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BRIEF COMMUNICATION

Brassinosteroid regulates secondary metabolism in tomato towards enhanced tolerance to phenanthrene

G.J. AHAMMED[1], Y.H. ZHOU[1], X.J. XIA[1], W.H. MAO[1], K. SHI[1], and J.Q. YU[1,2]*

Department of Horticulture, Zijingang Campus, Zhejiang University, Hangzhou 310058, P.R. China[1]
Key Laboratory of Horticultural Plants Growth, Development and Quality Improvement, Agricultural Ministry of China, Hangzhou 310058, P.R. China[2]

Abstract

We investigated the role of 24-epibrassinolide (EBR) in the amelioration of phenanthrene (PHE) stress in tomato (*Solanum lycopersicum* L.). Exposure to PHE (300 μM) significantly decreased shoot and root length (19 and 16 %, respectively), fresh mass (35 and 43 %, respectively), contents of chlorophyll *a* (26 %), chlorophyll *b* (27 %), and carotenoids (18 %) in tomato plants. In addition, PHE increased the malondialdehyde (MDA) content (57 %) and activity of secondary metabolism related enzymes glutathione-S-transferase (GST), glucose-6-phosphate dehydrogenase (G6PDH), shikimate dehydrogenase (SKDH), phenylalanine ammonia-lyase (PAL), and cinnamyl alcohol dehydrogenase (CAD). The expression levels of *GST1, PPO, SKDH, PAL* and *CAD* genes were also induced by PHE. Importantly, EBR (0.1 μM) alone and in combination with PHE increased the growth, biomass and activity of those enzymes significantly over control and PHE alone, respectively. Consistent with enzyme activities, transcript levels of *GST1, PPO, SKDH, PAL*, and *CAD* were further increased in PHE+EBR over PHE alone. However, MDA content was remarkably decreased in PHE+EBR than PHE alone. Meanwhile, content of phenols, flavonoids, and antioxidant activity were increased by PHE and PHE+EBR further increased all those parameters. These observations suggest that EBR regulates secondary metabolism in tomato which might enhance tolerance to PHE.

Additional key words: CAD, chlorophyll, 24-epibrassinolide, G6PDH, GST, malondialdehyde, PAL, SKDH, *Solanum lycopersicum.*

Secondary metabolites are the natural compounds distinct from the intermediates and products of primary metabolism. Plant secondary metabolism covers all physiological and biochemical facets of "secondary products" including functional and evolutionary aspects (Iriti and Faoro 2009). Different elicitors regulate the degree of secondary metabolites production which are most often used for plant defense and stress tolerance (Zhao *et al.* 2005).

Brassinosteroids (BRs) regulate a wide range of physiological processes in plants (Bajguz and Hayat 2009). BRs have protective role in responses to various stresses including high and low temperature, drought, salinity, herbicides, organic pollutants, heavy metals, and pathogen attack (Bajguz and Hayat 2009, Choudhary *et al.* 2011, Li *et al.* 2011, Ahammed *et al.* 2012). In addition, BRs also increase production of various secondary metabolites like phenolics, shikonin, forskolin, *etc*. (Yang *et al.* 1999, Choudhary *et al.* 2011, Swamy and Rao 2011).

Phenanthrene (PHE) is a three ring polycyclic aromatic hydrocarbon (PAH) widely found in environment. It is considered to be a carcinogen (Luch 2005, Liu *et al.* 2009). PHE is often used as model compound that causes oxidative stress, growth inhibition, chlorosis, necrosis, and localized cell death in plants (Liu *et al.* 2009, Ahammed *et al.* 2012). Some stresses like osmotic stress, drought, salinity, high irradiance, and exposure to ozone could increase production of secondary metabolites

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Abbreviations: BRs - brassinosteroids; CAD - cinnamyl alcohol dehydrogenase; DPPH - 1,1-diphenyl-2-picrylhydrazyl; EBR - 24-epibrassinolide; G6PDH - glucose-6-phosphate dehydrogenase; GST - glutathione-S-transferase; MDA - malondialdehyde; MW - MilliQ water; OPPP - oxidative pentose phosphate pathway; PAHs - polycyclic aromatic hydrocarbons; PAL - phenylalanine ammonia-lyase; PHE - phenanthrene; PPO - polyphenol oxidase; ROS - reactive oxygen species; SKDH - shikimate dehydrogenase.
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* Corresponding author: fax: (+86) 571 88982381, e-mail: jqyu@zju.edu.cn

