RESEARCH ARTICLE

Rape (*Brassica chinensis* L.) seed germination, seedling growth, and physiology in soil polluted with di-*n*-butyl phthalate and bis(2-ethylhexyl) phthalate

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Abstract Phthalic acid esters (PAEs) pollution in agricultural soils caused by widely employed plastic products is becoming more and more widespread in China. PAEs polluted soil can lead to phytotoxicity in higher plants and potential health risks to human being. We evaluated the individual toxicity of di-*n*-butyl phthalate (DnBP) and bis (2-ethylhexyl) phthalate (DEHP), two representative PAEs, to sown rape (*Brassica chinensis* L.) seeds within 72 h (as germination stage) and seedlings after germination for 14 days by monitoring responses and trends of different biological parameters. No significant effects of six concentrations of PAE ranging from 0 (not treated/NT) to 500 mg kg^{-1} on germination rate in soil were observed. However, root length, shoot length, and biomass (fresh weight) were inhibited by both pollutants (except root length and biomass under DEHP). Stimulatory effects of both target pollutants on malondialdehyde (MDA) content, superoxide dismutase (SODase) activity, ascorbate peroxidase (APXase) content, and polyphenoloxidase (PPOase) activity in shoots and roots (SODase activity in shoots excluded) were in the same trend with the promotion of proline (Pro) but differed with acetylcholinesterase activity (except in shoots under DnBP) for analyzed samples treated for 72 h and 14 days. Responses of representative storage compounds free amino acids (FAA) and total soluble sugar (TSS) under both PAEs were raised. Sensitivity of APXase and Pro in roots demonstrates their possibility in estimation of PAE phytotoxicity and the higher toxicity of DnBP, which has also been approved by the morphological photos of seedlings at day 14. Higher sensitivity of the roots was also observed. The recommended soil allowable concentration is 5 mg $DnBP kg^{-1}$ soil for the development of rape. We still need to know the phytotoxicity of DEHP at whole seedling stage for both the growing and development; on the other hand, soil criteria for PAE compounds are urgently required in China.

Keywords Phytotoxicity · Oxidative stress · PAEs · Rape (*Brassica rapa*) · Seed germination · Seedling growth

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Background, aims, and scope

Phthalic acid ester (PAE) compounds are the most widely used plasticizers worldwide, also well-known as vital endocrine-disrupting chemicals (Chen et al. 2008; Wagner and Oehlmann 2009). There have been ubiquitous contaminations in every environmental matrix because of their weak binding forces with other components of environmental compartments (Blair et al. 2009). Di-*n*-butyl phthalate (DnBP) and bis(2-ethylhexyl) phthalate (DEHP), two representative PAEs in soils and sediments of China (Tan 1995; Wang et al. 2006; Liu et al. 2009b), are causing increasing concern because of their harmful effects on urease, phosphatase, catalase, microorganisms, animals, and the microbial community in contaminated soils (Chen et al. 2004;

Gao and Chen 2008; Xie et al. 2009; Gao 2010). Phytotoxicity tests using higher plants are frequently employed for pollutants but there have been few such studies on PAEs. Investigations have shown that DnBP and DEHP in soil can decrease the vitamin C content in the fruit of peppers (Capsicum spp.) (An et al. 1999; Yin et al. 2002); significant accumulation and differences in expression of six protein spots in the leaf tissue of Chinese cabbage (Brassica rapa var. chinensis) have also been observed (Liao et al. 2009).

In agricultural soils, the main anthropogenic sources of PAEs are atmospheric from paint spraying, plastic garbage incineration, agricultural film plasticizers volatilization, industrial dusts sedimentation, sewage sludge and biosolids, chemical fertilizers, organic fertilizers and pesticides application, and rainwater leaching of piles of plastic waste (Li et al. 2010; Guo and Kannan 2012). Soil concentrations of DnBP and DEHP have been reported to range from 2.75 to 29.37 and from 1.15 to 7.99 $mg\,kg^{-1}$, respectively (Xu et al. 2008). Concentrations of DnBP and DEHP in vegetables sold in Guangzhou, South China are around 4.72 and 4.98 $mg\,kg^{-1}$ respectively, and in the Pearl River Delta, it is between 0.07 and 11.22 $mg\,kg^{-1}$ for the sum of DnBP and DEHP (Cai et al. 2003; Zeng et al. 2005, 2006). Six PAE compounds have been nominated by US Environmental Protection Agency (US EPA) as priority pollutants, and DnBP has been ranked by the European Union as a priority pollutant. PAEs may induce cancer and gene mutations after long-term contact at low concentrations and may be transferred from contaminated soils and crops to the human food chain, leading to considerable public concern (Hu et al. 2007).

