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RESEARCH PAPER

Role of brassinosteroids in alleviation of phenanthrene–cadmium co-contamination-induced photosynthetic inhibition and oxidative stress in tomato

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Abstract

Heavy metal pollution often occurs together with organic contaminants. Brassinosteroids (BRs) induce plant tolerance to several abiotic stresses, including phenanthrene (PHE) and cadmium (Cd) stress. However, the role of BRs in PHE+Cd co-contamination-induced stress amelioration is unknown. Here, the interactive effects of PHE, Cd, and 24-epibrassinolide (EBR; a biologically active BR) were investigated in tomato plants. The application of Cd (100 μM) alone was more phytotoxic than PHE applied alone (100 μM); however, their combined application resulted in slightly improved photosynthetic activity and pigment content compared with Cd alone after a 40 d exposure. Accumulation of reactive oxygen species and membrane lipid peroxidation were induced by PHE and/or Cd; however, the differences in effect were insignificant between Cd and PHE+Cd. The foliar application of EBR (0.1 μM) to PHE- and/or Cd-stressed plants alleviated photosynthetic inhibition and oxidative stress by causing enhancement of the activity of the enzymes and related transcript levels of the antioxidant system, secondary metabolism, and the xenobiotic detoxification system. Additionally, PHE and/or Cd residues were significantly decreased in both the leaves and roots after application of EBR, more specifically in PHE+Cd-stressed plants when treated with EBR, indicating a possible improvement in detoxification of these pollutants. The findings thus suggest a potential interaction of EBR and PHE for Cd stress alleviation. These results advocate a positive role for EBR in reducing pollutant residues for food safety and also strengthening phytoremediation.

Key words: Brassinosteroids, food safety, heavy metal, photosynthesis, phytoremediation, polycyclic aromatic hydrocarbons (PAHs).

Introduction

Polycyclic aromatic hydrocarbons (PAHs) and cadmium (Cd) are two widespread environmental pollutants, well recognized for their toxic, mutagenic, and carcinogenic properties (Goldman *et al*., 2001; Sun *et al*., 2011; Adams *et al*., 2012). The severity of environmental contamination is further shown to be aggravated with mixed pollution of heavy metals and organic pollutants, prevalent among contaminated sites around the world (Sandrin and Maier, 2003; Pilon-Smits, 2005). Shortage of arable land in many developing countries results in growing of vegetables on and/or around polluted sites. Such plants grown in these polluted

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areas usually take up PAHs and heavy metals from the contaminated soil and/or air. Cd toxicity has emerged as one of the major agricultural problems in many soils around the world (Hasan *et al*., 2011). Evidence of PAH contamination of agricultural crops is also rapidly emerging (Mo *et al*., 2009). For instance, vegetables grown in the Pearl River Delta of southern China have been reported to contain toxic concentrations of PAHs as high as 5353 μg kg^{-1} (Mo *et al*., 2009). Contamination of food commodities including vegetables with Cd has also become a matter of grave concern in sustaining healthy agricultural practices (Falcó *et al*., 2005). Besides direct exposure to contaminants, dietary intake of Cd and PAHs has an adverse impact on potential health risk, such as childhood asthma, skin cancer, and lung cancer (Goldman *et al*., 2001; Falcó *et al*., 2005). Previous findings have shown that vegetables are one of the main sources of the total dietary intake of PAHs and Cd (Falcó *et al*., 2005; Mo *et al*., 2009). Therefore, research focusing on the reduction of xenobiotic residues in food commodities has received much attention in the past decade.

Cd is considered to be a non-essential trace element, highly phytotxoic at higher concentrations (López-Millán *et al*., 2009; Zhang *et al*., 2011; Masood *et al*., 2012). Cd has been shown to interfere with the uptake, transport, and utilization of essential nutrients and water, to decrease photosynthesis, change enzyme activities, and also to cause symptoms such as chlorosis, necrosis, and root browning in tomato (López-Millán *et al*., 2009; Hasan *et al*., 2011). Similarly, PAHs have also been shown to decrease biomass, photosynthetic pigments, and photosynthesis, alter antioxidant enzyme activities, and induce oxidative stress in *Arabidopsis* and tomato (Liu *et al*., 2009; Ahammed *et al*., 2012*a*, *b*). Excessive production of reactive oxygen species (ROS) and subsequent oxidative damage may be the common mechanism of phytotoxicity induced by both Cd and PAHs in plants (Liu *et al*., 2009; Masood *et al*., 2012). ROS overload causes degradation of photosynthetic pigments and damage to photosynthetic machinery, which in turn decrease photosynthesis (Hasan *et al*., 2011; Ahammed *et al*., 2012*b*; Masood *et al*., 2012). Interestingly, additive, independent, synergistic, as well as antagonistic toxicity to plants has been reported following mixed pollution of PAHs and heavy metals (Babu *et al*., 2001; Lin *et al*., 2008; Sun *et al*., 2011; Zhang *et al*., 2011). For instance, Babu *et al*. (2001) observed synergistic effects of photooxidized PAHs and Cu on photosynthesis and plant growth in *Lemna gibba*. In contrast, stress-ameliorative effects of fluoranthene (a type of PAH) against Pb and Cu on the growth of *Triticum aestivum* have also been reported by Wetzel *et al*. (1994). These authors observed antagonistic toxicity, where the negative effects of metal solutions were diminished by simultaneous addition of fluoranthene. Likewise, pyrene has been shown to alleviate Cu-induced biomass reduction in *Zea mays* L. (Lin *et al*., 2008). However, to date, the physiological and molecular mechanisms behind this synergistic or antagonistic toxicity of metal and PAHs in mixed pollution to plants is poorly understood.

Phytoremediation is a promising green technology using higher plants to restore contaminated soils and water following exposure to toxic organic and/or inorganic compounds (Pilon-Smits, 2005). In recent years, phytoremediation of co-contamination has become an appealing research avenue (Sun *et al*., 2011; Zhang *et al*., 2011). However, remediation of mixed pollution offers a complex problem, as different pollutants may influence the remediation processes through their interaction (Sandrin and Maier, 2003; Sun *et al*., 2011; Zhang *et al*., 2011). Biodegradation of organic pollutants has been shown to be affected by the presence of metals in both aerobic and anaerobic systems (Sandrin and Maier, 2003). Accordingly, interactions between metal and organic pollutants may influence stress responses and corresponding stress tolerance in plants.

Plants, being sessile, inherently possess the ability to detoxify xenobiotics for their survival (Coleman *et al*., 1997; Pilon-Smits, 2005). However, plants themselves suffer from phytotoxicity under high levels of toxin exposure because of rate-limiting antioxidant and detoxification capacity. Most often, the plant's detoxification system is not very efficient in bringing about complete degradation of some recalcitrant foreign compounds (Dixit *et al*., 2011). Previous studies reveal that hormonal supplementation enhances plant tolerance and detoxification capacity under xenobiotic stress (Xia *et al*., 2009; Hasan *et al*., 2011; Ahammed *et al*., 2012*a*, *b*).

Brassinosteroids (BRs) are a class of essential plant hormones with multiple roles in the induction of tolerance to various abiotic stresses such as high or low temperature, moisture stress, drought, salinity, pesticide injury, PAHs, and heavy metal stresses (Divi and Krishna, 2009; Xia *et al*., 2009; Hasan *et al*., 2011; Ahammed *et al*., 2012*b*; Choudhary *et al*., 2012*a*, *b*). BRs increase biomass, chlorophyll contents, photosynthesis, and the potential of antioxidants and detoxification to improve plant tolerance under stress. Previously, BRs have been reported to alleviate Cd as well as PAH stresses in different plants including tomato (Hasan *et al*., 2011; Ahammed *et al*., 2012*b*). However, the role of BRs in mitigation of a co-contaminated system such as Cd+PAHs has not been studied yet. Keeping this in mind, it was hypothesized that BRs might have an anti-stress effect on the co-contamination by PAHs and Cd in tomato. Therefore, the present study was carried out to investigate the interactive effects of phenanthrene (PHE, a model PAH) and Cd on the growth, photosynthetic machinery, enzymes, and gene expression of the antioxidant and detoxification system, secondary metabolism, and pollutant residues in tomato plants. Foliar application of 24-epibrassinolide (EBR; an active BR) was used as a BR source to determine whether EBR could alleviate the PHE+Cd co-contamination-induced stress in tomato. This study will provide details of the underlying physiological and molecular mechanisms of the phytotoxicity of dual environmental pollution and the feasibility of EBR application to induce stress tolerance in tomato.

Materials and methods

Plant materials and growth conditions

Tomato (*Solanum lycopersicum* L. cv. Hezuo 903) seeds were purchased from the Zhejiang Academy of Agriculture, Hangzhou, China. Seeds were surface sterilized with 0.4% sodium hypochlorite for 15 min followed by repeated washings with Milli-Q water. Sterilized seeds were sown in a mixture of peat and vermiculite (7:3, v:v). The experiment was carried out in a multispan greenhouse at the Institute of Vegetable Sciences, Zhejiang University. The approximate conditions inside the

greenhouse during the experimental period were as follows: temperature 25/17 ºC (day/night), mean relative humidity 80%, photosynthetic photon flux density (PPFD) 800 µmol m^{-2} s^{-1}, and a photoperiod of 14/10 h (day/night). Upon the appearance of the first true fully expanded leaves, a group of eight seedlings was transplanted into a container (40 cm×25 cm×15 cm) filled with Hoagland's nutrient solution.