like phenols, flavonoids, antho-cyanins, alkaloids, cyanogenic glucosides, glucosinolates, *etc*. (Zhao *et al.* 2005). The enhanced production of secondary metabolites helps the plant tolerate stress as many of them are reactive oxygen species (ROS) scavengers. PHE stress increased ROS accumulation in *Arabidopsis* but its effects on secondary metabolism is unknown (Liu *et al.* 2009). Recently, we have demonstrated that BRs alleviate PHE phytotoxicity by enhancement of antioxidant enzyme activities and photosynthesis (Ahammed *et al.* 2012). We hypothesized that BRs induced PHE stress tolerance might also be associated with regulation of plant secondary metabolism. Therefore, we investigated the effects of PHE and 24-epibrassinolide (EBR) on growth and secondary metabolism in tomato plants.

Seeds of tomato (*Solanum lycopersicum* L. cv. Hezuo 903) were germinated and grown in a mixture of peat and *Vermiculite* (7:3, v/v) in a growth chamber maintaining following conditions: 12-h photoperiod, photosynthetic photon flux density (PPFD) of 600 μmol m^{-2} s^{-1}, and day/night temperature of 25/17 °C. Three weeks after sowing, groups of eight seedlings were transferred into a container (40 × 25 × 15 cm) filled with Hoagland's nutrient solution (pH 7.1 ± 0.2). Two weeks after transfer, shoots were sprayed either with 12 cm^3 EBR (0.1 μM) or MilliQ water (MW). Next day seedlings were exposed to freshly prepared nutrient solution with or without 300 μM PHE. EBR and PHE (*Sigma-Aldrich*, St. Louis, USA) were initially dissolved in ethanol and acetone respectively; then diluted by MW to desired concentration. The final ratio of ethanol/MW (v/v) was 1:10 000, whereas acetone/MW was 1:1 000. These concentrations of ethanol and acetone were simultaneously used for all treatments including control group. Nutrient solution with or without PHE were renewed every alternate day while EBR was sprayed every 7 d. The experiment was terminated after three weeks of PHE exposure. For physiological, biochemical, and molecular studies, roots were immediately frozen in liquid nitrogen and stored at -80 °C until analyses.

Leaf pigments were extracted pooling the 3rd leaves from three plants in 80 % acetone and their content was measured by spectrophotometer *UV-2410PC* (*Shimadzu*, Kyoto, Japan) according to Lichtenthaler (1987). MDA was measured in root as an end product of lipid peroxidation by 2-thiobarbituric acid (TBA) test according to the method of Hodges *et al.* (1999). Secondary metabolism related enzyme activities and content of metabolites were analyzed using 0.3 g of root tissue in each experiment. GST activity was determined spectrophotometrically following method of Wang *et al.* (2010). Enzymatic formation of 2,4-dinitro-phenyl-S-glutathione was monitored at 340 nm (ε_{340}= 9.6 mM^{-1} cm^{-1}) for 5 min and corrected for non-enzymatic controls. Method of Debnam and Emes (1999) was used to analyse G6PDH activity. The reduction of NADP to NADPH was measured as the rate of change of the absorbance at 340 nm for the initial 5 min. SKDH was determined according to Diaz *et al.* (2001) with few modifications. The reaction system contained 0.1 cm^3 of enzyme solution, 1 cm^3 of 200 mM Tris-HCl (pH 9), 0.8 cm^3 of 5 mM shikimic acid and 0.1 cm^3 of 10 mM NADP. Changes of A_{340} was read at 20-s intervals and absorption coefficient of 6.22 mM^{-1} cm^{-1} was used. PAL activity was assayed by an adaptation of methods described by Ruiz *et al.* (1999) and Nguyen *et al.* (2003) based on the yield of cinnamic acid and estimated at 290 nm in the presence and absence of L-phenylalanine. One unit of PAL activity was defined as the change in absorbance per 1 cm^3 of enzyme extract. CAD was measured following the oxidation of the appropriate hydroxycinnamyl alcohol at 30 °C according to Mitchell *et al.* (1994). Total soluble protein content was measured according to the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Frozen root tissues were ground in liquid nitrogen and total RNA was isolated according to manufacturer's protocol using *TRIzol* reagent (*Invitrogen*, Carlsbad, CA, USA). The entire procedures for RNA preparation, reverse transcription, and subsequent quantitative real time PCR (iCycler iQ^{TM}, *Bio-Rad*, Hercules, USA) have been described elsewhere (Wang *et al.* 2010). Gene-specific primers were designed based on the mRNA or EST sequences for the corresponding genes, as follows: *GST1* (F: 5'-CTCTGGTTTGGAGCAATTCA-3', R: 5'-AATTTCAGCTGGATGCCTTT-3'), *PPO* (F: 5'-CAC CCACCTCCTGATCTCTCA-3', R: 5'-GGGACGAAT ACGGAGCTTAG-3'), *SKDH* (F: 5'-ATATCTGGGTC ACCTTTGGC-3', R: 5'-AGATAAGGCCTCAGCTC CAA-3'), *PAL* (F: 5'-AGGAGATCGACAAGGTGT-3', R: 5'-TAGCAGATTGGAAGAGGA-3'), *CAD* (F: 5'-GCCGCTGACTCACTTGATTA-3', R: 5'-TTCCAT CAAGCTTCAACAGC-3'), and *actin* gene (F: 5'-TGG TCGGAATGGGACAGAAG-3', R: 5'-CTCAGTCAG GAGAACAGGGT-3') was used as an internal control. 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. Quantification of mRNA levels has been performed following a method of Livak and Schmittgen (2001).

