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RESEARCH ARTICLE

# Effects of bisphenol A on chlorophyll synthesis in soybean seedlings

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**Abstract** Bisphenol A (BPA), as an emerging environmental pollutant, is potentially harmful to plant growth. Chlorophyll (Chl) is critical in photosynthesis that provides matter and energy for plant growth. How BPA affects the chlorophyll content remains largely unknown. Here, the effects of BPA on Chl synthesis in soybean seedlings were investigated. Exposure to 1.5 mg/L BPA decreased the 5-aminolevulinic acid (ALA) content and increased protoporphyrin IX (Proto IX), magnesium protoporphyrin, and protochlorophyll contents and 5-aminolaevulinic acid dehydratase, porphobilinogen deaminase, uroporphyrinogen III synthase, uroporphyrinogen III decarboxylase, and protoporphyrinogen oxidase activities. Exposure to 17.2 and 50.0 mg/L BPA exerted the opposite effects on these four intermediates and five enzymes. Following the withdrawal of BPA exposure, the aforementioned parameters gradually recovered, except magnesium protoporphyrin content in exposure to 50.0 mg/L BPA. Our findings revealed that exposure to low-concentration BPA increased the Chl content in soybean seedlings through improving Chl synthesis, especially the conversion from ALA to Proto IX, whereas exposure to high-concentration BPA decreased the Chl content through inhibiting Chl synthesis, especially the conversion from ALA to Proto IX. The dual effects of BPA were largely reversed following the withdrawal of BPA exposure.

**Keywords** Bisphenol A · Chlorophyll · Intermediates · Soybean seedlings

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## Introduction

Bisphenol A [2,2-bis(4-hydroxyphenyl)propane; BPA] is an intermediate compound used to produce polycarbonate plastics, flame retardants, epoxy resins, and coatings (Goodson et al. 2002), and its global production is approximately 3,700,000 tons per year (Mihaich et al. 2009). Global consumption of BPA in 2011 exceeds 5,500,000 tons (Flint et al. 2012). The increasing applications have led to the large release of BPA into the environment in many countries and regions, especially in the USA, Germany, Japan, and Taiwan (Huang et al. 2012), and more than 555 tons BPA released into the environment in the USA only in 2008 (Environmental Protection Agency 2001). Therefore, BPA is ubiquitous in the environment (Santos et al. 2001; Singh et al. 2010; Staples et al. 2010). BPA is discharged into the environment primarily through wastewater treatment plants, BPA production facilities, hazardous waste landfill sites, as well as other sources (Gassara et al. 2013). The continuous discharge of BPA has gradually increased its concentration in various ecosystems, including rivers, oceans, and soils (Asakura et al. 2004; Bian et al. 2010; Matsumoto et al. 2005). For example, the content of BPA in hazardous landfill leachates ranges from 1.3 to 17,200 ng/mL (Yamamoto et al. 2001), the content of BPA in American rivers ranges from 4.4 to 19,000 ng/mL (Singh et al. 2010), and the average content of BPA in soil of Brazilian sanitary landfills was 21.30 μg/g of soil (Vieceli et al. 2011).

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Environmental pollution with BPA (Pan et al. 2013; Speranza et al. 2011; Zoeller et al. 2005) has caused extensive concern, and numerous studies have reported that the ubiquity of BPA in the environment negatively affects the reproductive functions and the health of invertebrates and vertebrates, including humans (Yi et al. 2011; Yigit and Daglioglu 2010). In contrast to the vast literature regarding the effects of BPA on animals, only a few reports have been published regarding the effects of BPA on plants, which can rapidly absorb and transfer the compound (Adamakis et al. 2012; Nakajima et al. 2002). Previous studies have demonstrated that low concentrations of BPA (0.01 and 1.5 mg/L) stimulate the growth of carrot (*Daucus carota* L.) and soybean (*Glycine max* L.) (Qiu et al. 2013; Sun et al. 2013; Terouchi et al. 2004), whereas high concentrations of BPA (10.0, 17.2, and 50.0 mg/L) inhibit the growth of broad bean (*Vicia faba* L.), durum wheat (*Triticum durum* Desf.), lettuce (*Lactuca sativa* L.), tomato (*Lycopersicon esculentum* Mill.) (Ferrara et al. 2006), and soybean (*Glycine max* L.) (Qiu et al. 2013; Sun et al. 2013). The mechanism of BPA action on plants has primarily been investigated in light of photosynthesis, which is the basis of plant growth and development (Qiu et al. 2013). However, how BPA affects photosynthesis remains largely unknown.