Treatments and biomass analysis

At the fourth leaf stage, the whole foliar region of the seedlings was sprayed with EBR and/or Milli-Q water (containing an equal ratio of ethanol used for the preparation of EBR solution). The working solution of 0.1 µM EBR (Sigma-Aldrich, St. Louis, MO, USA) was prepared by dissolving the solute in ethanol followed by dilution with Milli-Q water [ethanol:water (v/v)=1:10 000]. At 24 h after EBR pretreatment, seedlings were exposed to freshly prepared nutrient solution with or without PHE and/or Cd. PHE (purity 98%, Sigma-Aldrich, China) was initially dissolved in acetone and then diluted with Milli-Q water containing nutrient solution to a final concentration of 100 µM [acetone:water (v/v)=1:1000]. Controls as well as the rest of the treatments were exposed to the same ratio of acetone. Cd (100 µM) was supplied as $CdCl_2 \cdot 2.5H_2O$ (analytical grade) from a stock solution prepared in Milli-Q water. The concentrations of EBR, PHE, and Cd were selected on the basis of a preliminary experiment, previous studies, and others published reports (López-Millán *et al*., 2009; Xia *et al*., 2009; Ahammed *et al*., 2012*a*, *b*). Thus, the experiment consisted of eight treatments with three replications (each replication means one container, containing eight seedlings). Nutrient solution was changed every 5 d with or without PHE and/or Cd to avoid metabolic effects of root exudates as well as to maintain the stable concentration. EBR was sprayed every 10 d. Forty days after imposition of stress, the experiment was terminated and plants were harvested after the determination of gas exchange and chlorophyll fluorescence parameters. For physiological, biochemical, and molecular analysis, leaves were immediately frozen in liquid nitrogen and stored at –80 °C until further analyses. Eight plants from each treatment were randomly selected and divided into shoots and roots. Selected plants were dried in an oven at 80 °C for 24 h and then weighed to record their dry weights.

Determination of PHE and Cd contents

PHE was extracted from freeze-dried plant samples with dichloromethane and methanol (9:1, v:v) mixture by ultrasonication. The extracts were concentrated and cleaned up by passing them through an anhydrous Na_2SO_4/Florasil column, and eluted with dichloromethane. Elutes were concentrated and analysed by gas chromatography–mass spectrometry (Shimadzu GCMC-QP2010One Ultra System, Kyoto, Japan) following the method of Sun *et al*. (2011). To analyse Cd residue, dried leaf and root samples were ground to a fine powder and digested with HNO_3/$HClO_4$ (3:1, v:v). The Cd content was determined by atomic absorption spectrophotometry (Shimadzu AA-6300, Japan) as described in Masood *et al*. (2012).

Gas exchange measurements

Gas exchange parameters such as the CO_2 assimilation rate (P_n), stomatal conductance (g_s), and the intercellular CO_2 concentration (C_i) were determined on the third fully expanded leaves using an infrared gas analyser (IRGA) portable photosynthesis system (LI-COR 6400, Lincoln, NE, USA). The measurement was performed within the time period 8.00–11.00 h maintaining the air temperature, air relative humidity, CO_2 concentration, and PPFD, at 25 °C, 80–90%, 400 µmol mol^{-1}, and 1000 µmol m^{-2} s^{-1}, respectively.

Chlorophyll a fluorescence quenching and chlorophyll content measurements

Chlorophyll fluorescence parameters were measured on the third fully expanded leaves after 30 min of dark adaptation using an imaging pulse amplitude-modulated (PAM) fluorimeter (IMAG-MAXI; Heinz Walz, Effeltrich, Germany). Chlorophyll fluorescence parameters were measured and calculated as described by Xia *et al*. (2009) and Jiang *et al*. (2012). The maximum photochemical efficiency of photosystem II (PSII; F_v/F_m), the quantum efficiency of PSII (Φ_{PSII}), and the photochemical quenching coefficient (qP) were determined for the same leaves as an area of interest. Photosynthetic pigments were quantified by pooling third leaves from at least three plants per treatment. Leaf chlorophyll (Chl *a* and Chl *b*) and carotenoids (Carts) were extracted in 80% acetone and their contents (µg/g FW) were analysed spectrophotometrically according to Lichtenthaler and Wellburn (1983).

Determination of H_2O_2 content and membrane damage (lipid peroxidation), and histochemical detection of H_2O_2 and $O_2^{\cdot-}$ in leaves

H_2O_2 content in leaves was determined spectrophotometrically by a peroxidase assay according to Willekens *et al*. (1997). Accumulation of H_2O_2 in leaves was visually detected by staining with 3,3-diaminobenzidine (DAB) using the method of Thordal-Christensen *et al*. (1997). The $O_2^{\cdot-}$ accumulation was visualized using the nitroblue tetrazolium (NBT) staining procedure. NBT- and DAB-stained leaves were photographed using a digital camera (Canon DS126201, Japan). The level of lipid peroxidation in leaves was determined by quantifying the malondialdehyde (MDA) equivalents using 2-thiobarbituric acid (TBA) as described by Hodges *et al*. (1999).

Assay of antioxidant enzymes

The activities of antioxidant enzymes were assayed in leaves using spectrophotometric methods. Superoxide dismutase (SOD) activity was determined following the method of Giannopolitis and Ries (1977) based on the photochemical reduction of NBT. One unit of SOD activity was defined as the amount of enzyme required to inhibit 50% of the reduction rate of NBT as monitored at 560 nm. The catalase (CAT) activity was assayed as described by Cakmak and Marschner (1992). Ascorbate peroxidase (APX) activity was measured according to the method described by Nakano and Asada (1981).

Determination of detoxification-related enzymes and glutathione content in the leaves

Glutathione *S*-transferase (GST) activity was assayed spectrophotometrically using a GST activity assay kit (Jiancheng Bio Co., Nanjing, China) following the manufacturer's instructions (Xia *et al*., 2009). Peroxidase (POD) activity was assayed using guaiacol as substrate, as described by Cakmak and Marschner (1992). The method of Foyer and Halliwell (1976) was followed to determine glutathione reductase (GR) activity by monitoring the glutathione-dependent oxidation of NADPH at 340 nm.

The glutathione content was determined according to Sgherri and Navari-Izzo (1995) by an enzymatic recycling method. Total glutathione was sequentially oxidized by 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and reduced by NADPH in the presence of GR. Oxidized glutathione (GSSG) was assayed by derivatizing reduced glutathione (GSH) with 2-vinylpyridine. The GSH content was then calculated by subtracting GSSG from total glutathione.

Determination of secondary metabolism-related enzyme activity in leaves

The activity of phenylalanine ammonia-lyase (PAL) was assayed by a combination of the methods of Ruiz *et al*. (1999) and Nguyen *et al*. (2003) based on the yield of cinnamic acid. One unit of PAL activity was defined as the change in absorbance at A_{290} ml^{-1} enzyme extract. Polyphenol peroxidase (PPO) activity was assayed by measuring the increase in absorbance at 370 nm with caffeic acid as a substrate (Ruiz *et al*., 1999). The activity of shikimate dehydrogenase (SKDH) was

determined as described by Diaz *et al*. (2001). Cinnamyl alcohol dehydrogenase (CAD) was assayed spectrophotometrically according to Mitchell *et al*. (1994) based on the oxidation of the appropriate hydroxycinnamyl alcohol at 30 °C. Protein contents were quantified according to Bradford (1976). All spectrophotometric analyses were conducted on a 96-well Enspire™ 2300 Multilabel Reader (PerklinElmer, Singapore).

Total RNA extraction, cDNA synthesis, and quantitative real-time PCR (qRT-PCR) analyses

Total RNA was extracted from ~100 mg of leaf tissue using the Total RNA Miniprep Kit (Axygen Biosciences, CA, USA) according to the manufacturer's protocol. Genomic DNA was removed with the RNeasy Mini Kit (Qiagen, Hilden, Germany). A 1 µg aliquot of total RNA was reverse-transcribed for the synthesis of cDNA using the ReverTra Ace qPCR RT Kit (Toyobo, Japan), following the manufacturer's instructions. Gene-specific primers for qRT-PCR were designed based on the mRNA or expressed sequence tag (EST) for the corresponding genes as follows: *Fe-SOD* (F, 5'-GTATCACAGGGCGTATGTCG-3'; R, 5'-GGGCTTCATAGATTCCCAGA-3'), *CAT1* (F, 5'-TGATCGC GAGAAGATACCTG-3'; R, 5'-CTTCCACGTTCATGGACAAC-3'), *APX* (F, 5'-TCTGAATTGGGATTTGCTGA-3'; R, 5'-CGTCTAAC GTAGCTGCCAAA-3'), *GST1* (F, 5'-CTCTGGTTTGGAGCAATT CA-3'; R, 5'-AATTTCAGCTGGATGCCTTT-3'), *POD* (F, 5'- TGA TCGCGAGAAGATACCTG-3'; R, 5'-ATCACCATTGGCTTCTGA CA-3'), *GR* (F, 5'-TTGGTGGAACGTGTGTTCTT-3'; R, 5'-TCTC ATTCACTTCCCATCCA-3'), *GSH1* (F, 5'-TTGCTTATGCATGTTG CTCA-3'; R, 5'-ACAACCTCGGCTACTTCGTT-3'), *PAL* (F, 5'-AG GAGATCGACAAGGTGT-3'; R, 5'-TAGCAGATTGGAAGAGGA-3'), *PPO* (F, 5'-CACCACCTCCTGATCTCTCA-3'; R, 5'-GGGACGAA TACGGAGCTTAG-3'), *SKDH* (F, 5'-ATATCTGGGTCACCTTTG GC-3'; R, 5'-AGATAAGGCCTCAGCTCCAA-3'), *CAD* (F, 5'-GC CGCTGACTCACTTGATTA-3'; R, 5'-TTCCATCAAGCTTCAACA GC-3'), and the *actin* gene (F, 5'-TGGTCGGAATGGGACAGAAG-3'; R, 5'-CTCAGTCAGGAGAACAGGGT-3') as an internal control. For qRT-PCR, PCR products were amplified in triplicate using the SYBR Green PCR Master Mix (Applied Biosystems) in 25 µl qRT-PCRs in an iCycler iQ™ 96-well real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The PCR conditions consisted of denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. The software provided with the PCR system was used to calculate threshold cycle values, and quantification of mRNA levels was performed according to the method of Livak and Schmittgen (2001). The threshold cycle (Ct) value of actin was subtracted from that of the gene of interest to obtain a ΔCt value. The Ct value of the untreated control sample was subtracted from the ΔCt value to obtain a ΔΔCt value. The fold changes in expression level relative to the control were expressed as $2^{-\Delta\Delta Ct}$.