Total phenol content was determined according to Singleton and Rossi (1965) based on Folin phenol reagent reduction. The antioxidant activity of root ethanolic extract was determined using 1,1-diphenyl-2-picryl-hydrazyl (DPPH) according to Tadolini *et al.* (2000) and flavonoid content was determined according to Zhishen *et al.* (1999). Analyses were carried out spectrophotometrically. Data were expressed as means ± standard deviation and analyzed using one-way *ANOVA*. Significant differences between groups were at $P \leq 0.05$. Tukey's test was used to compare means.

Activities of enzymes related to secondary metabolism *viz.* GST, G6PDH, SKDH, PAL, and CAD in tomato roots were increased by PHE application (Fig. 1). Addition of EBR on PHE-stressed plants further increased all those enzyme activities. Plant GSTs are well known for their roles in detoxification of wide range

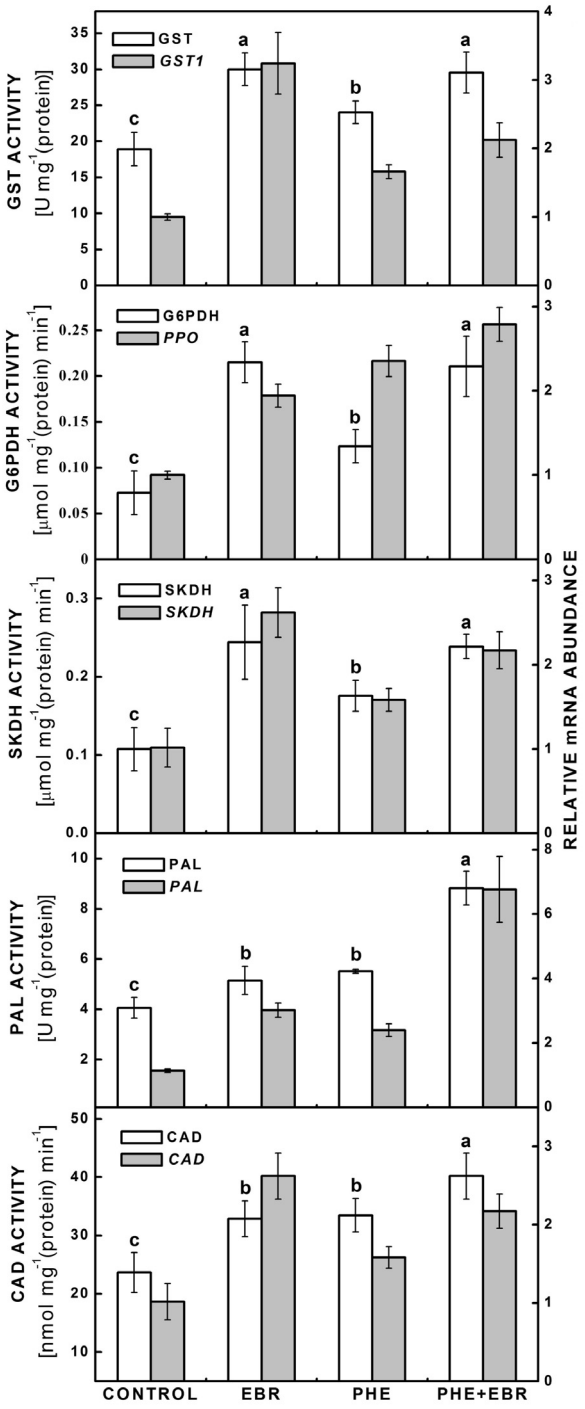

Fig. 1. Activities of secondary metabolism related enzymes *viz*. glutathione S-transferase (GST), glucose-6-phosphate dehydrogenase (G6PDH), shikimate dehydrogenase (SKDH), phenylalanine ammonia-lyase (PAL), and cinnamyl alcohol dehydrogenase (CAD) as well as transcript levels of *GST1, PPO, SKDH, PAL*, and *CAD* genes in response to phenanthrene (PHE) and 24-epibrassinolide (EBR) application. Assays were carried out using root tissues collected after 21 d of respective treatments. Values represent the average of three replicates obtained from separate experiments. Different letters indicate significant differences ($P < 0.05$) according to Tukey's test.