The germination and prime seedling stages are considered crucial for the subsequent vegetative and reproductive growth of higher plants. They have developed an efficient anti-oxidant defense system that helps them to survive under adverse environmental conditions (Mishra et al. 1993). In plants, malondialdehyde (MDA) formation describes the oxidative stress caused by lipid peroxidation (Dionisio-Sese and Tobita 1998); superoxide dismutase (SODase) is the most valuable compound in the protective system of higher plants to survive oxidative stress (Asada and Takahashi 1987; Schoner and Krause 1990), and this constituent has long been used in the assessment of environmental toxicity (Mishra et al. 1993); ascorbate peroxidase (APXase) is one of the two major scavengers that control of excessive levels of H_2O_2 (Wang et al. 2010); polyphenoloxidase (PPOase) could be important for the induced defense of plants (Constabel and Ryan 1998); acetylcholinesterase (AChEase) alternation could indicate the contamination of environment (Ferreira et al. 2006); free proline (Pro) content could also be considered a biomarker of organic pollution (Qu et al. 2006). Short-term effects of pollutants on initial growth (germination, root elongation, shoot elongation, and biomass), anti-oxidase activity, critical amino acids, such as total amino acid (free amino acids (FAA); more commonly utilized in the demonstration of crop quality) and storage compounds, such as total soluble sugar (TSS) have been investigated in representative model crops for the assessment of target pollutants. Subchronic toxicity test were also conducted to check the relatively longer exposure damage of target pollutants and the reliability of sensitive indicators. Rape (B. rapa) has been selected following the recommendation of Organization for Economic Co-operation and Development (1984) and US EPA (Fletcher et al. 1985) for the separate phytotoxicity testing of DnBP and DEHP.

In the present study, prediction of the potential toxicity of DnBP and DEHP on rape seedlings (at 3 and 14 days after sowing) was determined using a range of physiological tests, and the recommended allowable soil concentrations were also proposed for the pollutant with higher toxicity.

Materials and methods

Chemicals and reagents

DnBP (99.1 %) and DEHP (99.6 %) were obtained from AccuStandard®, Inc., New Haven, CT. Nitro blue tetrazolium (NBT), ascorbic acid (AsA), trichloroacetic acid (TCA), thiobarbituric acid (TBA), Pro, alanine, sulfosalicylic acid, ninhydrin, catechol, anthrone, polyvinylpyrrolidone (PVP), monosodium orthophosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), hydrogen peroxide (H_2O_2), o-methoxyphenol, riboflavin, EDTA-Na$_2$, sodium hydroxide, glacial acetic acid, and acetone used in the tests were all analytical reagents purchased from the National Pharmaceutical Group Chemical Reagent Co., Ltd., Shanghai. Catalog number A204 for AChEase assay kits were purchased from Jiancheng Bioengineering Institute, Nanjing, China.

Soil preparation

The test soil consisted of the surface layer (top 20 cm of the soil profile) of an uncontaminated area at Qixia, Nanjing, China, and is classified as an Alfisol (soil with more clayey and fine particulates, brownish reddish, and more calcium) according to the US Department of Agriculture (US DA 2011) classification system. The soil pH was 7.4, with 1.67 % clay, 6.22 % silt, 2.11 % sand, 14.6 % organic matter, and available nitrogen, phosphorus, and potassium concentrations of 96.8, 14.4, and 102.8 %, respectively. Soil was passed through a 2-mm sieve before use and the background concentrations of the two target pollutants were

determined to be 142.6±4.19 μg DnBP and 194.5±1.09 μg DEHP kg^{-1} soil (oven dry basis).

For acute toxicity test, batches of soil (250 g×4) were adjusted to concentrations of DnBP or DEHP of 0, 1, 5, 20, 100, and 500 mg kg^{-1} soil by spraying with stock solutions in acetone (5 ml). For subchronic toxicity test, pots of soil (2.5 kg×4) were adjusted to concentrations of DnBP or DEHP of 0, 1, 5, 20, 100, and 500 mg kg^{-1} soil by spraying with stock solutions in acetone (50 ml). The same volumes of pure acetone without PAE pollutants were sprayed on every not treated (NT) soil sample in each toxicity test group. After evaporation of acetone, the soils were mixed thoroughly and the soil moisture was amended to 70–75 % (60 % of water holding capacity) before use.