Statistical analyses

Data were statistically analysed and expressed as mean ±SD. Analysis of variance (ANOVA) was performed and treatment differences were compared using Tukey's test ($P < 0.05$).

Results

Effects of EBR on biomass accumulation and PHE and Cd content

Exposure to PHE and/or Cd was observed to decrease the biomass accumulation in terms of dry weight significantly when compared with the untreated control (Fig. 1A). The inhibitory effects of Cd on biomass accumulation were much stronger than those of PHE (Fig. 1A). EBR application to PHE-, Cd-, and PHE+Cd-stressed plants was able to improve the dry weight by

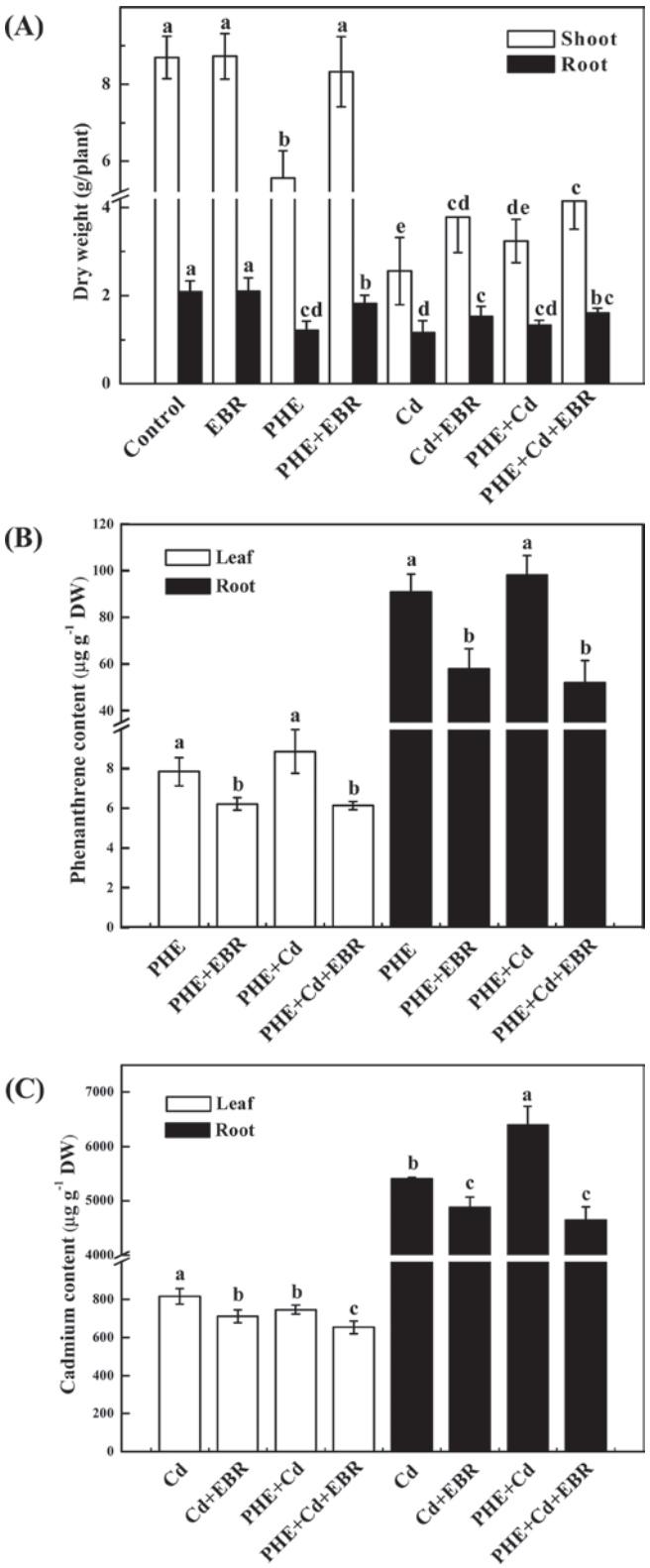

Fig. 1. Interactive effects of PHE, Cd, and EBR on (A) plant biomass, (B) PHE residue, and (C) Cd content in tomato plants after 40 d of the respective treatments. The culture solution was co-contaminated with 100 µM PHE and 100 µM Cd. EBR (0.1 µM) was applied on the foliar portion. Bars of the same colour with different letters are significantly (P <0.05) different according to Tukey's test. Data are the means, and the error bar indicates ±SD (n=8 for dry weight analysis and n=3 for pollutant residue analysis).

49, 48, and 28%, respectively, in shoot, and 50, 32, and 21%, respectively, in root when compared with plants stressed only by PHE-, Cd-, and PHE+Cd (Fig. 1A). These EBR-induced increments were all statistically significant, with the exception of only PHE+Cd+EBR in root.

The leaves and roots showed no significant difference in terms of PHE accumulation when compared with plants exposed to PHE alone or the PHE+Cd combination (Fig. 1B). Application of EBR to plants stressed by only PHE was able to reduce PHE accumulation significantly in leaves by 21% over PHE alone treatment. Interestingly, EBR applied to PHE+Cd-stressed plants showed a 31% reduction in PHE accumulation in leaves in comparison with PHE+Cd-only treatments. It is worth mentioning that EBR when applied to PHE+Cd-treated plants could reduce PHE accumulation in leaves by a factor of 10% when compared with EBR applied to plants treated with PHE alone. EBR application was found to be more effective in decreasing the PHE accumulation in root by 32% and 50% compared with PHE- and PHE+Cd-stressed plants, respectively (Fig. 1B).

Analysis of Cd content revealed that addition of PHE with Cd in nutrient solution decreased Cd accumulation in leaves by 8.5%, but increased Cd content in roots by 18.3% when compared with Cd accumulation in Cd alone-treated plants (Fig. 1C). In contrast, EBR application to Cd-stressed plants remarkably decreased the Cd content in both the leaves and roots compared with Cd alone. EBR-induced reduction in Cd content was 10% and 27% in roots following Cd+EBR and PHE+Cd+EBR treatments, respectively, when compared with the respective pollutant-only treatments (Fig. 1C). These results provide evidence that EBR and PHE co-application to Cd only-stressed plants could significantly lower the Cd content when compared with PHE only treatments of Cd-stressed plants.

Effects of PHE–Cd co-contamination on leaf gas exchange of tomato plant and role of EBR

Leaf gas exchange parameters such as P_n was significantly decreased by PHE and/or Cd stress (Fig. 2A). Compared with control, P_n was decreased by 14, 62, and 56% following PHE, Cd, and PHE+Cd treatments, respectively. Application of EBR to PHE-, Cd-, and PHE+Cd-stressed plants was able to improve the P_n significantly when compared with their respective treatments alone. It was interesting to find that co-application of EBR and PHE to Cd-stressed plants showed more significant improvement in the P_n value (50%) when compared with PHE applied alone to Cd-stressed plants (Fig. 2A).

A significant reduction in g_s was noted for Cd- and PHE+Cd-stressed plants when compared with untreated controls. Application of EBR alone to PHE-stressed plants produced no significant increase in g_s value when compared with PHE alone-stressed plants. Feeding of EBR to Cd-stressed plants showed a significant improvement in the g_s value, and co-application of EBR with PHE to Cd-stressed plants was able to improve g_s values more significantly compared with either Cd-stressed or PHE+Cd-stressed plants (Fig. 2A).

In contrast, the C_i value was increased by 16, 17, and 20% for PHE-, Cd-, and PHE+Cd-stressed plants, respectively, when compared with untreated control. Application of EBR to PHE-stressed plants produced a significant decrease in C_i, however, no significant change in C_i was recorded for Cd-stressed plants. The co-application of EBR and PHE was able to decrease C_i significantly when compared with plants stressed with PHE+Cd alone (Fig. 2A).

EBR influences PHE–Cd co-contamination effects on chlorophyll fluorescence and pigments in tomato leaves

Changes in chlorophyll fluorescence parameters in response to PHE and/or Cd are shown in Figs 2B and 3A. A significant decrease in F_v/F_m and Φ_{PSII} in PHE-, Cd-, and PHE+Cd-stressed plants was observed when compared with untreated control. The maximum decrease in F_v/F_m, Φ_{PSII}, and qP was recorded under Cd alone, followed by PHE+Cd and PHE. The respective pseudocoloured images for F_v/F_m (Fig. 3A) also confirm the significant impact of the pollutants under study on F_v/F_m values. Application of EBR to Cd and PHE+Cd-stressed seedlings was able to increase F_v/F_m and Φ_{PSII} significantly over Cd and PHE+Cd alone treatments. EBR application to PHE+Cd-stressed plants produced a significant increase in F_v/F_m compared with PHE+Cd- and Cd+EBR-stressed plants. However, Φ_{PSII} values showed no significant difference for PHE+Cd+EBR versus plants stressed with Cd+EBR alone.