Chlorophyll (Chl) is vital for photosynthesis, which allows plants to absorb light energy that is used to synthesize photosynthetic products (Alberte et al. 1977). Our previous study suggested that low concentrations of BPA increase, whereas high concentrations of BPA decrease, the Chl content in soybean seedlings (Qiu et al. 2013). However, little information is available regarding how BPA affects the Chl content in plants. It has been reported that the Chl content in plants tightly related to its biosynthesis, and Chl is synthesized from its first committed precursor, 5-aminolevulinic acid (ALA) (Bollivar 2006). ALA subsequently converts to protoporphyrin IX (Proto IX) by 5-aminolaevulinic acid dehydratase (ALAD), porphobilinogen deaminase (PBGD), uroporphyrinogen III synthase (UROS), uroporphyrinogen III decarboxylase (UROD), and protoporphyrinogen oxidase (PPO) (Tripathy and Pattanayak 2012) to provide substrate for synthesis of Mg-protoporphyrin IX (Mg-Proto IX) and protochlorophyll (Pchl) (Bollivar 2006). In the present work, the effects of BPA on the Chl synthesis in soybean seedlings were investigated by determining the contents of four intermediates (ALA, Proto IX, Mg-Proto IX, and Pchl) and the activities of five key enzymes (ALAD, PBGD, UROS, UROD, and PPO) in Chl synthesis of soybean seedlings exposed to BPA. The results would provide references for further understanding the effect mechanism of BPA on plant photosynthesis and for scientifically evaluating the potential ecological risks of BPA in the environment.

## Materials and methods

### Preparation of BPA solution

Based on the BPA concentrations used in previous studies about the effects of BPA on plants and animals (Ferrara et al. 2006; Mandich et al. 2007; Mihaich et al. 2009; Saiyood et al. 2010), the current pollution situation and development trend of BPA in developing countries, and one particular accident that released a large amount of BPA into the environment, three representative concentrations of BPA (1.5, 17.2, 50.0 mg/L) were chosen for study. In these concentrations, 1.5 mg/L is the safety concentration of drinking water calculated based on the upper limit of safety for people as reported by the United States Environmental Protection Agency (Geens et al. 2011), 17.2 mg/L is the concentration of the hazardous landfill leachates (Yamamoto et al. 2001), and 50.0 mg/L is the concentration of BPA in soil suddenly polluted by BPA as well as the concentration of BPA selected in the other reports (Ferrara et al. 2006; Saiyood et al. 2010). At 25 °C, BPA is a solid compound with low volatility and water solubility ranging from 120 to 300 mg/L (Ferrara et al. 2006; Mihaich et al. 2009). BPA solutions at different concentrations (1.5, 17.2, and 50.0 mg/L) were prepared by dissolving appropriate quantities of BPA (Sinopharm Chemical Reagent Co., Ltd, China) in Hoagland's solution (pH 7.0) with continuous stirring and/or sonication at 25 °C. All reagents used were of analytical grade.

### Plant culture

Soybean seeds (Zhonghuang 25, Wuxi Seed CO., Ltd., China) were surface-sterilized in $HgCl_2$ (0.1 %) solution for 5 min and rinsed with distilled water several times. Then, the seeds were placed in a dish containing three layers of gauze and germinated in the incubator at 25± 1 °C. When the radicle length was approximately 1 cm, the seedlings were transplanted into plastic pots (15 cm in diameter; three plants per pot) filled with distilled water, which was changed every day. After the second true leaf had developed (approximately 10 days after germination), the seedlings were cultured in one-half strength Hoagland's solution (pH 7.0) in a greenhouse. The photosynthetic photon flux density provided by the incandescent lamps in the greenhouse was 300 μmol/$m^2$/s, which was measured using a photometer (Fluke 941, Fluke Corporation, USA). The nutrient solution was aerated twice each day using an electronic air pump, and distilled water was added to maintain the solution volume (ZrL 2002). The nutrient solution was renewed every 3 days to maintain a stable pH.

### Treatment with BPA

After the third true leaf had developed (approximately 30 days after germination), the soybean seedlings were transplanted into BPA solutions at various concentrations (1.5, 17.2, and 50.0 mg/L, pH 7.0). Control soybean seedlings were cultured in the one-half strength Hoagland's solution (pH 7.0) without BPA. All treatments were performed in triplicate, and the solutions were renewed every 3 days. Soybean seedlings were cultured in the BPA solutions for 7 days and then in one-half strength Hoagland's solution (pH 7.0) without BPA for 7 days to self-recover. After the 1-, 4-, 7-, 8-, 11-, and 14-day treatments, the leaves were sampled for the determination of the content of Chl, the contents of four intermediates (ALA, Proto IX, Mg-Proto IX, and Pchl), and the activities of five key enzymes (ALAD, PBGD, UROS, UROD, and PPO).

### Determination of the Chl content

The leaves of soybean seedlings were soaked in 80 % acetone, and then the chlorophyll was extracted. The extract was centrifuged at 5300×*g* for 10 min. Then, the absorbance of the supernatant was read at 645 and 663 nm, respectively. The content of chlorophyll was calculated according to the equation $20.2A_{645}+8.02A_{663}$ (Lichtenthaler 1987).