Plant inhibition experiment

Seeds of rape (*B. chinensis* L.) recommended by the Organization for Economic Co-operation and Development (1984) as a test species in terrestrial environmental assessment were obtained from the Chinese Academy of Agricultural Sciences. Seeds were stored in a refrigerator at 4 °C before use. Seeds were surface sterilized by immersion in 10 % sodium hypochlorite solution for 10 min (US EPA 1996), rinsed three times with deionized water, soaked in deionized water for 2 h, and finally sown in the prepared soils. Plastic equipment was avoided throughout the procedure to eliminate PAE background contamination. All the glass Petri dishes were washed and baked in an oven at 400 °C before use (Ma et al. 2012).

For acute toxicity test, 250 g of test soil spiked with different concentrations of DnBP or DEHP were placed in 150×20 mm glass Petri dishes. Four replicates of each treatment were set up and 50 pretreated soaking seeds of uniform size were sown with equal spacing in each Petri dish. The dishes were covered, sealed with tape and placed in the dark in a growth chamber at 25±1 °C for 72 h before the germination test was halted. The germination status of each treatment was checked, seedling root and shoot lengths were measured, and the biomass in each dish was determined by fresh weight (FW). Root length was defined as the length from root tip to root radicle. Seeds were considered to have germinated when the root length was >5 mm (Wang et al. 2001), and subchronic toxicity test, 2.5 kg of test soil spiked with different concentrations of DnBP or DEHP were placed in 25×10 cm straw pressing pots. Four replicates of each treatment were set up and 25 germinated seedlings of uniform height were transplanted with equal spacing in pots and cultivated under the same chamber condition as before. After 11 days, root and shoot were also harvested after photographing for the analysis of selected sensitive parameters.

Determination of enzymes and critical compounds in plant defense mechanism

All analyses were performed on seedling of shoots and roots of days 3 and 14 separately. Excess induced oxidative damage was estimated by measuring MDA levels. About 0.5 g of fresh sample was homogenized in 5 ml of 0.1 % (*w/v*) TCA solution. After centrifugation (15 min, 6,000 rpm), 2 ml of the supernatant was added to 2 ml 0.5 % TBA in 20 % TCA and heated in a boiling water bath for 30 min. After fast cooling on ice the mixture was centrifuged at 6,000 rpm for 5 min. The absorbance of the supernatant at 532 and 600 nm was read to calculate the MDA content (Amor et al. 2005).

Fresh samples of about 0.5 g were ground in a mortar to extract with ice-cold 50 mM potassium phosphate-buffered saline (PBS; pH 7.8). The homogenates were centrifuged at 6,000 rpm at 4 °C for 20 min and the supernatant was collected for SODase assay, following the method of Giannopolitis and Ries (1977) by measuring its ability to inhibit the photochemical reduction of NBT. One unit of SODase activity is defined as the amount of enzyme inhibiting 50 % of the initial reduction of NBT under light. The enzymatic activity was expressed as units (U) per milligram FW.

The same enzyme extraction method was used as for SODase and APXase assay, except for pH condition of PBS buffer (pH 7.0), was performed using the method of Koricheva et al. (1997) in which the 3 ml of reaction mixture consisted of 50 mM PBS (pH 7.0), 0.3 mM AsA, 0.06 mM H$_2$O$_2$, 0.1 mM EDTA, and 0.1 ml enzyme (sample). The reaction was initiated by the addition of H$_2$O$_2$ and the decrease in absorbance at 290 nm within 30 s was measured with a UV spectrophotometer.

PPOases are enzymes that catalyze the O$_2$-dependent oxidation of monophenols or o-diphenols. Fresh plant material was ground in a mortar with ice-cold 0.1 M PBS buffer (pH 7.8) containing 1 % solid PVP. After centrifugation at 15,000×*g* for 15 min at 4 °C, the supernatant was used for the PPOase assays. The reaction was initiated by the addition of 30 μM of catechol. PPOase activity was measured by the disappearance of catechol spectrophotometrically at 525 nm every 30 s for 5 min at 25 °C.

Both analyses were performed on seedling shoots and roots. The instructions for the AChEase assay kit were followed (Nwosu et al. 1992) based on the principle that acetyl cholinesterase can hydrolyze acetylcholine to acetic acid and choline and produce yellow trinitrobenzene in the presence of thiol reagent. AChEase activity was quantified by colorimetry by production of a yellow compound at 412 nm. Pro content was determined by the method of Li et al. (1995) with modification to expand the test weight and volume in proportion.