Application of PHE and Cd alone or in combination significantly reduced the photosynthetic pigments Chl *a*, Chl *b*, and Carts (Fig. 3B). When compared with untreated control, the maximum decrease in Chl *a* was recorded under Cd alone (93%), followed by PHE+Cd (81%) and PHE alone (13%). Chl *b* and Carts showed a similar trend following exposure to Cd and PHE pollutants. Observations made with the naked eye (leaf symptoms) clearly indicated severe chlorosis under Cd and PHE+Cd treatment, which was consistent with the respective pigment data (Fig. 3A, B). In contrast, EBR application together with Cd and PHE+Cd was able to increase the photosynthetic pigments significantly compared with Cd- and PHE+Cd only treatments. Similar to the chlorophyll fluorescence results, the most significant improvement contributed by EBR was observed under Cd+EBR. In this treatment Chl *a*, Chl *b*, and Cart contents were increased by 197, 237, and 79%, respectively, compared with Cd alone.

Effects of PHE–Cd co-contamination on $O_2^{\cdot-}$ and H_2O_2 accumulation and lipid peroxidation in tomato leaves, and role of EBR

ROS accumulation and MDA contents were remarkably increased in leaves following exposure to PHE and/or Cd when compared with untreated control (Fig. 4). About 87% H_2O_2 accumulation was recorded under Cd treatment, followed by 83% under PHE+Cd and 40% under PHE alone over untreated control (Fig. 4B). EBR applications to PHE-, Cd-, and PHE+Cd-stressed plants showed a significant reduction in H_2O_2 when compared with their respective treatments without EBR. The H_2O_2 content was decreased by 21, 25, and 24% following PHE+EBR, Cd+EBR, and PHE+Cd+EBR (Fig. 4B), respectively. It was interesting to observe that co-application of EBR and PHE was

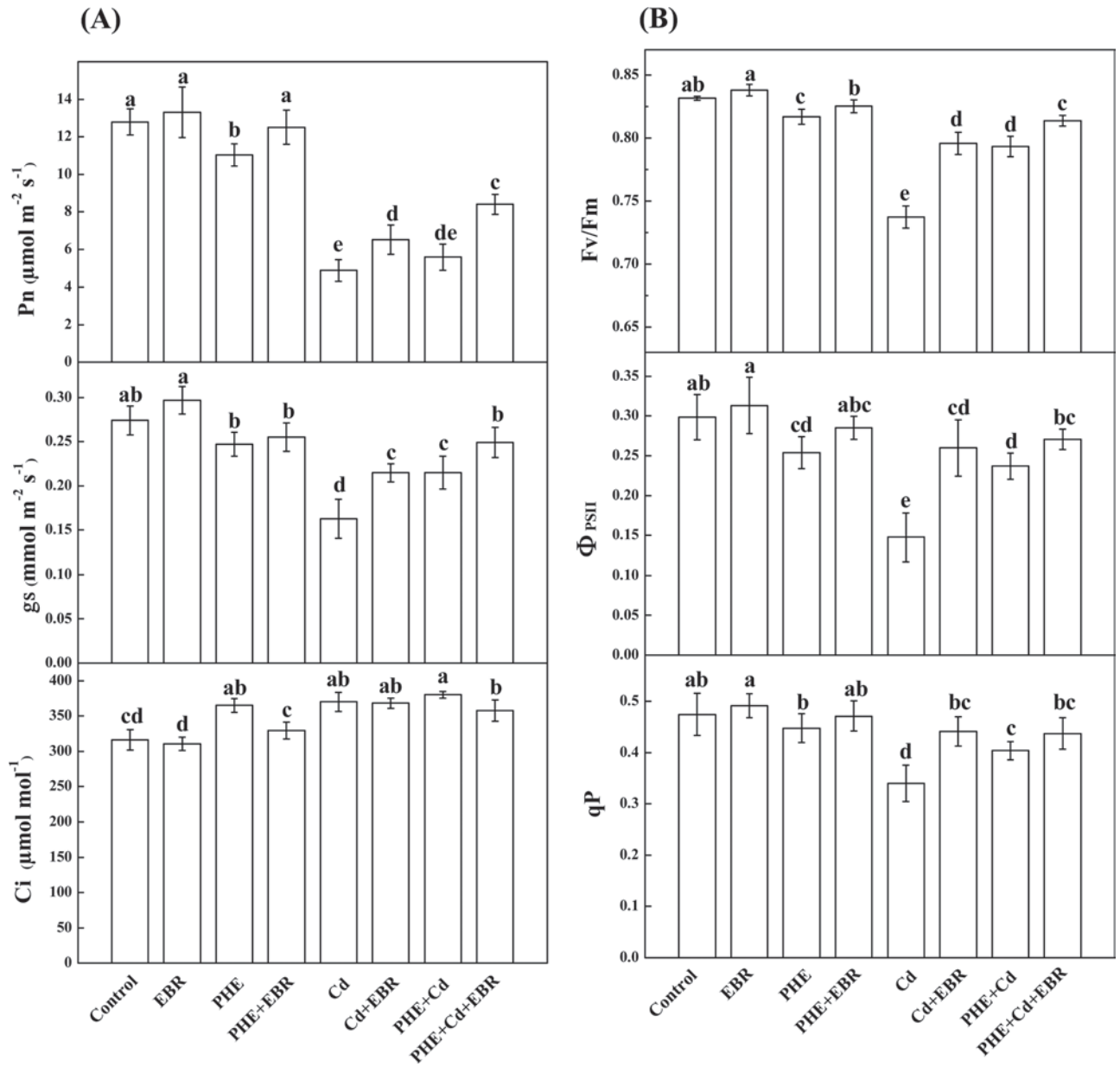

Fig. 2. Interactive effects of PHE, Cd, and EBR on (A) leaf gas exchange and (B) chlorophyll fluorescence quenching parameters in tomato. All measurements were taken on the third fully expanded leaves after 40 d of the respective treatments. The culture solution was co-contaminated with 100 μM PHE and 100 μM Cd. EBR (0.1 μM) was applied on the foliar portion. Data are the means of six biological replicates (±SD). Means denoted by the same letter did not differ significantly at $P < 0.05$ according to Tukey's test.

more effective in reducing the H_2O_2 content when compared with plants stressed with PHE+Cd alone (Fig. 4B). Histochemical observations for H_2O_2 detection in leaves with DAB staining are also fairly in agreement with the biochemical data; however, slightly increased H_2O_2 was visualized under Cd-only treatment compared with PHE+Cd (Fig. 4A lower panel). A similar trend for the $O_2^{\cdot -}$ accumulation in leaves was also observed (Fig. 4A upper panel). Likewise, MDA contents were increased by 24, 22, and 8% compared with untreated control following PHE+Cd, Cd, and PHE, respectively. However, EBR application on stressed plants significantly decreased MDA contents by 9, 9, and 5% compared with their respective treatments without EBR (Fig. 4C). Corresponding NBT- and DAB-stained leaves also showed less ROS accumulation following EBR application in Cd-, PHE-, and PHE+Cd-stressed plants (Fig. 4A).

EBR induces changes in the effects of PHE–Cd co-contamination on antioxidant enzymes and gene expression in tomato leaves

Tomato plants stressed with Cd and PHE showed significant changes in antioxidant enzyme activities. The activity of SOD and APX was significantly increased by PHE and/or Cd compared with untreated control. Compared with control, SOD was increased by

