Aquatic Toxicology 109 (2012) 166–175

Contents lists available at SciVerse ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Oxidative effects and metabolic changes following exposure of greater duckweed (*Spirodela polyrhiza*) to diethyl phthalate

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ARTICLE INFO

Article history:
Received 25 August 2011
Received in revised form
27 September 2011
Accepted 4 October 2011

Keywords:
Diethyl phthalate
Oxidative stress
Allelochemical
Spirodela polyrhiza

ABSTRACT

The toxicity and effects of diethyl phthalate (DEP), a potent allelochemical, on the growth of greater duckweed were studied. Biochemical analyses and physiological methods were combined to investigate oxidative stress, adverse effects and their mechanisms in greater duckweeds grown in 0–2 mM of diethyl phthalate (DEP) after cultivation for 7 days. The results showed that J-shaped concentration response curves were displayed in hydrogen peroxide (H_2O_2), ascorbic acid (ASA) and dehydroascorbate (DHA) levels, and ascorbate peroxidase (APX) and gualacol peroxidase (POD) activities, indicating reduced oxidative stress and toxic effect. The inverted U-shaped curves were exhibited in relative growth rate (RGR), fresh weight/dry weight (FW/DW) ratio, total chlorophyll content, total soluble thiols, and glutathione reductase (GR) activity, revealing beneficial effect in plant growth. The inverted U-shaped curves were also found in malondialdehyde (MAD) and superoxide radical ($O_2^{\bullet -}$) contents with the increasing concentration of DEP, indicative of enhanced oxidative stress. The results suggest that DEP is toxic to the greater duckweed by inducing oxidative stress and antioxidative enzymes may play important roles in the defense strategy against DEP toxicity.

1. Introduction

Diethyl phthalate (DEP), one of the low molecular weight phthalate esters (PAEs), is used as plasticizers to increase the flexibility of high molecular weight polymer (Staples et al., 1997) in many products, including automotive components, herbicides, toys, cosmetic formulations, medical treatment tubing, plastic packing films, and as an ingredient in aspirin coating (McCarroll, 2006). As many other PAEs, DEP is not chemically bound to the polymer products and migrates readily from the plastics to the environment. Therefore, DEP is frequently detectable worldwide in surface water as well as in drinking water, leading to a significant exposure of organisms to this compound (Langer et al., 2010; Leitz et al., 2009; Sekizawa et al., 2003). In addition, even though DEP is biodegraded rapidly in soil (Cartwright et al., 2000), concern has been raised about the effects on human health from the absorption of DEP from soil to plants (Kapanen et al., 2007). The fate of DEP in environments has been investigated intensively. However, its effects on higher plant growth and development have not been so well described. Herring and Bering (1988) reported that DEP caused a significant inhibition of plant germination and seedling growth. At concentration of 0.1% DEP, spinach (*Spinacia oleracea*) and pea (*Pisum sativum*) root length is decreased and seedlings were desiccated during development. In their investigation of the toxicity and effects on protein synthesis of the PAEs in radish seedlings, Saarma et al. (2003) found that DEP caused retardation of growth in radish (*Raphanus sativus*). *In vitro* protein labeling coupled with two-dimensional gel electrophoresis studies revealed that heat shock proteins (HSPs) were not affected by DEP; however, certain HSPs can be used as an indicator of DEP-stress. Interestingly, there are proteins that were found only in DEP-treated radish root tissues (Saarma et al., 2003). Barnyard grass competes with crops and causes reduction in rice yields worldwide (Junaedi et al., 2010). DEP was identified as one of the 15 potentially allelopathic compounds from the grass (Xuan et al., 2006). The amount of DEP in root exudates of barnyard grass was estimated to be 2.7 $\mu g\,ml^{-1}$. In general, DEP concentration at 405.5 μM showed great growth inhibition of 6 indicator plants, suggesting that DEP and PAEs derivatives may be derived from plants and act as plant phytotoxins (Xuan et al., 2006).