A fresh sample of about 0.5 g was homogenized with 5 ml of 3 % sulphosalicylic acid before incubation at 100 °C for 10 min. Then 2 ml of the supernatant was added to a mixture of 2 ml of glacial acetic and 2 ml of 2.5 % (w/v) acidic ninhydrin before boiling at 100 °C for a further 25 min. After the solution was cooled, 5 ml of toluene was added for extraction, prior to reading the absorbance of the upper red layer (toluene) at 520 nm and calculating the content of Pro (in milligrams per gram FW).

Determination of storage compounds (FAA and TSS)

Both FAA and TSS were analyzed on the whole harvested seedlings. FAA content was estimated following the method of Shukla et al. (2002) with minor modifications to expand the test weight and volume in proportion. Fifty milligrams of ice-water bath ground fresh sample was stirred in 5 ml 10 % acetic acid (v/v) solution overnight and distilled water was added to adjust the total volume to 50 ml. After filtration, 2 ml of the extract, 3 ml 1 % ninhydrin (in 0.5 M citrate buffer, pH 5.5) and 0.1 ml 1 % ascorbic acid were added and mixed before heating in a boiling water bath for 15 min. After cooling, FAA content was determined by the absorbance at 570 nm. Alanine was used as the FAA standard.

TSS content was estimated according to Moya et al. (1993) with slight modification. Eighty milliliters of 80 % ethanol was added to 1.0 g of fresh sample and extracted in a water bath at 80 °C for 30 min. The extraction was repeated twice and the mixture was centrifuged at $12,000 \times g$ for 10 min. All the supernatants were combined and made up to a final volume of 25 ml with 80 % ethanol. Ten-milliliter extract was steamed to dry over a boiling water bath; 0.5 ml of H_2SO_4-anthrone reagent was added and the TSS content was determined by spectrophotometry at 620 nm. Glucose was used as the standard for quantification.

Data analysis

The fifty percent inhibitory concentration (IC_{50}) values of the different parameters were calculated using the SPSS v.14.0 software package (Sundarammal et al. 2012). All results are presented as mean values±standard error (SE) of four replicates with 50 seeds each (for both growth and biochemical assays). One-way analysis of variance was used to assess significant differences with a p value less than 0.05.

Results

Effects of DnBP and DEHP on germination and growth

Little effect of either pollutant was observed on the germination rate of rape after incubation for 72 h, even

at the highest concentration studied (500 $mgkg^{-1}$ soil) (Table 1). In contrast, inhibition of root elongation, shoot elongation, and biomass in seedlings exposed to DnBP were more apparent (but not root elongation and biomass when exposed to DEHP). Among all the estimated growth parameters, root elongation may be considered the most sensitive physical parameter with the lowest IC_{50} under exposure to DnBP, with a value of <500 $mgkg^{-1}$ soil, and with less sensitivity to DEHP (<1,500 $mgkg^{-1}$ soil).

Effects of DnBP and DEHP in acute toxicity test

As shown in Fig. 1a, compared with the NT, all doses of both pollutants (and especially, >5 $mgkg^{-1}$), showed significant positive effects on the MDA content of roots but there were only small negative effects on the shoots which became significant at 100 $mgkg^{-1}$ under DEHP. SODase (Fig. 1b) showed similar trends in the roots and inhibition in the shoots under DEHP treatment. The APXase activity responses (Fig. 1c) in both roots and shoots were positive except at 1 $mgkg^{-1}$ DEHP and 5 $mgkg^{-1}$ DnBP in the shoots. Both DnBP and DEHP were associated with significant increases in PPOase (Fig. 1d) in both roots and shoots except at 20 $mgkg^{-1}$, but exposure to DnBP led to more PPOase activity than DEHP when the concentrations of the target pollutants were relatively high (>100 $mgkg^{-1}$ soil). Figure 1e shows that AChEase activity was lowered after DnBP treatment in both shoots and roots. However, DEHP appeared to stimulate activity in the shoots and inhibit AChEase in the roots. Pro accumulated in the shoots except at 1 and 5 $mgkg^{-1}$ DnBP (Fig. 1f). There was a slight tendency for Pro accumulation to decline in roots at higher DnBP concentrations. The contents of FAA and TSS were all significantly higher with exposure to both target pollutants except for only a slight increase FAA and TSS in roots (Fig. 2).