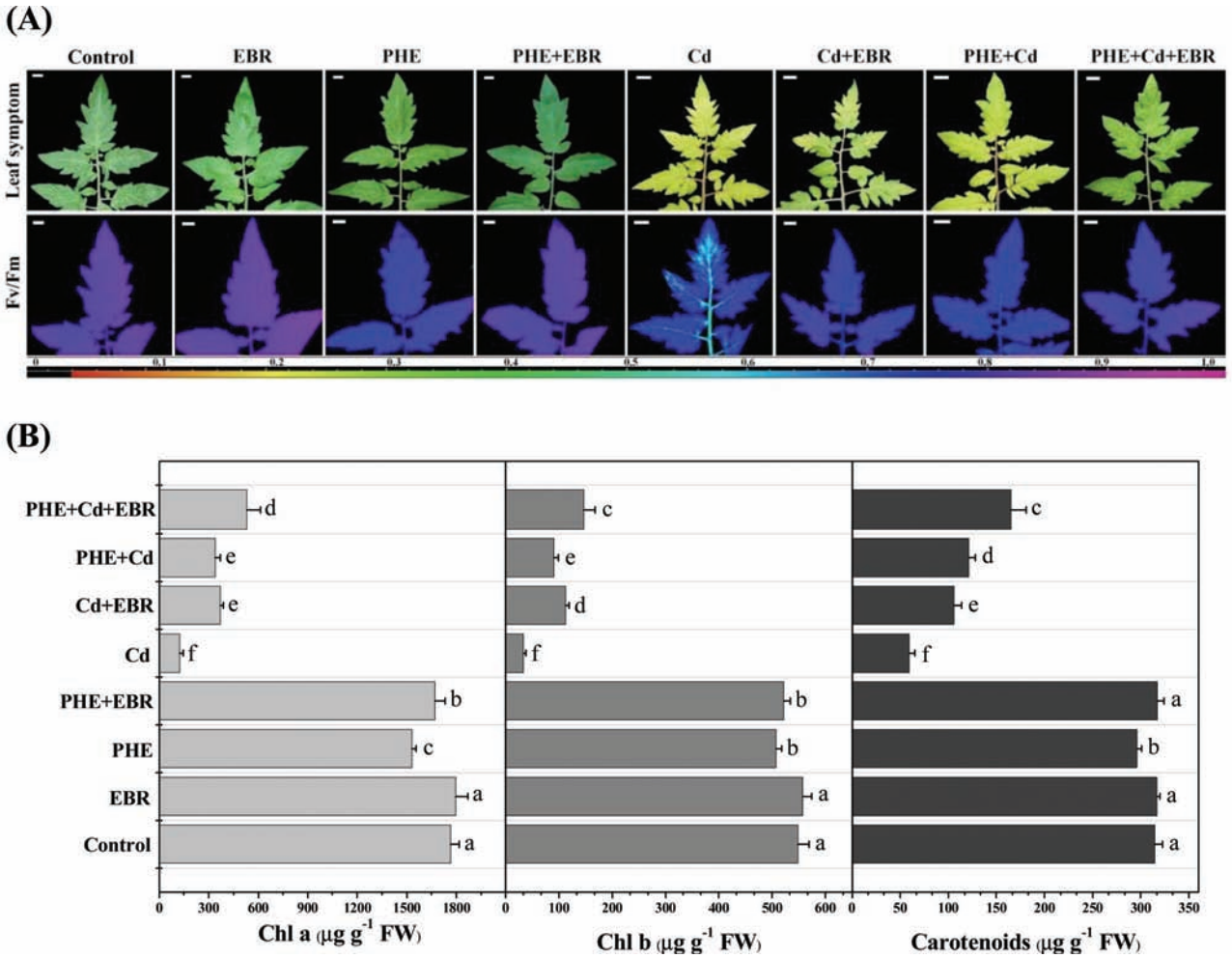

Fig. 3. Effects of PHE, Cd, and EBR alone or in combination on the maximum photochemical efficiency of PSII (F_v/F_m) and chlorophyll contents in tomato leaves after 40 d of the respective treatments. (A) Original image of the third leaves (upper panels) and pseudocolour image of F_v/F_m (lower panels); (B) photosynthetic pigment contents under different treatments. Data are the average of three replicates and are presented as the mean ±SD. Means denoted by the same letters did not differ significantly at $P < 0.05$ according to Tukey's test. Horizontal bars=1 cm.

10, 21, and 55% following PHE, Cd, and PHE+Cd, respectively; while the respective increases for APX were 59, 61, and 174% when compared with untreated control (Fig. 5A). CAT was induced by PHE and PHE+Cd (49% and 59%, respectively), but was decreased by Cd alone (by 15%) compared with untreated control (Fig. 5A). Application of EBR alone or in combination with Cd, PHE, and PHE+Cd produced increased SOD, CAT, and APX compared with untreated control or their respective Cd, PHE, and PHE+Cd alone treatments. The EBR supplemented increases were highest under Cd+EBR treatment compared with Cd alone, which were 23, 25, and 43% higher for SOD, CAT, and APX, respectively (Fig. 5A).

qRT-PCR analysis also supported the changes in the activities of SOD, CAT, and APX induced under pollutant stress and EBR applications (Fig. 5B). The *Fe-SOD*, *CAT1*, and *APX* transcript levels were shown to be induced by PHE and/or Cd, while EBR application was able to enhance expression further over the respective pollutant alone treatments (Fig. 5B). *Fe-SOD* and *APX* transcript levels were maximum under EBR alone (2-fold) followed by PHE+EBR and PHE, while the highest *CAT1* transcript level was observed under PHE+Cd+EBR (6-fold) followed by Cd+EBR and PHE+Cd (Fig. 5B).

Effects of PHE–Cd co-contamination and EBR on detoxification-related enzyme activity and gene expression

The activities of detoxification-related enzymes such as GST, POD, and GR were increased by PHE and/or Cd compared with untreated control (Fig. 6A). Similarly, PHE and/or Cd treatments induced the expression of *GST1*, *POD*, and *GR1* genes compared with untreated control (Fig. 6B). EBR application alone or with pollutants further increased GST, GR, and POD activity as well as the related gene expression compared with untreated control and pollutant-only treatments, respectively. The highest induction by EBR was observed in Cd+EBR for GR, which was 55% over Cd alone. For POD and GST, the maximum increments by EBR was recorded in PHE+EBR, which were 42% and 27%, respectively, compared with PHE alone.

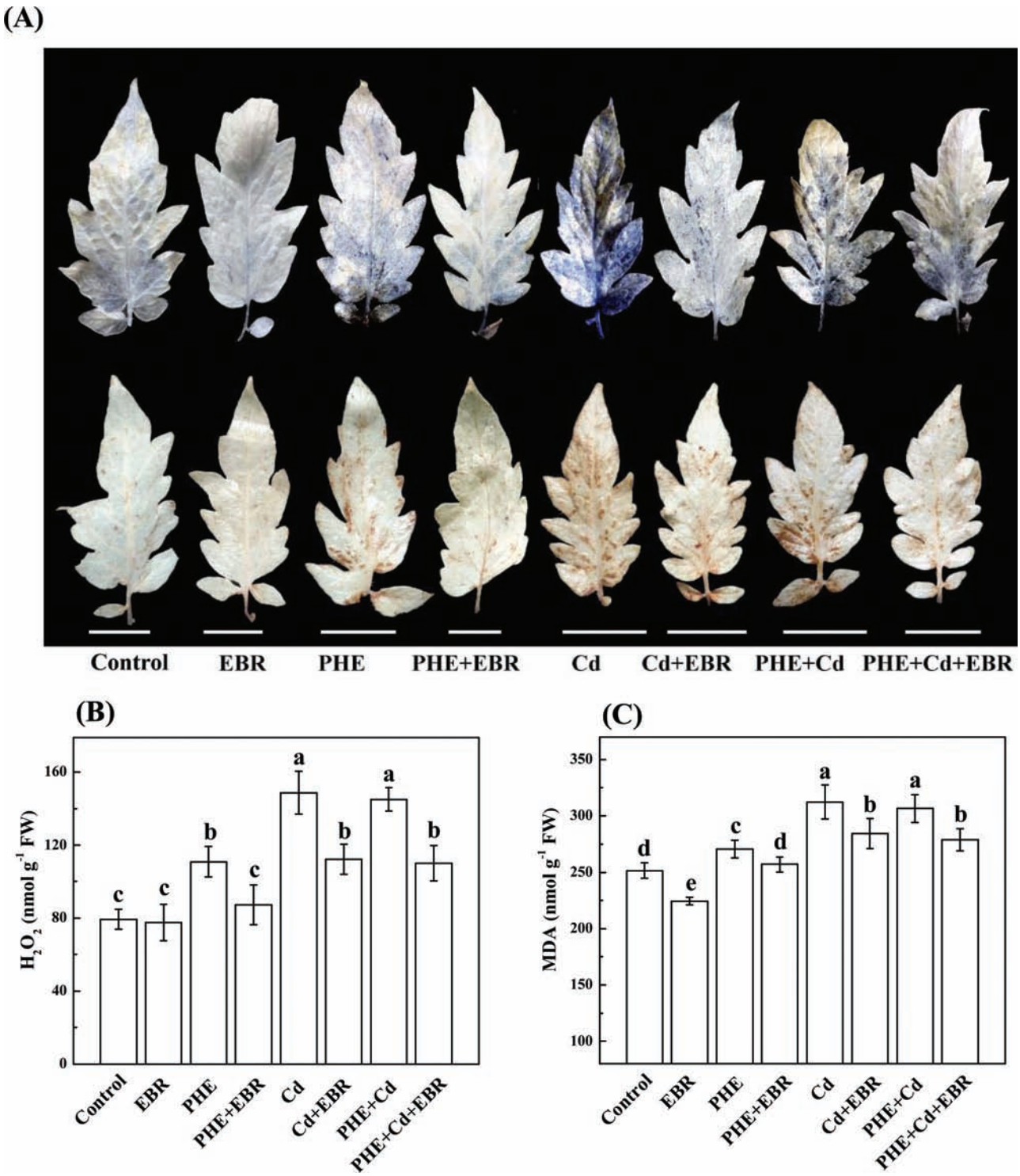

Fig. 4. Interactive effects of PHE, Cd, and EBR on (A) $O_2^{\cdot -}$ (upper panel) and H_2O_2 accumulation (lower panel), (B) H_2O_2 contents, and (C) MDA contents in tomato leaves after 40 d of the respective treatments. Accumulation of $O_2^{\cdot -}$ and H_2O_2 in leaves was detected by NBT and DAB staining, respectively. Each value in the graph shows the mean with the standard deviation of three replicates. Means denoted by the same letters did not differ significantly at $P < 0.05$ according to Tukey's test. Horizontal bars=2 cm.

The GSH content was increased by 39, 220, and 237% following PHE, Cd, and PHE+Cd treatments (Fig. 6A), whereas the *GSH1* transcript level was induced by 24, 36, and 24%, respectively, over that of untreated control (Fig. 6B). EBR application to PHE- and Cd-stressed plants further increased GSH content by 39, 17, and 22% following PHE+EBR, Cd+EBR, and PHE+Cd+EBR treatments, respectively, whereas the *GSH1* transcript level was induced by 44, 52, and 21%, respectively, compared with their respective pollutant alone treatments.