### Determination of the contents of ALA, Proto IX, Mg-Proto IX, and Pchl

The content of ALA was determined using the previous method with minor modifications (Wang et al. 1995). Fresh samples (2 g) were homogenized with 6 mL of acetic sodium buffer (pH 4.6) in an ice-cold water bath. The homogenates were extracted in boiling water bath for 15 min and centrifuged at l0,000×*g* for 20 min. The pellet was extracted twice with 4 mL of buffer. The supernatants were collected to determine the content of ALA. Ethyl acetoacetate (four drops) was added to 1 mL of extract and kept in a boiling water bath for 15 min, and then 4 mL Ehlrich reagent was added. The mixture was kept for 15 min, and its absorption was detected at 533 nm. The content of ALA (nmol/$g_{FW}$) was calibrated using the standard of ALA-HCl (Sigma) (Yu et al. 2006).

Fresh samples (2 g) were homogenized with 90 % chilled ammonical acetone (acetone 0.1 N $NH_4OH$=9:1) (10 mL) and centrifuged at 12,000×*g* for 10 min at 4 °C. The supernatant was mixed in a separating funnel with an equal volume of ice-cold hexane. This was properly mixed, and the two layers were allowed to separate. The lower layer was re-extracted with 1/3 volume of hexane. The extract, i.e., the hexane-extracted acetone-residue solvent mixture (HEAR), was then used to measure fluorescence emission spectra. Fluorescence emission spectra were recorded at room temperature in a photon-counting SLM 8000 spectrofluorometer in the ratio mode. A tetraphenylbutadiene block was used to adjust the voltage to 20,000 counts per second in both the reference and the sample channels when excited at 348 nm, and the emission was monitored at 422 nm. The slit widths of both the excitation and emission monochromators were set at 4 nm. Rhodamine B was used as a quantum counter in the reference channel. Hexane-extracted acetone residue was placed in the sample chamber, and fluorescence emission spectra were recorded from 580 to 700 nm. Samples were excited at 400 nm ($E_{400}$) and 440 nm ($E_{440}$), and fluorescence emission spectra were corrected for instrument response. Proto IX content was calculated from fluorescence emission spectra ($E_{400}$ and $E_{440}$) as described: Proto IX ($E_{400}F_{633}$)=($E_{400}F_{633}$ − 0. 25× $E_{400}F_{622}$ − 0.24×$E_{440}F_{640}$)/0.95 (Tripathy et al. 2007). The relative content of Mg-Proto IX was $E_{440}F_{595}$. Here, $E_{400}F_{633}$ represents that the fluorescent properties at 400 nm exciting light and others are the same.

The content of Pchl was determined according to the methods in previous reports (Chia and He 1999; Hodgins and Van Huystee 1986). Fresh samples (0.3 g) were homogenized with 25 mL 80 % alkaline acetone and ground. Then, the samples were extracted to determine its absorption Pchl at 575, 590, and 628 nm. The content of Pchl (nmol/g) was calculated by the equation Pchl=(0.0563×$A_{628}$+0.007225× $A_{590}$ − 0.02955×$A_{575}$)×$10^3$.

### Determination of the activities of ALAD, PBGD, UROS, UROD, and PPO

The ALAD extraction was carried out based on the previous method (Scarponi et al. 1985). Leaf tissues (2.5 g) were homogenized with 0.05 M Tris–HCl buffer (pH 8.2) containing 0.1 mM dithiotheritol (DTT, 0.5 mL/g fresh weight). The homogenate was filtered through four layers of cheese cloth, and the filtrate was centrifuged at 10,000×*g* for 1 h at 4 °C. The supernatant was assayed for the activity of ALAD. One milliliter of enzyme extract was incubated with 0.27 mL of 1 mg/mL ALA, 1.35 mL of 0.05 M Tris–HCl buffer (pH 8.2) containing 0.1 mM DTT, and 0.08 mL of 0.2 M $MgCl_2$ for 2.5 h at 37 °C. The reaction was terminated by adding 0.3 mL of 3.0 M trichloroacetic acid. After cooling, samples were centrifuged at 2000×g for 10 min. The supernatant was mixed (1:1) with Ehrlich's reagent, and then the absorbance was determined at 555 nm after 10 min against zero time blank. The concentration of porphobilinogen (PBG) was calculated based on previous method (van den Boogert et al. 1998). The activity of ALAD was expressed as nmol of PBG formed/ $g_{FW}$/2.5 h and was calculated by the equation ($V_t$×$\Delta A$/$\Delta t$)/ ($E_{FW}$×$V_s$), where $V_t$ was the volume of the reaction solution, $\Delta A$ was the change of absorbance, $\Delta t$ was 2.5 h, and FW was the fresh weight.

The PBGD, UROS, and UROD extraction was carried out according to the previous method (Felix and Brouillet 1990;

Frydman and Frydman 1979). The leaf tissues (300 g) were homogenized in 10 mM Tris–HCl buffer solution (pH, 8.0) containing 5 mM 2-mercaptoethanol, 2 mM phenylmethanesulfonyl fluoride, and 2 mM EDTA-$Na_2$ (3 mL/g fresh weight). The homogenate was filtered through several layers of nylon cloth and centrifuged at 20,000×*g* for 15 min. All operations were performed at 4 °C. The supernatant was used as crude enzyme for the activity measurements.