Effects of DnBP and DEHP in sub-chronic toxicity test

Photos of rape seedlings under treatment of 0, 5, and 10 mg kg^{-1} DnBP and DEHP for 14 days reveal the severe exterior change of rape seedlings under the treatment of DnBP (at 10 $mgkg^{-1}$ so that the withered seedlings could not be harvested) and the stable look of seedlings under DEHP (Fig. 3). APXase and Pro were selected for further verifying of their sensitivity to DnBP and DEHP (Fig. 4), and compared with the NT, samples under the treatment of 5 and 10 $mgkg^{-1}$ of both pollutants showed significant positive increase for APXase and Pro. Especially at 10 $mgkg^{-1}$, seedlings of rapes were so withered that the seedlings were hard to collect and the two sensitive parameters could not be analyzed.

Table 1 Dose-dependence linear regression fitting of two PAE compounds on growth of rape seedlings in test soil

Compound	End point	Regression parameter				IC$_{50}$ (mgkg^{-1})
		a	b	R	P value	
DnBP	Germination rate	−0.0026	96.24	0.5002	0.1372	38,621
	Shoot elongation	−0.0003	0.0533	0.9973	0.0227	1,489
	Root elongation	−0.0005	0.2673	0.9412	0.0389	465.4
	Biomass	−0.0001	0.1323	0.9467	0.0416	3,677
DEHP	Germination rate	−0.0016	96.25	0.5398	0.1525	5,9843
	Shoot elongation	−0.0002	0.0973	0.8866	0.0489	2,014
	Root elongation	0.0004	−0.0705	0.8973	0.0462	1,426
	Biomass	0.0001	0.1233	0.9982	0.0337	3,767

Each point is the mean of 200 seeds. Significant differences are defined as $p<0.05$

Discussion

Effects on germination, shoot elongation, root elongation, and biomass at 72 h

The virtual absence of any effect on germination may be related to development of the dicotyledons which derive most of their nutrition from their own hypertrophic cotyledons rather than from the soil during germination (Shu et al. 1999). Consequently, no significant differences were found for test rape seeds in germination percentage even when exposed to a range of concentrations of the two target PAE pollutants. Stimulatory effects on root elongation and biomass under DEHP and inhibition of DnBP to different initial growth parameter supports the conclusion that DnBP has higher phytotoxicity because the plant cannot develop fully without favorable growth conditions during the initial stages of growth. In addition, the lower IC$_{50}$ of DnBP to root elongation indicates a higher toxicity to rape seedlings during the initial growth stages. However, rape produces a family of plant hormone compounds named brassinosteroids which play an important regulatory role in root elongation, shoot elongation, and the development of other organs (Yokota 1997). Also as a kind of ester, DEHP may have put on similar positive effects on roots and biomass of test plant, which may be viewed as mimicking the effects of plant hormones. In the roots the initial increase in MDA was followed by a decline at the highest dose/s, which also occurred in response to SODase. This may indicate some weakness in the antioxidant defense system.

Effects on antioxidase and critical compounds at 72 h

Inhibition of MDA content when exposed to DnBP at concentrations of >5 mgkg^{-1} indicates both the sensitivity of
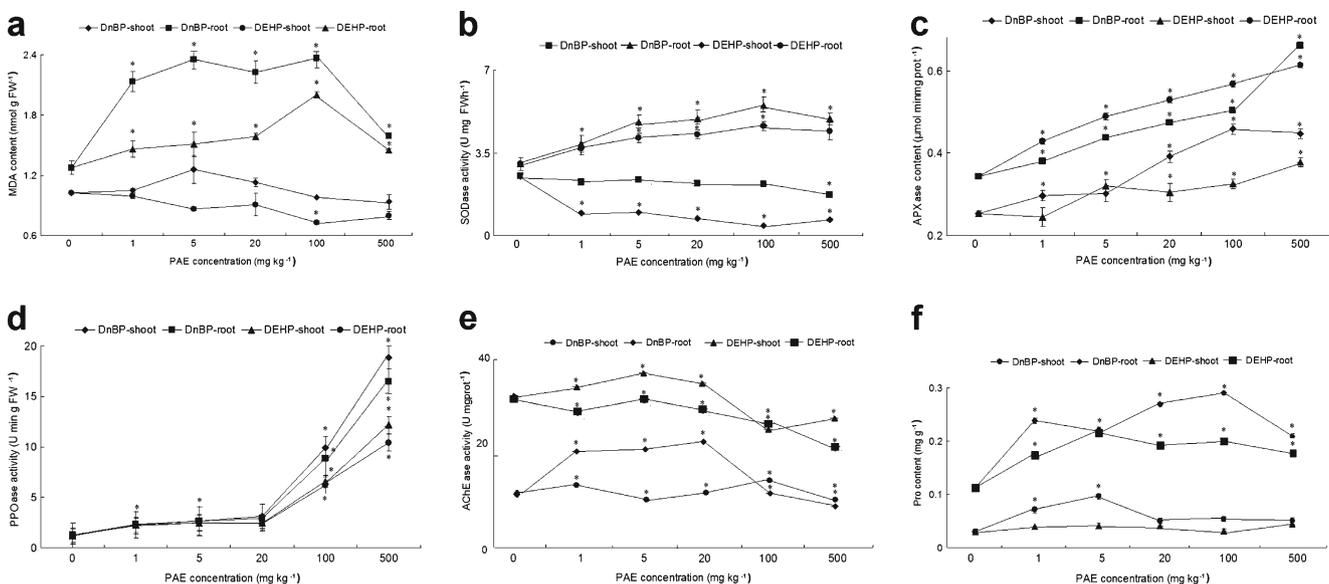