DEP was demonstrated unequivocally to affect plant growth by affecting seed germination, root growth, and shoot development (Herring and Bering, 1988; Saarma et al., 2003; Xuan et al., 2006). However, details are lacking on whether growth retardation effects and interference with any biochemical processes are mediated through production of reactive oxygen species (ROS). If

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doi:10.1016/j.aquatox.2011.10.003

so, how plant antioxidative systems respond to DEP-stress and at what level antioxidant mechanisms are triggered in the plant tissues. Greater duckweed (*Spirodela polyrhiza*) was employed as a target plant in this study because it is very sensitive to various pollutants (Appenroth et al., 2010) and easy to culture and handle in laboratory. Studies were conducted to investigate the biochemical changes occurring in toxic and adverse effects of DEP in greater duckweed. In view of the oxidative stress caused by DEP, antioxidative parameters were the focus of the current studies.

2. Materials and methods

2.1. Plant materials and diethyl phthalate treatment

S. polyrhiza (L). Schleiden, clone DR (geographic isolate) plants were collected from a local stream at Tainan city in 2009. Axenic plants were cultured under control conditions as previously described (Cheng, 2011). Ten days (10 ± 2 days) before experiments, plants were further acclimated to modified SH growth medium (without 1% sucrose) (Schenk and Hildebrandt, 1972; precultivation). Experimental cultures were started by inoculation of 45 healthy colonies with 3 fronds from pre-cultivation cultures. These 10–12-day-old plants from pre-cultivation were treated with diethyl phthalate. For concentration-dependent experiments, DEP was given at concentrations 0 (control), 0.25, 0.5, 1.0 and 2.0 mM and plants were harvested 7 days after treatment. For duration-dependent experiments, plants were cultured in SH medium alone or treated with 1 mM DEP and were harvested at 0 (control), 1, 2, 4, and 7 days after treatment. Throughout the experiments, plant cultures were maintained in the medium at 25 ± 1 °C, under a 16:8 (light/dark) photoperiod with cool white fluorescent tubes (Philips 18 W, TLD, Thailand) at irradiance of 70 $\mu E\, m^{-2}\, s^{-1}$.

2.2. Growth parameters

Greater duckweed growth was determined by measuring frond number (FN) and fresh weight/dry weight ratio (FW/DW). Plant growth was monitored for 7 days by counting the frond numbers at the start of the experiments ($t = 0$), and 2, 4, and 7 days after the start of the exposure. All visible fronds were counted. Relative growth rate (RGR) was calculated from the following equation with the measured parameter x (FNt) and the start of the test (FNt_0) for each replicate separately: $\mathrm{RGR} = (\ln \mathrm{FN}t_1 - \ln \mathrm{FN}t_0)/(t_1 - t_0)$ (Radić et al., 2011). To measure plant biomass, greater duckweed plants were surface-dried with paper towels and the fresh weight was determined. To measure dry weight, plant tissues were dried at 80 °C for >24 h up to constant weight. Fresh to dry weight ratio (FW/DW) was calculated according to: fresh weight (mg)/dry weight (mg).

2.3. Determination of photosynthetic pigments

A 0.05 g of *Spirodela* frond was homogenized on ice with a Polytron homogenizer (Art-Miccra D-8, Mullhelm, Germany) in 2 ml of cold 80% acetone. The homogenate was centrifuged at $10{,}000 \times g$ (Hettich Centrifuge Universal 16R, Tuttlingen, Germany) at 2 °C for 5 min and the absorbance of the supernatant was measured at 470, 645, and 663 nm using a UV–vis spectrophotometer (Unicam Helios β, Cambridge, United Kingdom). The chlorophyll *a*, *b*, *a*/*b* and carotenoid concentrations were calculated as described by Lichtenthaler (1987).