The PPO extraction was carried out based on the previous method (Jacobs and Jacobs 1981). The leaf tissues were homogenized in HEPES buffer (pH 7.8) containing 0.05 M HEPES, 0.5 M sucrose, 1 mM dithiotheritol, 1 mM $MgCl_2$, 1 mM EDTA, and 2 % albumin from bovine serum. The homogenate was filtered through 100 meshes of nylon cloth. The filtrate was centrifuged at 800×*g* for 2 min at 4 °C, and then the supernatant was centrifuged at 17,000×*g* for 6 min at 4 °C. The precipitation was dissolved in 0.05 M Tris–HCl buffer (pH 7.3) containing 2 mM EDTA, 20 % (*v*/*v*) glycerin and used to determine the activity of PPO.

The enzyme activity assay was performed by using the method of Elisa kit. The 10-μL crude enzyme solution was incubated with 40 μL dilute solution containing 1 % albumin from bovine serum, 5 % polyoxyethylene sorbitan fatty acid esters, and Tween 20 for 1 h at 37 °C. After adding 50 μL enzyme-labeled antibody (horseradish peroxidase) buffer (pH 7.4) containing 2 M NaCl, 0.15 M $KH_2PO_4$, 0.81 M $Na_2HPO_4 \cdot 12H_2O$, 0.27 M KCl, 0.31 M $NaN_3$, and 0.05 % Tween 20 and incubating for another 1 h at 37 °C, the mixture was colored with 0.1 mL 3,3′,5,5′-tetramethylbenzidine solution containing 0.2 M $Na_2HPO_4$, 0.1 M citric acid, and 0.75 % $H_2O_2$. The reaction was stopped by adding 50 μL of 3 M $H_2SO_4$. The enzyme activity was measured at 450 nm and expressed as mean OD units corrected for a substrate blank and calculated according to the standard curve.

### Statistical analysis

Significant differences between the treatments were analyzed by one-way analysis of variance (ANOVA) using SPSS 16.0. Student's *t* test was used to determine the significance of the differences between the treatments ($p < 0.05$) (Ke et al. 2003).

## Results

### Effects of BPA on the Chl content in soybean seedlings

Table 1 shows the Chl contents in soybean seedlings treated with different concentrations of BPA. During the stress period (from the first to the seventh day of the experiment), the Chl content in soybean seedlings exposed to 1.5 mg/L BPA was higher than that of the control; this effect first became weaker and then remained unchanged with increasing exposure time. After soybean seedlings were exposed to 17.2 mg/L BPA, the Chl content was higher than in the control on the first day and lower than the control after longer exposure. After soybean seedlings were exposed to 50.0 mg/L BPA, the Chl content gradually decreased compared with that of the control, and this effect was more evident than that of 17.2 mg/L BPA exposure.

During the recovery period (from the eighth to the 14th day of the experiment), the Chl content in soybean seedlings exposed to 1.5 mg/L BPA did not obviously change from the eighth to the 11th day and then increased on the 14th day. The Chl content in soybean seedlings exposed to 17.2 mg/L BPA also recovered to the control level. After treatment with 50.0 mg/L BPA, the Chl content in soybean seedlings was maintained at a lower level than in the control. These data suggested that the changes in Chl content induced by BPA exposure were alleviated following the withdrawal of BPA, except for that of the 50.0 mg/L BPA treatment.

### Effects of BPA on the ALA content in soybean seedlings

The changes in ALA content in soybean seedlings exposed to different concentrations of BPA during the stress and recovery periods are shown in Fig. 1a. During the stress period, the ALA content in soybean seedlings exposed to 1.5 mg/L BPA continuously decreased compared with that of the control. The opposite effect was observed in soybean seedlings treated with 17.2 mg/L BPA. After soybean seedlings were exposed to 50.0 mg/L BPA, the ALA content in soybean seedlings increased from the first to the fourth day and then decreased from the fourth day to the seventh day. Additionally, the ALA content remained above that of the control for the entire stress period.

During the recovery period, the ALA content in soybean seedlings exposed to 1.5 mg/L BPA gradually increased to the control level. The ALA contents in soybean seedlings exposed to 17.2 or 50.0 mg/L gradually decreased from the eighth to the 14th day but did not decrease to the control level. Our data suggested that the changes in ALA content induced by BPA exposure were reversed following the withdrawal of BPA.

### Effects of BPA on the Proto IX content in soybean seedlings

As shown in Fig. 1b, exposure to 1.5 mg/L BPA during the stress period increased Proto IX content in soybean seedlings over that of the control, and this effect remained nearly unchanged as exposure time was prolonged. After exposure to 17.2 mg/L BPA, the Proto IX content in soybean seedlings decreased relative to that of the control, and the decrease was more evident at the seventh day than that at the first day. Greater decreases in the Proto IX content in soybean seedlings were observed after exposure to 50.0 mg/L BPA.