Fig. 1 MDA content, SODase activity, APXase content, PPOase activity, AChEase activity, and Pro content of rape shoots and roots after germination for 72 h under 0 (*NT*), 1, 5, 20, 100, and 500 mgkg^{-1} of DnBP and DEHP. **a** MDA content, **b** SODase activity, **c** APXase content, **d** PPOase activity, **e** AChEase activity, and **f** Pro content. Each point is the mean of four replicates from four Petri dishes. *Bars* are SE and *asterisks* denote significant differences from NT
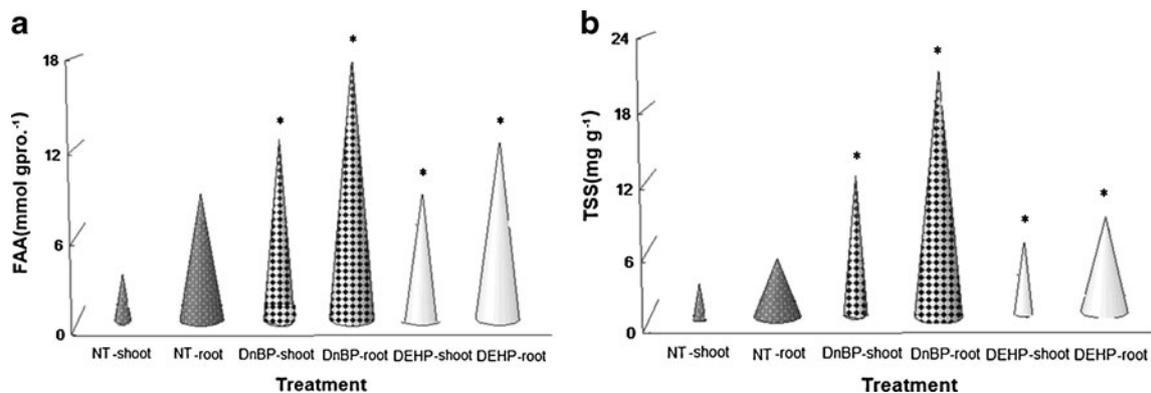
Fig. 2 Mean FAA and TSS contents of rape shoots and roots under treatment of 500 mgkg^{-1} DnBP and DEHP after germination for 72 h. **a** FAA and **b** TSS contents. Each point is the mean of four replicates

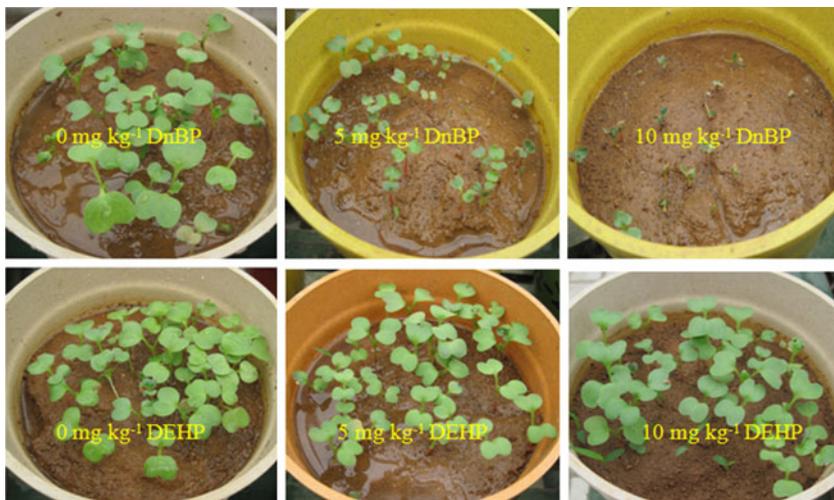from four Petri dishes. *Bars* are SE and *asterisks* denote significant differences from NT