2.4. Estimation of ROS

The level of $O_2^{\bullet -}$ was assayed spectrophotometrically by measuring the reduction of exogenously supplied nitroblue tetrazolium (NBT) according to Doke (1983). Five-plants were immersed in 1.5 ml of mixture containing 0.01 M sodium phosphate buffer pH 7.8, 0.05% NBT and 10 mM NaN_3. After 60 min of incubation, 1.0 ml of the reaction solution was transferred into a test tube and heated at 85 °C for 15 min. The solution was then cooled and its absorbance at 580 nm was measured. Reducing activity of NBT was expressed as the increase in A_{580} per hour per gram dry weight (DW). H_2O_2 content was measured according to Velikova et al. (2000) and Sumithra et al. (2006). Fifty milligrams of tissue was homogenized with a mortar and pestle under liquid nitrogen conditions in cold 0.1% TCA and the homogenate was centrifuged at $10{,}000 \times g$ at 2 °C for 15 min. The reaction mixture contained 0.5 ml supernatant of frond extract, 0.5 ml potassium phosphate buffer (10 mM, pH 7.0) and 1 ml KI (1 M). The reaction mix was allowed to stand in the dark for 1 h and the absorbance was recorded at 390 nm. The amount of hydrogen peroxide was calculated using a standard curve that was prepared with known concentrations of H_2O_2.

2.5. Extraction and estimation of malondialdehyde, ascorbic acid, and total thiols

The level of lipid peroxidation was estimated indirectly *in vitro* by the formation of malondialdehyde (MDA) (the main aldehyde produced) that reacts with thiobarbituric acid reactive substance (TBARS) as described by Ortega-Villasante et al. (2005). Plant tissue samples (0.1 g) were homogenized with 2 ml of 80% (v/v) ethanol (Hodges et al., 1999), followed by centrifugation at $10{,}000 \times g$ at 2 °C for 10 min. A 1-ml aliquot of plant extract was added to a test tube with 1 ml of 2-thiobarbiyuric acid (TBA) solution consisting of 20.0% (w/v) trichloroacetic acid (TCA), 0.65% TBA and 0.01% butylated hydroxytoluene (BHT). Samples were then mixed vigorously, heated at 95 °C in water bath for 25 min, cooled, and centrifuged at $1630 \times g$ for 3 min. Absorbance of the supernatant was measured at 532 nm, and the value of nonspecific turbidity at 600 nm was subtracted. MDA equivalents were calculated using extinction coefficient of 155 $mM^{-1}\, cm^{-1}$ (Heath and Packer, 1968).

Ascorbic acid content was measured according to Tewari et al. (2006) and Law et al. (1983) with modifications. Plant material (0.05 g) was homogenized on ice with a Polytron homogenizer in 2 ml of cold 10% (w/v) TCA. The homogenate was centrifuged for 5 min at $10{,}000 \times g$ at 2 °C and supernatant was collected. The assay is based on the reduction of Fe^{3+} to Fe^{2+} by ascorbic acid (ASA) and subsequently the formation of Fe^{2+} and bipyridyl complex (giving a pink color) that absorbs at 525 nm. The reaction mixture contained 0.2 ml of the supernatant, 0.2 ml sodium phosphate buffer (150 mM, pH 7.4), 0.2 ml of distilled water, 0.4 ml 10% TCA, 0.4 ml 44% phosphoric acid, 0.4 ml 4% bipyridyl (in 70% ethanol), and 0.2 ml $FeCl_3$. The mixtures were incubated in a water bath for 1 h at 37 °C. After incubation, the absorbance was recorded at 525 nm and the amount of ASA was calculated using a standard curve that was prepared with known concentrations of ascorbic acid. Total ascorbate (ASC) was measured after reducing dehydroascorbate (DHA) to ASA by dithiothreitol (DTT) in the supernatant. The reduction mixture contained 0.2 ml of the supernatant, 0.2 ml sodium phosphate buffer (150 mM, pH 7.4), and 0.1 ml of DTT (10 mM). The mixtures were incubated in an incubator for 15 min at 25 °C. After incubation, total ascorbate (ASC) was measured as ASA. DHA content was calculated from the difference between ASC and ASA. The levels of ASA and DHA were expressed as $\mu M\, g^{-1}$ of fresh weight.

Total thiols were extracted and measured according to Malec et al. (2010) and Ellman (1959) with modifications. Plant material (0.05 g) was homogenized with a mortar and pestle under liquid nitrogen in a 1 ml of cold Tris–HCl buffer (100 mM, pH 7.8). The homogenate was centrifuged for 25 min at $15{,}000 \times g$ at 2 °C (centrifugation I) and supernatant was collected. The pellet was re-extracted with 1 ml of cold Tris–HCl buffer and centrifuged