2018). The mitigation effects of EB on water stress induced reduction in plant growth could have been because of improvement in chlorophyll content and Fv/Fm as well as nutrient elements



**Fig. 3.** Hydrogen peroxide [ $H_2O_2$ ; (A)], malondialdehyde [MDA; (B)], electrolyte leakage [EL; (C)], superoxide dismutase [SOD; (D)], catalase [CAT; (E)] and peroxidase [POD; (F)] in the leaves of pepper plants grown under well water (WW) and water stress (WS) sprayed with  $1.0 \mu\text{M}$  24-epibrassinolide (EB)/ $0.1 \text{ mM}$  scavenger of NO, cPTIO or non-sprayed (NS). Data are means  $\pm$  S.E of three replications. Mean values carrying different letters differ significantly ( $P \leq 0.05$ ) based on Duncan's multiple range test.



**Fig. 4.** Ascorbate [AsA; (A)] and glutathione [GSH; (B)] on fresh weight (FW) basis in the leaves of pepper plants grown under well water (WW) and water stress (WS) sprayed with  $1.0 \mu\text{M}$  24-epibrassinolide (EB)/ $0.1 \text{ mM}$  scavenger of NO, cPTIO or non-sprayed (NS). Data are means  $\pm$  S.E of three replications. Mean values carrying different letters differ significantly ( $P \leq 0.05$ ) based on Duncan's multiple range test.

including  $\text{Ca}^{2+}$  and  $\text{K}^+$ , which were suppressed by WS. These findings displayed that EB might play a crucial role in the response of plants to WS, as earlier proposed in tomato and grape seedlings by Yuan et al. (2010) and Wang et al. (2015), respectively.

To gain an insight into whether endogenous NO contributes to the EB-induced tolerance of WS-pepper plants, the scavenger of NO, cPTIO, was sprayed to the leaves of WS-plants during the experimentation period to ascertain whether it could reverse the EB induced NO content. The cPTIO led to reverse the increased NO content, so that EB application could not be able to sustain tolerance to water stress of pepper plants. These results suggest that NO in leaves might have contributed to EB-induced tolerance to water stress in pepper plants. The effect of

NO application has been investigated in different plants under drought stress such as in barley (Gan et al., 2015) and sugarcane (Silveira et al., 2017) as well as under other stresses such as salinity stress in maize (Kaya et al., 2015), mungbean (Salahuddin et al., 2017), and chickpea (Kumari et al., 2017), and cadmium stress in mungbean (Nahar et al., 2016). However, no study was deciphered from the available literature providing data on the role of EB-induced NO in improving tolerance to water stress. So, this seems to be the first report providing an insight to understand the underlying mechanism of EB in improving water stress tolerance.