the roots and the higher toxicity of DnBP compared with DEHP. Antioxidant enzymes and certain inducible metabolites in oxidative stress play a vital role in the adaption and ultimate survival of plants under stress conditions. Generally, the formation of MDA in plants reflects the oxidative stress as the result of lipid peroxidation. Usually, an increase in MDA content exhibits protective mechanisms and the consumption of MDA indicates slight damage to plant organs. An increase in MDA in the root indicates their sensitivity compared with the shoots and is also due to direct contact between the roots and pollutants in the soil. MDA in the shoots may require longer exposure times before it responds to the PAE.

Antioxidant status is an indicator of physiological equilibrium and is critically important for understanding the responses of organisms to contaminated environments. In particular, increases in SODase in plant roots at concentrations of DnBP at >5 mgkg^{-1} are important. SODase is extremely important antioxidant enzymes in the understanding of stress response mechanisms in cells in connection with the production of reactive oxygen species. A decline in

SODase activity in the aboveground parts indicates a higher adaptability in adverse conditions. Anti-oxidation and detoxification are the first line of defense to environmental stress (Wilhelm 2007), but increasing pollutant concentrations may lead to the breakdown of these processes and the stimulation of other relevant responses (Zhou et al. 2010). Reduced levels of SODase activity indicate a loss of protective capacity against cellular superoxide toxicity which might be caused by consumption of SODase over the short term and result in a transient shortage of SODase.

The trend in APXase activity represents the NT of excessive levels of H_2O_2 in the test plants because APXase is one of the two major scavengers of H_2O_2. APXase is present throughout the cell and has high substrate affinity when ascorbate acts as a reductant. The increase in APXase activity in the roots does not agree with the other reports on heavy metal stress in which leaves have been shown to produce more APXase than roots (Zhang et al. 2008; Zhang et al. 2010) but is in accord with our earlier conclusion that roots were more sensitive than shoots. Moreover, during germination APXase plays an important role in

Fig. 3 Photos of rape seedlings under treatment of 0, 5, and 10 mgkg^{-1} DnBP and DEHP for 14 days. Each photo stands for the general status of four replicate pots
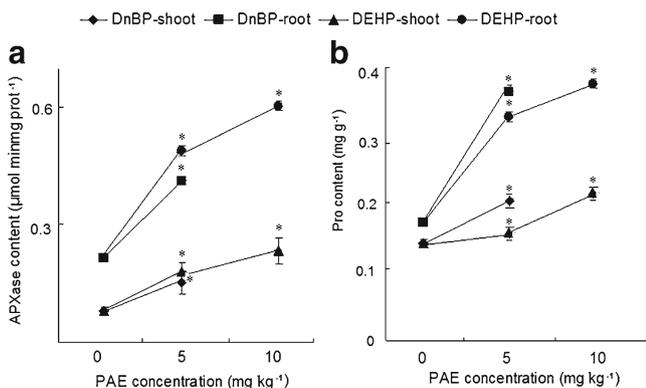
Fig. 4 APXase content and Pro content of rape seedling shoots and roots after germination for 14 days under 0 (NT), 5, and 10 mgkg^{-1} of DnBP and DEHP. **a** APXase and **b** Pro contents. Each point is the mean of four replicates from four pots. *Bars* are SE and *asterisks* denote significant differences from NT. The data of rape treated with DnBP under 10 mgkg^{-1} are omitted because the seedling withered at day 14, which is proved by photos in Fig. 3

avoidance of H_2O_2 toxicity and the roots may possess a higher capacity to scavenge excess H_2O_2 during contact with contaminated soil. The sensitive reaction of APXase in root of the rape, under the treatment of 1 mgkg^{-1} of DnBP or DEHP, entitles its recommendation as a biological indicator.

It has been over a century since the first report on catechol oxides as PPOases in plants (Bertrand 1896), but their specific functions have not been well explained until recently. However, their important roles as plant protection products (Constabel and Ryan 1998) have been widely accepted. Plants can accelerate PPOase activity as a defense against both biotic and abiotic stresses (Kwak et al. 1996). PPOases are documented to be defense-related proteins in tomato plants submitted to either wounding and/or to methyl jasmonate (MeJa) vapour treatment (Constabel et al. 1995). In the present study the induction of PPOase in rape was especially high in the shoots compared with the roots, and this is not in agreement with the common assumptions that roots are more sensitive than shoots due to the higher content of PPOases in both chloroplasts and mitochondria.