**Table 1** Effects of BPA on the content of Chl in soybean seedlings

| BPA (mg/L) | 1 days | 4 days | 7 days | 8 days | 11 days | 14 days |
|---|---|---|---|---|---|---|
| 0.0 | 1.23±0.04b (100.00) | 1.17±0.03a (100.00) | 1.15±0.18a (100.00) | 1.24±0.07a (100.00) | 1.29±0.04a (100.00) | 1.44±0.18b (100.00) |
| 1.5 | 1.46±0.04a (118.70) | 1.30±0.04a (111.11) | 1.24±0.02a (107.83) | 1.29±0.06a (104.03) | 1.34±0.04a (103.88) | 1.70±0.03a (118.06) |
| 17.2 | 1.43±0.01a (116.26) | 0.94±0.01b (80.34) | 0.93±0.09b (80.87) | 1.23±0.03a (99.19) | 1.29±0.01a (100.00) | 1.33±0.19bc (92.36) |
| 50.0 | 1.13±0.05c (91.87) | 0.76±0.15c (76.00) | 0.86±0.08b (74.78) | 1.05±0.03b (84.68) | 1.03±0.08b (79.84) | 1.14±0.07c (79.17) |

Values are means±standard deviation errors, $n$ =3

Significant differences at $p$ <0.05 are shown with different letters in each column

During the recovery period, the Proto IX content in soybean seedlings exposed to 1.5 mg/L BPA continuously increased and remained above the control level. The Proto IX contents in soybean seedlings exposed to 17.2 and 50.0 mg/L BPA gradually approached that of the control, although the Proto IX content in soybean seedlings exposed to 50.0 mg/L BPA remained lower than that in soybean seedlings exposed to 17.2 mg/L BPA. The data suggested that the decrease in Proto IX contents induced by exposure to 17.2 and 50.0 mg/L BPA was reversed following the withdrawal of BPA.

## Effects of BPA on the Mg-Proto IX content in soybean seedlings

The changes in the Mg-Proto IX contents in soybean seedlings exposed to different concentrations of BPA during the stress and recovery periods of the experiment are shown in Fig. 1c. During the stress period, the Mg-Proto IX content in soybean seedlings exposed to 1.5 mg/L BPA consistently remained above that of the control, and this degree of increase was nearly unchanged with the prolonging of exposure. Exposure to 17.2 and 50.0 mg/L BPA decreased the Mg-Proto IX contents in soybean seedlings, and the degree of this decrease became larger over the course of treatment. In addition, the decrease in the Mg-Proto IX content in soybean seedlings exposed to 50.0 mg/L BPA was higher than that in soybean seedlings exposed to 17.2 mg/L BPA.

During the recovery period, the Mg-Proto IX content in soybean seedlings exposed to 1.5 mg/L BPA remained higher than that of the control group; the content increased from the seventh to the eighth day and then decreased. The Mg-Proto IX contents in soybean seedlings exposed to 17.2 and

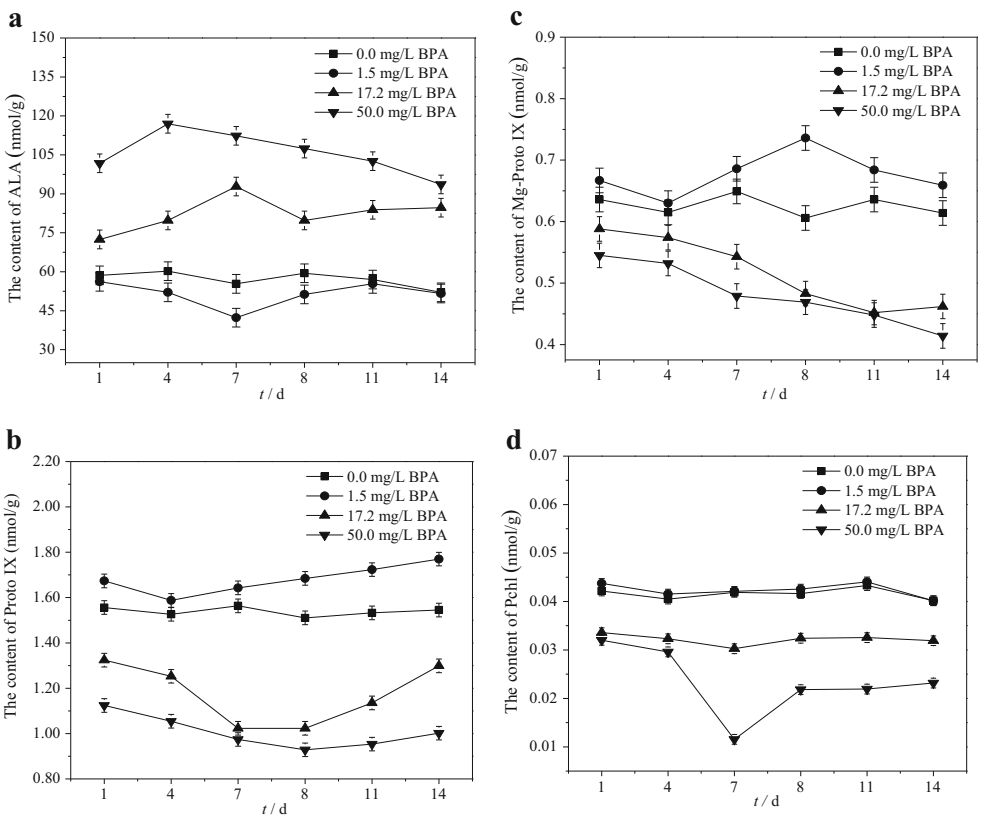


**Fig. 1** Effects of BPA on the contents of ALA (**a**), Proto IX (**b**), Mg-Proto IX (**c**), and Pchl (**d**) in soybean seedlings