#### 4.2. The contribution of NO in EB-improved photosynthetic traits under water stress

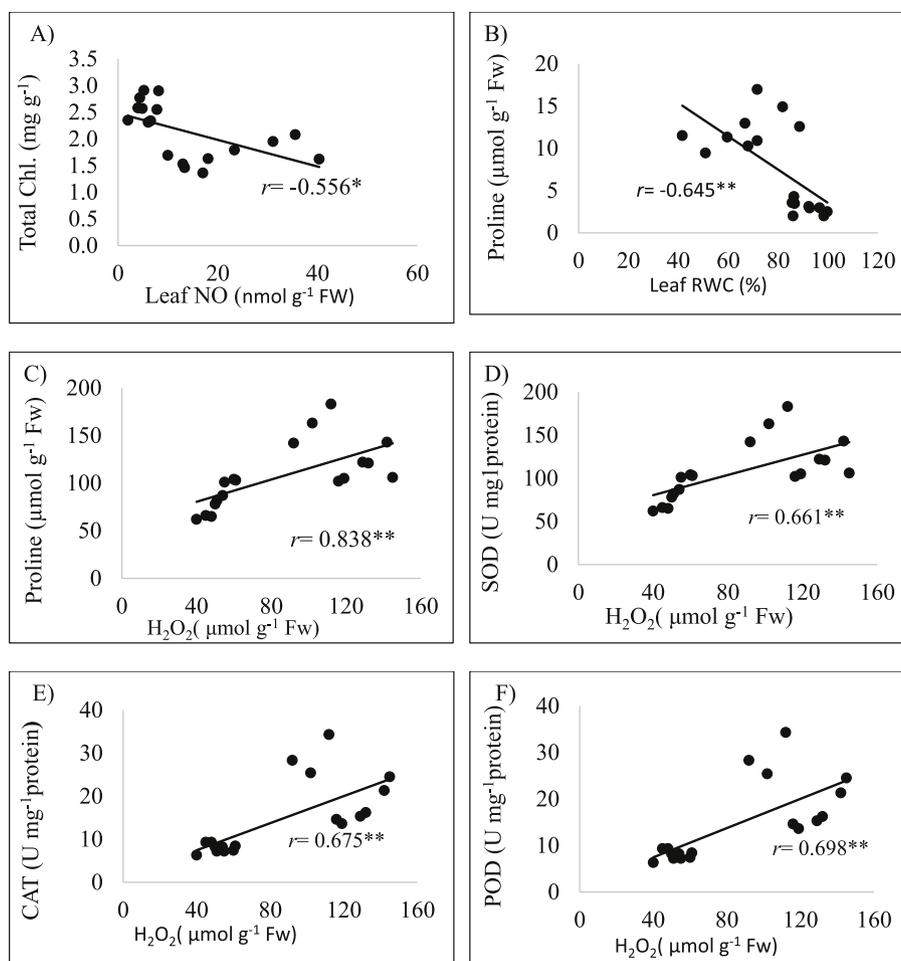
Plant chlorophyll content and maximum photochemical efficiency ( $F_v/F_m$ ) are both key traits, which indicate the status of photosynthesis activity, but these attributes are affected deleteriously under stress conditions (Sharma et al., 2015; Kalaji et al., 2016). Our results showed that WS significantly reduced both these traits. In earlier studies, the possible reason of reduction in these traits due to stress conditions might be primarily related to the excess accumulation of  $H_2O_2$  in plants (Gomes et al., 2016; Nxele et al., 2017). Our results similarly display that the lowered chlorophyll levels and  $F_v/F_m$  might be related to accumulation of  $H_2O_2$  at high levels in WS-pepper plants (Fig. 1C and D and Fig. 3A). Corresponding to that, linkage of chlorophyll content and  $F_v/F_m$  with  $H_2O_2$  content in the plants under water stress, EB enhanced chlorophyll content and  $F_v/F_m$  and reduced  $H_2O_2$  levels in WS-plants, suggesting that EB is involved in relieving the damaging effects of water stress on chlorophyll and  $F_v/F_m$ , possibly by reducing the accumulation of  $H_2O_2$ .

The endogenous NO was found to be further elevated in WS-plants by EB application. The scavenger of NO, cPTIO, was used to scavenge endogenous NO and to assess whether EB was still effective in increasing chlorophyll content and  $F_v/F_m$  in the plants in the case of reduced NO content in the WS-plants. The results showed that application of cPTIO reversed the increased NO induced by EB, which led to reverse improved chlorophyll content and  $F_v/F_m$ , suggesting that NO may play a critical role in EB-improved chlorophyll content and  $F_v/F_m$ .

There is a significant ( $P \leq 0.05$ ) nonlinear correlation ( $r = -0.556$ ) between endogenous NO and chlorophyll content (Fig. 5A), as has been previously observed that exogenously applied NO increased chlorophyll content in rice plants subjected to arsenic stress (Singh et al., 2016), in cotton crop exposed to saline stress (Kong et al., 2016) and chilling stressed-walnut shoots (Dong et al., 2018).

#### 4.3. The contribution of NO in EB-induced improvement in leaf water status and nutrient elements under water stress

Water stress has been reported to cause the imbalance between water status and nutrient acquisition (Zhao et al., 2015; Vurukonda et al., 2016; Bowles et al., 2016), which might be the reason for reduction in leaf water potential, relative water content (RWC),  $K^+$  and  $Ca^{2+}$  in the plants under water stress, but these traits were improved by the application of EB under water stress conditions (Fig. 1E and F; Fig. 2D and E). It has been reported that application of EB enhanced relative water content in chilli pepper plants under drought (Khamsuk et al., 2018). Increased water status of plants by EB under water stress is likely because of increased proline rather than total soluble sugar (TSS) content. Since EB enhanced proline content and reduced TSS content in plants, so this indicates that proline is the main source of osmotic adjustment. Analogous to our findings, in another study EB triggered the proline content to maintain plant water content (Rattan et al., 2012). The findings of the correlation analysis showed that RWC significantly ( $P < 0.01$ ) and nonlinearly ( $r = -0.645$ ) linked with proline content (Fig. 5B). This suggests that when plants show low RWC under water