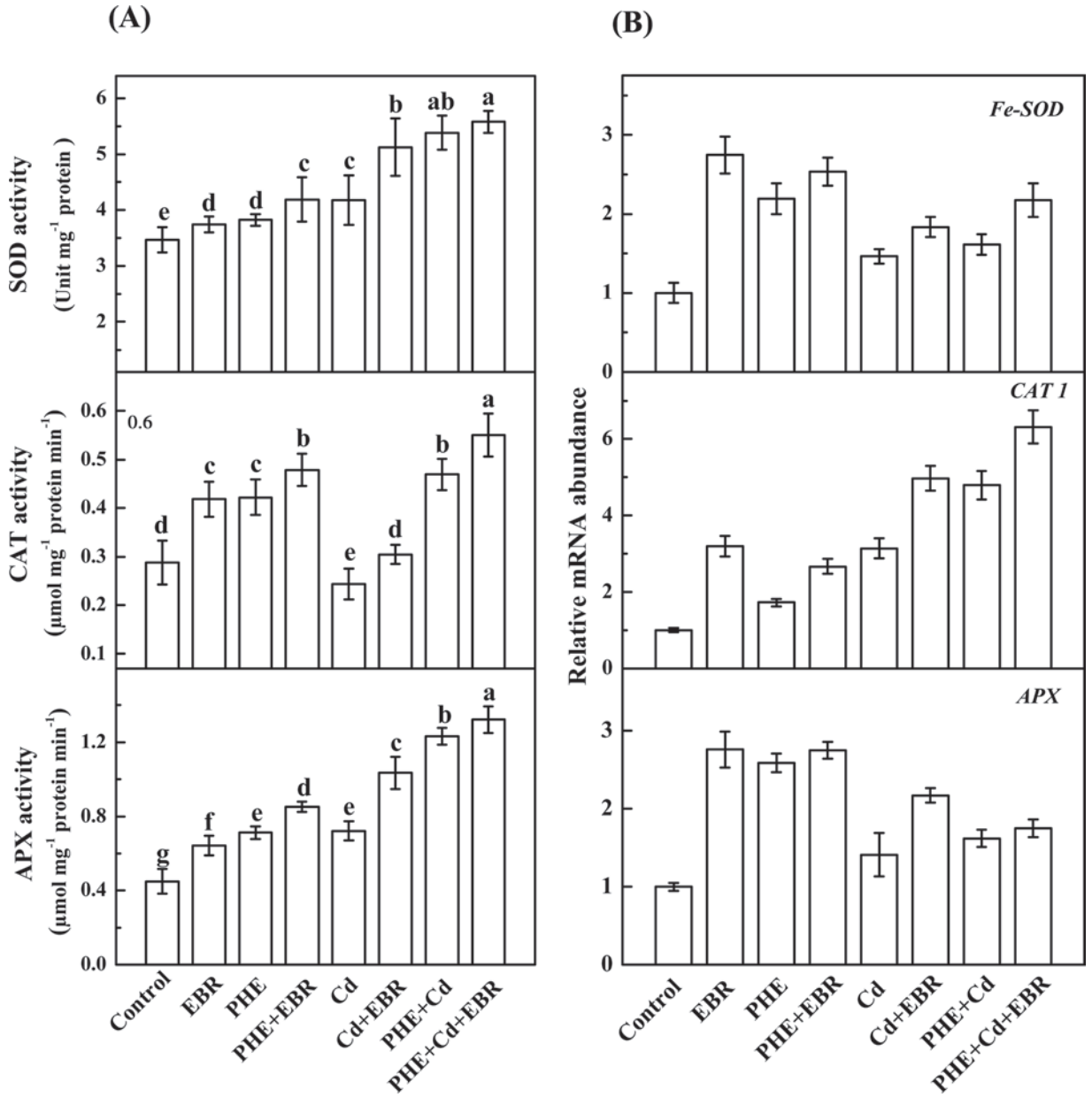

Fig. 5. Interactive effects of PHE, Cd, and EBR on (A) antioxidant enzyme activities and (B) related gene expression. Each value in the graph shows the mean with the standard deviation of three replicates. Means denoted by the same letters did not differ significantly at $P < 0.05$, according to Tukey's test.

Interactive effects of PHE, Cd, and EBR on plant secondary metabolism-related enzyme activity and gene expression in tomato leaves

As shown in Fig. 7A, PAL and PPO activities were induced by PHE and/or Cd. Compared with pollutant-only treatments, the PAL activity increased by 8.1, 13.8, and 15.6% following PHE+EBR, Cd+EBR, and PHE+Cd+EBR, respectively, whereas PPO activity was induced consecutively by 12.8, 16.6, and 12.6%. These results show that EBR when applied to PHE+Cd-stressed plants could improve PAL activity more significantly than in PHE+Cd- and Cd+EBR-stressed plants. A synergistic induction of SKDH and CAD activities was also observed by co-application of PHE and Cd. When compared with pollutant-only treatments, EBR-induced increases in SKDH and CAD activity were 19.4% and 32.3%, respectively, following PHE+Cd+EBR (Fig. 7A). EBR applied with PHE to Cd-stressed plants produced a significant increase in the transcript level of *CAD* by 44% (compared with PHE+Cd) and by 17.5% (compared with Cd+EBR), thereby showing the positive interplay of EBR and PHE in the induction of *CAD* expression under Cd and PHE stress (Fig. 7B). The *SKDH* transcript level was also found to be enhanced with EBR applied to PHE+Cd-stressed plants by 71% with regard to PHE+Cd and 8.3% with regard to Cd+EBR. In line with the enzyme activity, the transcript levels of *PAL* and *PPO* were induced by PHE and/or Cd, and application of EBR further increased their expression levels. EBR-induced up-regulation of *SKDH* and *CAD* transcripts was remarkable when EBR was applied with Cd and PHE+Cd (Fig. 7B).

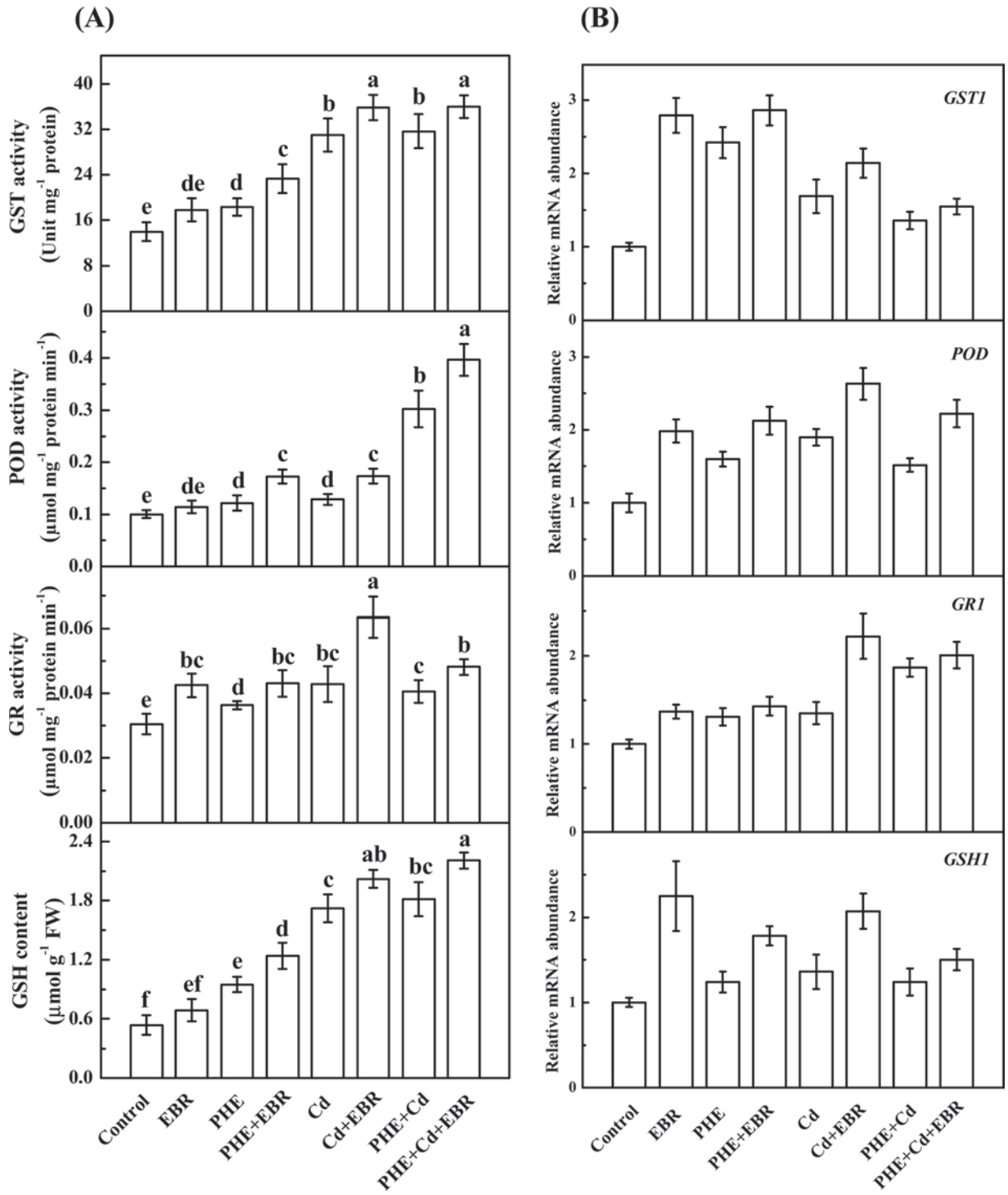

Fig. 6. Effects of EBR on PHE and Cd detoxification-related enzyme activities, glutathione content, and related gene expression. Each value in the graph shows the mean with the standard deviation of three replicates. Means denoted by the same letters did not differ significantly at $P < 0.05$ according to Tukey's test.