of xenobiotics. Apart from this, they are involved in the synthesis of sulfur-containing secondary metabolites and conjugation, transport, and storage of reactive oxylipins, phenolics, and flavonoids (Dixon *et al.* 2010). GST activity in tomato was increased by 59 and 27 %, respectively, when EBR and PHE were applied alone. It was further increased by 23 % in PHE+EBR compared to PHE alone. Enhanced GST activity suggested increased synthesis of sulfur-containing secondary metabolites which might be beneficial for plant tolerance. G6PDH is the key regulatory enzyme in the oxidative pentose phosphate pathway (OPPP; Kletzien *et al.* 1994). Application of EBR almost doubled the activity of G6PDH which might suggest an increased carbon flow through OPPP. In addition, G6PDH plays vital role in modulating antioxidants especially content of reduced glutathione, which helps to scavenge ROS and thereby enhance plant stress tolerance (Wang *et al.* 2008).

Furthermore, EBR alone increased the activity of SKDH, PAL, and CAD by 127, 27, and 39 %, respectively over control; those increments were 63, 36, and 42 % for PHE alone (Fig. 1). Interestingly, EBR application on PHE-stressed plants further increased those activities by 36, 60, and 20 % over PHE alone. SKDH is one of the important enzymes in the shikimate pathway that links metabolism of sugars to biosynthesis of aromatic compounds including L-phenylalanine. Thus, EBR-induced SKDH activity would increase the synthesis of L-phenylalanine (Wang *et al.* 2011). PAL is the first enzyme that catalyzes the elimination of NH_3 from L-phenylalanine to produce trans-cinnamate. Increased PAL activity enhances subsequent reactions in phenylpropanoid pathway to produce specific phenylpropanoid derivatives including simple phenols and flavonoids (Dixon and Paiva 1995). Similarly, NaCl stress in chamomile or Pb treatment of *Vallisneria natans* increased SKDH and PAL activities (Kovacik *et al.* 2009, Wang *et al.* 2011). CAD catalyzes the synthesis of cinnamyl alcohols from their corresponding cinnamaldehydes and is considered to be a highly specific marker for lignifications (Dos Santos *et al.* 2006).

To understand the molecular response to PHE and/or EBR, we investigated the transcript levels of *GST, PPO, SKDH, PAL*, and *CAD* genes (Fig. 1). Compared with control, their expression levels were increased by 3.6-, 1-, 1.6-, 1.7-, and 1.4-fold, respectively, in EBR alone treatment, whereas PHE alone caused 1.5-, 1.4-, 0.6-, 1.1-,and 1.3-fold increase, respectively. EBR application together with PHE further increased the expression levels of those genes. The transcript levels of different genes under different treatment were in agreement with physiological data (Fig. 1).

Consistently with enzyme activities, secondary metabolites like phenols and flavonoids were accumulated in plants treated by EBR, PHE, and PHE+EBR (Table 1). Increased total phenolics and flavonoids were also observed in root suspension cultures of *Panax ginseng* exposed to Cu (Ali *et al.* 2006). Choudhary *et al.* (2011) reported that EBR could increase

Table 1. Effects of PHE and EBR treatments on plant growth, contents of leaf pigments, malondialdehyde (MDA), phenols and flavonoids and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) activity in tomato root after 21 d of respective treatments. Means ± SD of 6 replicates for morphological parameters and 4 replicates for biochemical parameters. Different letters indicate significant differences ($P < 0.05$) according to Tukey's test.