**Fig. 5.** Correlation of leaf nitric oxide (NO) with total chlorophyll (A), correlation of proline with leaf water potential [ $\Psi$ ; B]), correlation of hydrogen peroxide ( $H_2O_2$ ) with proline (C), superoxide dismutase [SOD; (D)], catalase [CAT; (E)] and peroxidase [POD; (F)] in the leaves of pepper plants under water stress sprayed with 24-epibrassinolide (EB)/scavenger of NO, cPTIO. \*\* and \*: Correlation is significant at  $P \leq 0.01$  and  $P \leq 0.05$ , respectively.

stress, plants generate proline to maintain osmotic adjustment. Furthermore, proline suppressed the oxidative stress by lowering the accumulation of free radicals in plants (Szabados and Savoure, 2010.). Similarly, in the present experiment, there is a significant linear correlation ( $P \leq 0.001$ ) between proline content and  $H_2O_2$  content ( $r = 0.838$ ) in leaves (Fig. 5C). In our experiment, proline content was elevated in WS-plants. Its accumulation is likely to be useful in improving leaf water status and reducing oxidative impairment induced by water stress in pepper plants.

Another key strategy developed by plants to acclimate to water stress is the regulation of antioxidant defence system by up-regulating ascorbate (AsA) and glutathione (GSH) compounds, thereby playing crucial functions in eliminating ROS and sustaining cellular redox potential (Shi et al., 2016; Zechmann, 2018). It has previously been reported that AsA is the most key scavenging compound for the elimination of  $H_2O_2$  in plant cells (Zhang et al., 2015). Similarly, GSH is also a main non-enzymatic scavenger of ROS in plants. It is involved in controlling  $H_2O_2$  contents (Asgher et al., 2017). The present results obviously show that the levels of AsA and GSH increased against accumulation of  $H_2O_2$  to eliminate oxidative damage in WS-plants.

Nutrient elements are necessary for a variety of crucial physiological functions and they must be accumulated in plants at sufficient amount to maintain stability of plant structure and crucial metabolic events, so any deficiency of nutrient element uptake by plants can noticeably disturb plant metabolism (Liang et al., 2018). Brassinosteroid is one of the plant hormones which significantly controls the levels of nutrient elements in plants and it can alleviate stress by increasing nutrient elements (Yuan et al., 2015). As an example, treatment of EB considerably improved  $K^+$  and  $Ca^{2+}$  contents in salinity stressed-tomato plants (Soylemez et al., 2017) and in *Eucalyptus urophylla* subjected to iron deficiency (Lima et al., 2018). Our findings suggest that EB improved tolerance to water stress of pepper plants by improving water status as well as leaf  $Ca^{2+}$  and  $K^+$  contents.

Application of cPTIO along with EB led to reverse the improved RWC,  $Ca^{2+}$  and  $K^+$  contents as well as proline content in pepper plant leaves. This shows that EB triggered NO synthesis to induce tolerance to water stress by improving RWC, proline, and leaf  $Ca^{2+}$  and  $K^+$  contents. Previously, exogenously applied NO has been observed to enhance RWC in the leaves of rapeseed (Hasanuzzaman et al., 2017) and lettuce plants (Sánchez-Romera et al., 2018) exposed to water stress. In our previous investigation, it was also observed that NO increased leaf  $Ca^{2+}$  and  $K^+$  levels in tomato plants under boron toxicity (Kaya and Ashraf, 2015).

#### 4.4. EB triggers nitric oxide to improve tolerance to water stress of pepper plants

Nitric oxide (NO) is reported to play a role in response of plants to abiotic stresses. Pepper plants under water stress produced higher NO relative to that in well-watered plants (Fig. 2B). Similarly, it has been reported that water stress enhanced NO content in sugarcane (Silveira et al., 2017) plants under WS conditions and thus these findings suggest that NO could play a significant role as a signal molecule in some crucial metabolic processes of WS-plants. Moreover, exogenously applied EB led to a further elevation in NO synthesis in WS-pepper plants. EB is one of the several bio-stimulators taking part more efficiently in the synthesis of endogenous NO as has been reported in salt stressed-*Nicotiana benthamiana* (Zhu et al., 2016) and in *Arabidopsis thaliana* infected by cucumber mosaic virus (Zou et al., 2018). So, it is pertinent to note that the exogenously applied EB might induce endogenous NO, possibly acting as an antioxidant to enhance plant tolerance to a stress (Beligni et al., 2002; He et al., 2019). However, NO accumulation at high level can injure plants (Corpas et al., 2011; da Silva et al., 2018). Hence, a reasonable level of NO should be in the plant cell to enhance tolerance of plants to stress conditions. Generally, the mode of action of NO is to provoke post-translational modifications (PTMs) of enzymes,