Discussion

Plants possess an inherent capacity to degrade or sequester a large number of toxic compounds (Coleman *et al*., 1997). To date it is not clear to what extent PAH exposure can trigger the stress signalling pathway that is common to other abiotic or biotic stressors in plants. There is no concrete evidence for the existence of stress signalling components specific to PAH stress in plants (Alkio *et al*., 2005). Importantly, plant tolerance to heavy metals such as Cd is conferred by a specific physiological mechanism that jointly enables the plant to function normally even in the presence of high levels of potentially toxic elements (Pilon-Smits, 2005). In recent years, an extensive body of work has been published on BR-induced plant tolerance to

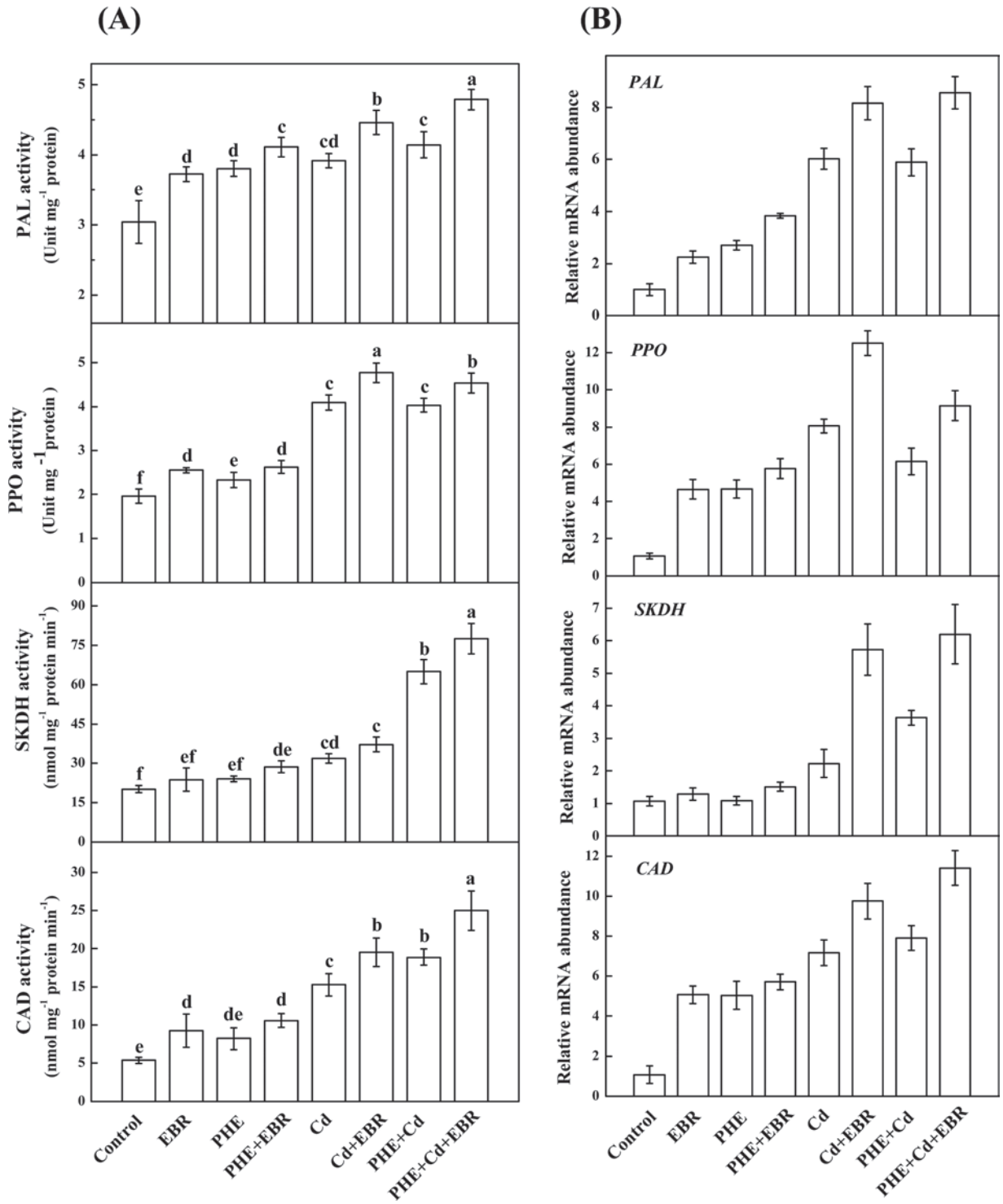

Fig. 7. Interactive effects of PHE, Cd, and EBR on secondary metabolism-related (A) enzyme activities and (B) gene expression. Each value in the graph shows the mean with the standard deviation of three replicates. Means denoted by the same letters did not differ significantly at $P < 0.05$ according to Tukey's test.

various abiotic stresses including temperature, drought, salinity, organic pollutants, and heavy metals (Xia *et al*., 2009; Cui *et al*., 2011; Hasan *et al*., 2011; Ahammed *et al*., 2012*a*, *b*; Choudhary *et al*., 2012*a*, *b*). So far the implication of BRs in the mitigation of mixed environmental pollution has not been studied. Here, an enhanced tolerance to and detoxification of PHE and Cd in combination by foliar application of EBR in tomato has been demonstrated.

In the current study, Cd showed a stronger effect on biomass compared with PHE in terms of dry weight reduction (Fig. 1). The findings can be supported by those of Zhang *et al*. (2011), who also observed similar phenomena in *Juncus subsecundus*.

The antagonistic interactions of PAHs and heavy metals can be explained by the report that a pyrene concentration of 50, 100, and 500 mg kg^{-1} in the soil tends to alleviate Cu- (200 mg kg^{-1} and 400 mg kg^{-1}) induced growth inhibition in *Z. mays* (Lin *et al*., 2008). Zhang *et al*. (2011) also inferred that PAHs might be used as a carbon source to stimulate microbial blooming or microbial composition modification in the rhizosphere, which might be the reason behind such stress ameliorative behaviour of PAHs against Cd recorded in the present investigation. Thus they concluded that a positive effect of PAH metabolites and/or phytohormones produced by microorganisms on plant growth might be the underlying reason for PAH-mediated Cd toxicity alleviation. Previous studies showed that BRs could alleviate the phytotoxicity of PHE or Cd alone in tomato (Hasan *et al*., 2011; Ahammed *et al*., 2012*a*, *b*). Similar results were recorded in the current study (Fig. 1). Moreover, it was noticed that application of EBR and PHE to Cd-stressed plants could alleviate Cd-induced biomass reduction more significantly than Cd+EBR- or PHE+Cd-only treatments. The positive impact of BRs on biomass production in the current study can be supported by the observations of Xie *et al*. (2011) who showed that BRs could induce cellulose biosynthesis by controlling the expression of *CELLULOSE SYNTHASE* genes which significantly contribute to the total biomass accumulation in *Arabidopsis*.

The leaf gas exchange study showed that both stomatal and non-stomatal factors were involved in the photosynthetic inhibition of PHE-stressed plants (Ahammed *et al*., 2012*a*, *b*). PHE as well as Cd has been reported to inhibit P_n and g_s in tomato, which supports the current observation (López-Millán *et al*., 2009; Hasan *et al*., 2011; Ahammed *et al*., 2012*b*). Thus, biomass reduction by PHE or Cd stress may be the consequence of reduction in chlorophyll content and consequent inhibition of photosynthesis (Ci *et al*., 2010; Ahammed *et al*., 2012*b*). It was also observed that PHE+Cd co-contamination could inhibit P_n, but the value of P_n was slightly higher than with Cd alone and lower than with PHE alone. Decreased P_n could be a consequence of the inhibition of selected reaction steps of the Benson–Calvin cycle and RuBisCO activity by these contaminants. Moreover, the reduction in the CO_2 assimilation rate might be attributed to altered stomatal opening and subsequent decline in g_s (Fig. 2A). It has previously been reported that application of exogenous EBR increases photosynthetic CO_2 assimilation in cucumber plants, which may provide an important mechanism for increased growth and yield in EBR-treated plants (Yu *et al*., 2004). In agreement with earlier studies, EBR follow-up treatment significantly increased CO_2 assimilation over pollutants alone in the current study (Xia *et al*., 2009; Ahammed *et al*., 2012*a*, *b*). EBR induces transcripts for photosynthetic genes and the activity of enzymes involved in the Benson–Calvin cycle, and thus improves photosynthetic capacity (Jiang *et al*., 2012). One interesting observation might be that Cd treatment reduced P_n and g_s of PHE-treated plants; however, when EBR is applied, this Cd-mediated reduction of PHE-stressed plants is slightly less for P_n or was not observable for g_s. It may suggest another piece of evidence that EBR application may protect stressed plants through physiological interaction with Cd and/or PHE.

PHE or Cd have been reported to decrease F_v/F_m, Φ_{PSII}, and qP in different plant species including tomato (López-Millán *et al*., 2009; Ci *et al*., 2010; Liu *et al*., 2011; Ahammed *et al*., 2012*a*, *b*). A decrease in F_v/F_m indicates the negative effects of these pollutants on the photochemical reactions which may affect the efficiency of PSII photochemistry by blocking electron transport. Interestingly, a significant effect of PHE on increasing F_v/F_m of Cd-treated plants was observed. This PHE-mediated increase was found to be slightly higher when EBR was co-applied with PHE. It might suggest a positive interaction of EBR and PHE to protect photosynthesis of plants against Cd stress. Leaf damage symptoms of these treatments are in line with this assumption (Fig. 3A). In addition, EBR seemed to protect against oxidative stress induced by Cd, but PHE did not exert such an effect. Moreover, the potential interaction of EBR and PHE could not be via a reduction of oxidative stress (measured as H_2O_2 and MDA contents). The value of Φ_{PSII} corresponds to the electron transport in the thylakoid membrane and thus a decrease in Φ_{PSII} is related to the electron transport chain in thylakoid membrane (Váňová *et al*., 2009). These phenomena revealed that inhibition of photosynthesis is the result of damage to the PSII reaction centre of the leaf. However, the current study showed that EBR application alone or with PHE and/or Cd could increase F_v/F_m (Fig. 2B). The promoting effects of BRs on F_v/F_m have been reported under different stresses such as PHE, pesticide, and chilling, in agreement with the present results (Xia *et al*., 2009; Cui *et al*., 2011; Ahammed *et al*., 2012*a*, *b*).