Parameters	Control	EBR	PHE	PHE+EBR
Phenols [mg g^{-1}(f.m.)]	0.59 ± 0.01 d	0.63 ± 0.01 c	0.68 ± 0.02 b	0.72 ± 0.01 a
Flavonoids [mg g^{-1}(f.m.)]	1.13 ± 0.06 c	1.49 ± 0.03 b	1.53 ± 0.07 b	1.69 ± 0.05 a
Shoot length [cm $plant^{-1}$]	46.12 ± 2.29 b	51.33 ± 3.31 a	37.27 ± 1.81 c	43.38 ± 1.59 b
Root length [cm $plant^{-1}$]	33.85 ± 1.80 b	36.03 ± 1.67 a	28.58 ± 1.73 c	32.33 ±1.19 b
Shoot fresh mass [g $plant^{-1}$]	29.79 ± 2.36 b	34.43 ± 2.58 a	19.34 ± 2.03 d	25.56 ± 2.17 c
Root fresh mass [g $plant^{-1}$]	3.57 ± 0.21 b	4.65 ± 0.21 a	2.03 ± 0.26 c	3.32 ± 0.22 b
Chl *a* [mg g^{-1}(f.m.)]	1.80 ± 0.05 a	1.81 ± 0.04 a	1.33 ± 0.06 b	1.80 ± 0.03 a
Chl *b* [mg g^{-1}(f.m.)]	0.67 ± 0.02 a	0.67 ± 0.02 a	0.49 ± 0.02 c	0.65 ± 0.02 b
Carotenoids [mg g^{-1}(f.m.)]	0.29 ± 0.01 ab	0.30 ± 0.01 a	0.24 ± 0.02 c	0.29 ± 0.01 b
MDA [µmol g^{-1}(f.m.)]	0.38 ± 0.04 c	0.37 ± 0.04 c	0.60 ± 0.05 a	0.52 ± 0.04 b
DPPH activity [%]	15.22 ± 0.64 d	18.44 ± 1.38 c	23.13 ± 1.85 b	26.74 ± 1.35 a

the total phenolics content under Cr stress. Importantly, phenolic compounds are involved in the detoxification of H_2O_2 and thus could protect cell from oxidative damage (Kovacik *et al.* 2009). Exposure to PHE significantly reduced growth parameters (length and fresh mass of shoot and root) as well as leaf pigments content (Table 1). Compared with control, the shoot and root length was decreased by 19 and 16 % and fresh mass by 35 and 43 %, respectively. PHE-induced growth reductions were consistent with previous reports on PAHs phytotoxicity (Liu *et al.* 2009, Ahammed *et al.* 2012). However, PHE+EBR increased shoot and root length by 16.4 and 13.2 % whereas fresh mass by 30.6 and 58.2 %, respectively, over PHE alone. The content of Chl *a*, Chl *b* and carotenoids were decreased by 26.2, 27.2, and 17.5 %, respectively, due to PHE application. Liu *et al.* (2009) also observed decreased leaf pigments content following PHE in *Arabidopsis*. PAHs induce abscisic acid (ABA) as well as ROS in plants which may promote chlorophyll degradation (Liu *et al.* 2009, Váňová *et al.* 2009). Decreased leaf pigments content would obviously affect the primary photochemical process and so photosynthesis (Ahammed *et al.* 2012). However, subsequent supply of EBR to stressed plants increased the leaf pigment contents (Table 1). EBR may have a protective effect on pigment-protein complexes which results in decreased degradation of chlorophyll (Li *et al.* 2011). EBR induced biomass and chlorophyll increments under various stresses have been extensively documented which are in agreement with our current findings (Bajguz and Hayat 2009, Choudhary *et al.* 2011, Li *et al.* 2011, Ahammed *et al.* 2012).

Exposure to PHE increased MDA content by 56.7 %, whereas EBR supplementation decreased it by 13.4 % below PHE alone (Table 1). Increased MDA is a consequence of oxidative damage under PHE stress (Liu *et al.* 2009). Accordingly, PHE stress increased anti-oxidant activity in terms of DPPH activity by 52 % over control (Table 1). EBR application on stressed plants further increased DPPH activity by 16 % over PHE alone. Choudhary *et al.* (2011) reported that EBR could increase DPPH activity under Cr stress which supports our current observation. Furthermore, Sasse (2003) interpreted that BRs influence the synthesis of proteins or enzymes by inducing the specific gene expression which may improve the overall metabolic activities of plants.

Taken together, it could be concluded that EBR application alleviated PHE-induced stress or damage which was clearly indicated by increased growth and biomass production. This improvement might be attributed to enhanced activity of secondary metabolism related enzymes, increased production of secondary metabolites, and subsequent ROS scavenging in response to EBR. To the best of our knowledge, these findings are novel and highly promising to regulate plant secondary metabolism using EBR towards enhanced tolerance. Further study using advanced molecular approach is required to better understand the mechanism of BRs-induced regulation of secondary metabolism and subsequent stress tolerance.

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