50.0 mg/L BPA remained lower than that of the control group and consistently decreased as the experiment proceeded, except for a slight increase in the soybean seedlings exposed to 17.2 mg/L BPA from the 11th to the 14th day. The decrease in the Mg-Proto IX content caused by 50.0 mg/L BPA was not recovered following the withdrawal of BPA.

### Effects of BPA on the Pchl content in soybean seedlings

The changes in the Pchl contents in soybean seedlings exposed to different concentrations of BPA during the stress and recovery periods of the experiment are shown in Fig. 1d. During the stress period, the Pchl content in soybean seedlings exposed to 1.5 mg/L BPA was not consistently changed compared to that of the control group. Exposure to 17.2 and 50.0 mg/L BPA continuously decreased the Pchl contents in soybean seedlings as treatment progressed, and the decrease in the 50.0 mg/L BPA group was significantly higher than that in the 17.2 mg/L BPA group.

During the recovery period, the Pchl content in soybean seedlings exposed to 1.5 mg/L BPA remained unchanged. The Pchl contents in soybean seedlings exposed to 17.2 and 50.0 mg/L BPA remained lower than those of the control but increased toward the control level as time progressed. Following the withdrawal of BPA, the decreases in the Pchl contents in soybean seedlings induced by exposure to 17.2 and 50.0 mg/L BPA were reversed.

### Effects of BPA on the activities of key enzymes in Chl synthesis of soybean seedlings

The changes in the activities of ALAD, PBGD, UROS, UROD, and PPO in soybean seedlings exposed to different concentrations of BPA during the stress and recovery periods of the experiment are shown in Fig. 2. During the stress period, the activities of ALAD, PBGD, UROS and UROD in soybean seedlings exposed to 1.5 mg/L BPA gradually increased compared with those of the control. Meanwhile, the activity of PPO was increased from the first to the fourth day and then decreased from the fourth day to the seventh day. Exposure to 17.2 mg/L BPA continuously decreased the activities of ALAD, PBGD, UROS, UROD, and PPO in soybean seedlings with prolonging the exposure time. Similar effects on the activities of PBGD, UROS, UROD, and PPO were observed in exposure to 50.0 mg/L BPA, which was more evident than that in exposure to 17.2 mg/L BPA. Nevertheless, the activity of ALAD was decreased from the first to the fourth day and then increased from the fourth to the seventh day. In addition, the activity of ALAD remained lower than that of the control during the whole stress period.

During the recovery period, the activities of ALAD, PBGD, UROS, UROD, and PPO in soybean seedlings exposed to 1.5, 17.2 or 50.0 mg/L BPA were gradually close to the control level.

### Correlation analysis

In order to understand how BPA affects the Chl content, a correlation analysis between the Chl content, each intermediate content, and the key enzyme activity in soybean seedlings was performed. As shown in Table 2, the Chl content was positively correlated to the Proto IX and Mg-Proto IX contents and negatively correlated to the ALA content. Furthermore, the ALA content was negatively related to the activities of ALAD, PBGD, UROS, and UROD.

## Discussion

Chl has a dual function in photosynthesis, both mediating light-driven charge separation and harvesting and transferring light energy to reaction centers (Rüdiger 2009). Thus, the Chl content of plant cells directly affects photosynthetic rate, indirectly changing plant quality and yield (Zhang et al. 2012). Our results showed that low-concentration BPA (1.5 mg/L) exposure increased the Chl content of soybean seedlings, and this effect gradually weakened with prolonging the exposure time of BPA. Middle- (17.2 mg/L) or high-concentration (50.0 mg/L) BPA exposure decreased the Chl contents, and this effect became lower or higher with prolonging the exposure time of 17.2 or 50.0 mg/L BPA. These effects became weaker following the withdrawal of BPA, and the Chl content in soybean seedlings exposed to the low concentration of BPA could recover to the control level. These results were in agreement with those of previous research (Qiu et al. 2013).