Leaf chlorosis is one of the common signs of Cd toxicity (Liu *et al*., 2011). Cd inhibits chlorophyll formation by interfering with protochlorophyllide production (Ci *et al*., 2010; Hasan *et al*., 2011). Heavy metals can also replace the central Mg from chlorophyll molecules in *in vivo* conditions. Moreover, enzymatic degradation of chlorophyll by chlorophyllase is also triggered by Cd (Hasan *et al*., 2011). PHE-induced leaf pigment loss may be a result of degradation and/or reduction in formation of photosynthetic pigments (Ahammed *et al*., 2012*a*, *b*). A decrease in chlorophyll content undoubtedly affects the photosynthetic efficiency. Importantly, EBR application on stressed plants significantly increased the chlorophyll content, possibly by stimulating transcription of genes implicated in the biosynthesis of pigments (Bajguz, 2011).

Overproduction of ROS and subsequent oxidative stress is the common mechanism of phytotoxicity for both PHE and Cd (Liu *et al*., 2009; Masood *et al*., 2012). Enhanced levels of ROS in cells resulted in pigment loss, reduction in photosynthetic CO_2 assimilation, and decreased protein and RNA levels (Mittler, 2002; Masood *et al*., 2012). Moreover, the critical enzyme of the Benson–Calvin cycle, RuBisCO, is directly fragmented by ROS (Ishida *et al*., 1999). As evidence of ROS generation, significantly increased $O_2^{\cdot -}$, H_2O_2, and MDA levels were observed under Cd and PHE+Cd treatments, followed by PHE alone (Fig. 4). MDA is an indicator of lipid peroxidation, signifying the damage due to harmful ROS. The current observations confirmed that the tomato plants were under oxidative stress following PHE and/or Cd treatments. However, $O_2^{\cdot -}$, H_2O_2, and MDA contents were not synergistically stimulated by PHE and Cd. EBR application was able to decrease the toxic $O_2^{\cdot -}$, H_2O_2, and MDA contents in the stressed plants, which is consistent with previous reports on BR-induced alleviation of stress due to heavy metals and PAHs (Hasan *et al*., 2011; Ahammed *et al*., 2012*a*, *b*). Oxidative

stress alleviation is generally attributed to enhanced antioxidant activity and subsequent ROS scavenging by BRs under stressful conditions (Cui *et al*., 2011). Indeed, the plant antioxidant system is activated to avoid oxidative damage by PHE- and/or Cd-induced ROS (Liu *et al*., 2009; Masood *et al*., 2012). Here in the present study, SOD and APX were induced by PHE and/or Cd, while CAT activities were only inhibited by Cd alone. Meng *et al*. (2009) observed similar phenomena under 100 µM Cd stress in *Brassica napus* L. In general, decreased SOD activity suggests that $O_2{\cdot}^-$ generation has exceeded the elimination ability of SOD, or the ROS might have inactivated enzymes. In the present study, co-application of PHE and Cd produced a positive effect on antioxidant enzyme activities, as much higher activity was observed following PHE+Cd than Cd alone. The transcript levels of *Fe-SOD*, *CAT1*, and *APX* were induced by PHE and/or Cd (Fig. 5B). However, co-application of PHE and Cd did not show an additive up-regulation of the expression level of *Fe-SOD* and *APX*, which suggested a post-transcriptional impact on the induction of the respective enzyme activity for ROS scavenging. Enhanced antioxidant enzyme activity has a positive role in minimizing the ROS level in order to maintain the normal plant physiological activity under stressful regimes (Mittler, 2002). Similar to the present observation, a synergistic up-regulation in SOD activity was observed under PHE plus arsenic co-contamination in *Pteris vitatta* (Sun *et al*., 2011). The present investigation showed that EBR application could further increase the activity of antioxidant enzymes and the transcript level of the related genes under PHE and/or Cd stress, which is in line with earlier reports (Hasan *et al*., 2011; Ahammed *et al*., 2012*a*, *b*). Recently, it has been shown that EBR-induced oxidative stress tolerance is associated with increased expression of genes encoding antioxidant enzymes in cucumber (Cui *et al*., 2011). Thus the increased transcript level of these enzymes might be the result of *de novo* synthesis and/or activation of the enzymes in response to BR-mediated transcription and/or translation of specific genes (Bajguz, 2000).

Plant GSTs are the key enzymes that catalyse the conjugation reaction of xenobiotics with GSH in phase II (Coleman *et al*., 1997). In the current experiments, GST activity and the *GST1* transcript level were induced by PHE and/or Cd, which were further increased by follow-up treatment with EBR (Fig. 6). GST activity was reported to be increased in *Arabidopsis* exposed to 100 µM Cd (Skorzynska-Polit *et al*., 2010). Enhanced GST and *GST1* suggested enforcement of the detoxification reaction. Moreover, enhanced POD activity due to EBR application might play a crucial role in the xenobiotic conversion, a part of the detoxification process (Xia *et al*., 2009). Likewise, an up-regulation in GR activity may reduce GSSG to GSH and supply more GSH to strengthen the detoxification process. Previously, GSH has been reported to be induced by both PHE and Cd (Ahammed *et al*., 2012*a*, *b*; Masood *et al*., 2012), which is in agreement with the current observation. It is well known that GSH and phytochelatins play a vital role in Cd detoxification and subsequent stress tolerance (Masood *et al*., 2012). The GSH content has been shown to increase positively under PHE plus arsenic co-contamination compared with PHE or arsenic alone in *P. vitatta* (Sun *et al*., 2011). The present study also observed a similar phenomenon for GSH content in PHE+Cd-treated tomato plants. EBR was reported to improve xenobiotic metabolism by enhancing the activities of GST, POD, and GR and related gene expression (Xia *et al*., 2009; Ahammed *et al*., 2012*a*). Consistent with previous reports, here it was shown that EBR-induced alleviation of PHE+Cd stress might be associated with enhanced activity of detoxification enzymes and related gene expression.

An enhancement of the activity of PAL may stimulate subsequent reactions in the phenylpropanoid pathway to produce specific phenylpropanoid derivatives such as phenols and flavonoids (Dixon and Paiva, 1995). Phenolic compounds play a vital role in the alleviation of oxidative stress as they are involved in the detoxification of ROS (Wang *et al*., 2011). The current observation of up-regulation of CAD activity and the *CAD1* transcript level signifies an enhancement in the synthesis of cinnamyl alcohols and is considered as a specific marker for lignification (Mitchell *et al*., 1994). Similarly, Wang *et al*. (2011) reported enhanced catalytic activities of PAL, PPO, and SKDH under Pb treatment, which supports the current data (Fig. 7). Secondary metabolism-related enzymes such as PAL, PPO, SKDH, and CAD as well as their transcripts were induced by EBR, PHE, and/or Cd (Fig. 7). The highest increments were recorded when pollutants were in combination. Importantly, EBR follow-up treatment on stressed plants further increased those activities which might influence the respective secondary metabolite synthesis required for active antioxidative protection (Fig. 7).

Earlier studies have shown that the combination of certain concentrations of metals (e.g. Cd) and PAHs could stimulate both metal and PAH uptake compared with metal alone, while growth and biomass were also increased (Lin *et al*., 2008; Zhang *et al*., 2011). Although some possible interpretations have been suggested, the underlying mechanism for this improved growth with high concentration of pollutants was not clear. Slightly increased PHE and Cd content accompanied by increased biomass was also observed following PHE+Cd compared with Cd alone (Fig. 1). As the content of PHE and Cd was not decreased, the improved growth could be contributed by the enhanced antioxidant potential and subsequent stress alleviation in the co-contaminated system. López-Millán *et al*. (2009) reported very high accumulation of Cd in leaves (1075 µg g^{-1} DW) and roots (4731 µg g^{-1} DW) following application of 100 µM Cd in tomato, which supports the current findings (Fig. 1C). On the other hand, EBR-induced biomass improvement may also be associated with enhanced degradation and detoxification of pollutant as PHE and Cd contents were significantly decreased in leaves and roots by EBR supplementation (Fig. 1B, C) (Ahammed *et al*., 2012*a*). It is to be noted that either PHE or EBR could reduce Cd content; however, the combined treatment (PHE+EBR) provoked a similar Cd content reduction. This indicates that the EBR and PHE effects on Cd accumulation were not additive. It is speculated that the reduction of leaf Cd content by EBR might be through PHE. In contrast, EBR reduced root PHE content, while this pollutant enhanced the amount of Cd in the root. However, EBR greatly decreased Cd content in the roots, which suggests that such a reduction in Cd level could be independent of PHE in roots.

In summary, it was observed that PHE and/or Cd treatment resulted in a decreased growth and photosynthetic capacity. PHE- and/or Cd-induced inhibition of photosynthesis can be attributed

to: (i) stomatal and non-stomatal limitation; (ii) reduction in photosynthetic pigments; and (iii) destruction of the photosynthetic apparatus. Single or mixed pollution induces oxidative stress. However, mixed pollution by PHE and Cd was less phytotoxic than Cd alone. EBR application alleviated the negative effects of Cd and PHE on growth and photosynthesis by improving antioxidant enzyme activity, xenobiotic detoxification capacity, and secondary metabolism. The findings also show positive interactions of EBR and PHE when applied together for Cd stress alleviation. All the studied parameters clearly indicate an anti-stress property of EBR against Cd and PHE co-contamination. These findings suggest the potential feasibility of BR application to reduce pollutant residues for food safety. Further studies using advanced molecular techniques and mutant analyses are required to better understand the detailed mechanism of BR-induced PHE+Cd stress tolerance in plants.

Acknowledgements

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