as well as antioxidative enzymes, through nitration and S-nitrosation (Corpas et al., 2011). It is believed that PTMs are effectively involved in regulating a number of physiological processes in plants (Astier and Lindermayr, 2012; Nabi et al. 2019). In our study, EB-mediated accumulation of NO was not higher than the level which can damage plants, so injurious effects on plant physiological processes was not observed. A similar statement was made in the case of *Camellia sinensis* grown at non-stressed conditions (Li et al., 2017). Here, it is likely that EB triggered NO to improve tolerance to water stress in pepper plants, but application of cPTIO combined with EB treatment eliminated the alleviating effect of EB by reducing endogenous NO. This reveals that the beneficial effect of EB on pepper plants subjected to water stress might relate to NO production. By evaluating the ameliorating role of EB on WS-induced plant growth inhibition and oxidative stress, this study provides a new insight into NO as a downstream signal molecule involving in EB-induced WS tolerance of pepper plants.

#### 4.5. EB triggers nitric oxide to improve antioxidant defence system

The present findings have revealed that EB reversed water stress-induced oxidative impairment in the pepper seedlings by lowering  $H_2O_2$  and malondialdehyde (MDA) content (Fig. 3A and B), and up-regulating the activities of antioxidant enzymes (Fig. 3D, E, F), which have also been reported by others, e.g., Abedi and Pakniyat (2010) in drought stressed-oilseed rape plants and Ramakrishna and Rao (2015) in radish plants under zinc toxicity. Plants naturally possess antioxidant enzymes some of which include CAT and POD to eliminate  $H_2O_2$  and so that they improve the capability of plants to endure under WS conditions (Hafez and Gharib, 2016). The reduced EL was associated with decreased MDA induced by the positive effect of EB, showing negligible injuries to the cell membrane, which has also been reported by Lima and Lobato (2017) in WS-cowpea plants. It has been reported that exogenously applied EB modifies antioxidant defence system under stress situations (Fariduddin et al., 2014; Lima and Lobato, 2017). The present study also assessed how far the antioxidant defence systems responded to EB application under water stress. Similarly, in our research, water stress enhanced these enzyme activities in the pepper plants. Increased antioxidant enzyme activities by EB decreased the  $H_2O_2$  content. The correlation graph displays that  $H_2O_2$  levels were significantly ( $P \leq 0.01$ ) and linearly correlated with SOD ( $r = 0.661$ ), CAT ( $r = 0.675$ ), and POD ( $r = 0.698$ ) (Fig. 5D, E, F). Conversely, application of cPTIO, a NO scavenger, in combination with EB treatment inverted these improvements, proposing that leaf NO is involved in EB-induced WS tolerance of pepper plants. These findings seem to be the first indicating that endogenous NO induced by EB is involved in improving WS tolerance of pepper plants. However, the positive role of NO application alone has been widely demonstrated in various plant species under environmental stress conditions such as in rapeseed under water stress (Hasanuzzaman et al., 2017). Likewise, it was verified that application of EB could stimulate the synthesis of NO which could be involved in enhancing antioxidant defence system and lowering  $H_2O_2$  and MDA to increase tolerance to WS stress of pepper plants as shown by the treatment of cPTIO, the NO scavenger, which significantly lowered endogenous NO and inverted the beneficial effects of EB-induced NO on all those parameters. Therefore, this suggests that NO is a downstream signalling molecule triggered by EB, which conferred tolerance in pepper plants to water stress.

## 5. Conclusions

Overall, EB induced the generation of nitric oxide which enhanced the tolerance to water stress in pepper plants. The findings reveal that NO might be a downstream signal molecule triggered by EB in pepper plants exposed to water stress. The application of cPTIO plus EB eliminated the alleviation effects of EB on plants under WS and caused EB as non-effective in enhancing tolerance to water stress. Therefore,

exogenously applied EB and endogenous NO are both jointly considered to be involved in enhancing WS tolerance in pepper plants. Moreover, endogenous NO triggered by EB could be a crucial signalling molecule in the up-regulation of antioxidant defence enzymes to reverse oxidative impairment in WS pepper plants. However, there is a need to study how the molecular system functions in the case of EB-mediated improved WS tolerance in plants. The roles of other signalling molecules are also needed to be studied in response of plants to water stress induced by EB.

#### Author Contributions

CK performed the experiments and wrote up the initial manuscript. MA and PA helped in designing the study and critically edited the whole manuscript. LW analyzed the data and helped in data compilation. All authors read and approved the final manuscript.

#### Compliance with ethical standards

#### Conflict of interest

The authors declare no conflict of interest regarding the publication of this paper.

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