How did BPA affect the Chl content of soybean seedlings? We speculated that the compound might impact Chl synthesis. Chl biosynthesis is subdivided into five key steps: (1) glutamic acid is converted to ALA, (2) eight molecules of ALA are linked to form Proto IX, (3) magnesium is inserted into Proto IX to form Mg-Proto IX (Tripathy and Pattanayak 2012), (4) Pchl is formed from Mg-Proto IX by the light-dependent enzyme NADPH/$H^+$-protochlorophyllide oxidoreductase (Von Wettstein et al. 1995), and (5) Pchl is transformed into Chl in the course of the dark reaction. In these steps, the synthesis and conversion of ALA are vital steps regulating Chl synthesis (Beale 1978; Bollivar 2006). Meanwhile, ALAD is the vital rate-limiting enzyme in the conversation of ALA, and the ALAD activity regulates the Chl content directly (Padmaja et al. 1990). In addition, other key enzymes, such as PBGD, UROS, UROD, and PPO, also play important roles in Chl synthesis (Frydman and Frydman 1979; Jiang et al. 2008). If any step of this pathway is blocked,

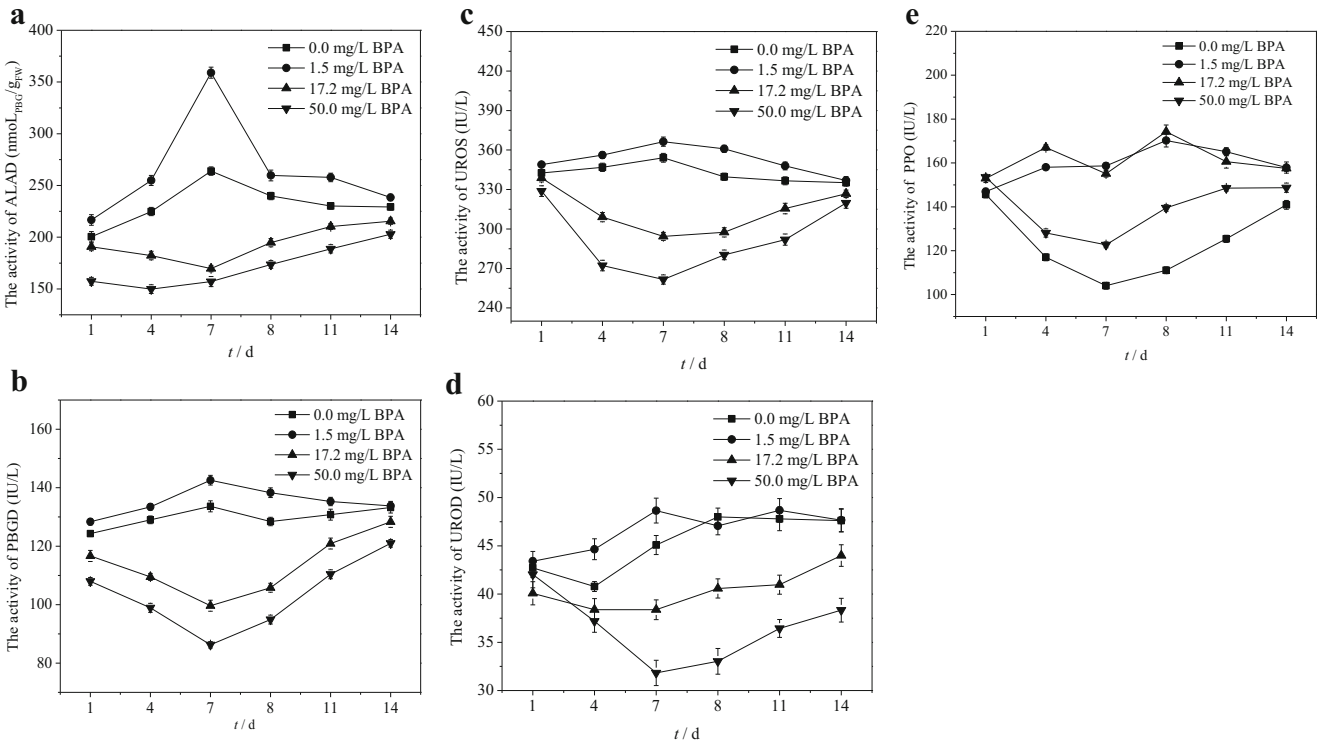


**Fig. 2** Effects of BPA on the activities of ALAD (**a**), PBGD (**b**), UROS (**c**), UROD (**d**), and PPO (**e**) in soybean seedlings

the enzyme activity before the blockage is expected to be inhibited, leading to the quick accumulation of intermediate before the blockage (Jones et al. 2004); meanwhile, the level of the subsequent intermediate would sharply decrease, and the activity of the enzyme regulating the content of this intermediate would change accordingly (Yu et al. 2006). Therefore, in this study, the effects of BPA on Chl synthesis in soybean seedlings were investigated by measuring the contents of key intermediates (ALA, Proto IX, Mg-Proto IX, and Pchl) and the activities of key enzymes (ALAD, PBGD, UROS, UROD, and PPO) in the pathway. Our results showed that BPA exposure caused similar changes in the Proto IX, Mg-Proto IX, and Pchl contents in soybean seedlings, indicating that these three intermediates responded consistently to BPA exposure. The order of these changes depended on the concentration of BPA exposure. Low-concentration BPA exposure (1.5 mg/L) promoted the Chl synthesis through improving the conversion of ALA to Proto IX, Mg-Proto IX, and Pchl, which was related with the increases in the activities of ALAD, PBGD, UROS, UROD and PPO. That is, low-concentration BPA exposure increased the activities of key enzymes in Chl synthesis, causing the increase in the content of Proto IX. The increased Proto IX provided enough substrate to synthesize Mg-Proto IX and Pchl and then promoted the Chl synthesis. These changes were consistent after a long exposure time, which might be due to the reduction in the effect of BPA on the key enzyme activities induced by the metabolism of BPA in the plants over prolonged exposure

**Table 2** Relationships between the Chl content, each intermediate, and the key enzymes' activity of soybean seedlings treated with BPA

| *y* | *x* | Linear regression equation | Correlation coefficient (*r*) |
|---|---|---|---|
| Chl content | ALA content | $y=-0.0055x+1.4613$ | −1.000** |
| | Proto IX content | $y=0.5048x+0.3883$ | 0.988* |
| | Mg-Proto IX content | $y=1.8680x-0.0057$ | 1.000** |
| | Pchl content | $y=11.278x+0.6904$ | 0.800 |
| ALA content | ALAD activity | $y=-0.3258x+152.91$ | −1.000** |
| | PBGD activity | $y=-1.2059x+215.2$ | −0.977** |
| | UROS activity | $y=-0.6533x+284.19$ | −1.000** |
| | UROD activity | $y=-4.3278x+253.07$ | −1.000** |
| | PPO activity | $y=-0.2854x+113.80$ | −0.244 |

*$p<0.05$; **$p<0.01$

(Noureddin et al. 2004). When the concentrations of BPA were increased to 17.2 and 50.0 mg/L, the activities of ALAD, PBGD, UROS, UROD, and PPO were inhibited to various extents, leading to the rapid accumulation and the decreases in the Proto IX, Mg-Proto IX, and Pchl contents (Fig. 1). These results indicated that high-concentration BPA exposure inhibited the activities of key enzymes in the conversion of from ALA to Proto IX, promoted the consumption of Proto IX, Mg-Proto IX, and Pchl, and thus inhibited Chl biosynthesis. The accumulation of ALA was unchanged, and the excess consumption of Proto IX, Mg-Proto IX, and Pchl was more evident after extended exposure to high concentrations of BPA. ALA is a potential plant growth regulator under stress conditions, serving as an essential biosynthetic precursor of tetrapyrrole compounds such as heme and cytochromes (Hotta et al. 1997). The accumulation of ALA might be used to synthesize other tetrapyrrole compounds and counteract the problems caused by BPA. Interestingly, the present results demonstrated that the decrease in Proto IX content was greatest among the contents of Proto IX, Mg-Proto IX, and Pchl, indicating Proto IX content was most sensitive to BPA exposure.

Following the withdrawal of low-concentration BPA exposure, the contents of ALA, Mg-Proto IX, and Pchl, as well as the activities of ALAD, PBGD, UROS, UROD, and PPO, recovered to their control levels, which might be due to the complete self-decomposition of BPA or the metabolism of BPA by plant enzymes (Fleisch et al. 2010; Nakajima et al. 2004). However, the Proto IX content consistently increased and did not recover after exposure. This result may be due to the greater sensitivity of Proto IX to BPA compared with other intermediates. Following the withdrawal of middle-concentration BPA exposure, ALA, Proto IX, Mg-Proto IX, and Pchl contents all recovered to their control levels, and the effects of BPA on ALAD, PBGD, UROS, UROD, and PPO activities gradually weakened. Previous studies concerning the effects of BPA in animals have reported that BPA can cause the excess accumulation of hydroxyl radicals (Obata and Kubota 2000), and we speculated that the same effect also occurred in soybean seedlings. Following the withdrawal of middle-concentration BPA exposure, peroxidase activity increased, not only accelerating BPA metabolism (Dogan et al. 2010) but also scavenging the excess free radicals induced by BPA stress (Obata and Kubota 2000). Following the withdrawal of high-concentration BPA exposure, the contents of ALA, Proto IX, and Pchl, as well as the activities of ALAD, PBGD, UROS, UROD, and PPO, all recovered to the control level, except for the Mg-Proto IX content. This exception may be related to the irreversible damage of the thylakoid membrane and chlorophyll molecules (Chereskin and Castelfranco 1982; Li et al. 2009).

In this study, the effect mechanism of BPA on the Chl content in soybean seedlings was understood from the view of Chl synthesis. However, previous studies have shown that the Chl content depends on a dynamic balance between Chl synthesis and Chl degradation (Rüdiger 1997; Santos 2004). Some reports showed that chloroplast structure also affects the Chl content (Santos et al. 2001). Therefore, apart from the Chl synthesis, the changes in the Chl content induced by BPA might be related with Chl degradation and chloroplast structure, which would be further investigated in the future.

## Conclusions

In conclusion, low-concentration (1.5 mg/L) BPA exposure promoted the biosynthesis of Chl through increasing the activities of the key enzymes converting ALA to Proto IX and then improving the conversion of ALA to Proto IX, Mg-Proto IX, and Pchl. High-concentration BPA exposure significantly inhibited the biosynthesis of Chl, which was through decreasing the activities of the key enzymes and blocking the conversion of ALA to Proto IX, Mg-Proto IX, and Pchl.

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