Biomarkers represent changes from the molecular to the organism level related to toxic effects of and/or exposure to contaminants. Moreover, responses of biomarkers appear prior to large variation in population and community levels and this is why they are employed as predictors of toxicity. AChEase has been used as a biomarker to assess the impact of heavy metal pollution in animal studies (Tsangaris et al. 2007), and it has been extracted from different plant species to treat human disease (Ingkaninan et al. 2003; Ferreira et al. 2006). The trends in Fig. 1e show that AChEase activity in both roots and shoots of rape exposed to both target pollutants was inhibited, especially in the roots at all PAE concentrations tested. High levels of AChEase inhibitory

activity usually appear in contaminated environments (Ferreira et al. 2006), but during the germination period AChEase is also bound up with the germination of seeds. The supplementary role of AChEase in seed germination is promotion of photophilous plants and restriction of non-photophilous plants. Rape does not require light during germination, and a decline in AChEase in shoots and roots is consistent with the characteristic of rape, which indicated a lack of effect of both target pollutants on germination rate. The role of AChEase and its responses in plants growth need elucidating under contaminated environment.

The Pro content of plants was formerly considered to act as a response to climatic change (Treichel et al. 1984) and heavy metal contamination (Rai et al. 2004; Sharma et al. 2005). Effects of organic pollutants such as pesticides on the free Pro content in *Euonymus japonica* also suggested a substantially increased free Pro content after pesticide application for 10 days (Qu et al. 2006). The stimulation of Pro content under both pollutants (Fig. 1f) over all treatments is in accordance with earlier studies on plants under adverse conditions. Under normal conditions the free Pro content in plants is relatively low. However, when drought, cold or high salinity occurs, the content of free Pro can increase substantially. The accumulation of Pro can be attributed to an enhancement of Pro synthesis, an inhibition of Pro oxidation or a decline in total protein synthesis accompanying the inhibition of Pro synthesis (Wong 2000). Pro can protect plant cells from adverse conditions by maintaining the integrity of cell membranes and preventing water loss from the cells (Zhang et al. 2003). Lower accumulation of Pro in the shoots of rape under DEHP may indicate the lower toxicity of this PAE compound (Fig. 1f). Both APXase and Pro in root are recommended as the most sensitive bio-indicators of rape under treatment of both DnBP and DEHP for their significant reaction at 1 mgkg^{-1} pollution level.

Effects on storage compounds at 72 h

The stimulation of FAA content and TSS content agrees with earlier investigations. Low levels of the herbicide trifluralin induced the elevation of free amino acids in melon seedlings (Starratt and Lazarovits 1999). TSS was also induced under drought, salinity and low temperatures and was often accompanied by increases in both FAA and Pro (Ren et al. 2001; Xu et al. 2001; Liu et al. 2007). Roots are believed to be classic reserve organs but much higher concentrations of storage compounds were observed in rape shoots (Fig. 2). One possible explanation is the increase in biomass because the shoots developed more rapidly than the roots.

In addition to the parameters monitored in this experiment, the transfer of target pollutants to test plants, which

has so much to do with the physical characteristics of compounds, may also significantly affect the expression of their toxicity. The internal relationship between accumulation of PAE target pollutants in plants and their phytotoxicity in longer-term studies beyond the seedling stage still requires further study.

Effects on seedling growth and sensitive compounds at 14 days

Photos of rape seedlings under treatment of 0, 5, and 10 mg kg^{-1} DnBP and DEHP for 14 days indicated the higher toxicity of DnBP to rape at 5 mg kg^{-1}, which could also be proved by the reaction of two sensitive parameters that both APXase and Pro content were significantly increased. Seriously withering of leaf margins have also been recognized which indicated the serious damage caused by DnBP to rape seedlings. In other documents, the appearance of leaf withered means severe trauma of plant growth mainly caused by environmental abnormal. Strong environmental pressure from metal contamination, like cadmium, Cr^{6+}, could result in both withering of leaves, root stock rotted, and intensification of metal detoxification processes (Pielichowska and Wierzbicka 2004; Szarek-Łukaszewska et al. 2004; Han and Hu 2005). NaF-treated plants showed alteration like withered appearance of the leaf tip within 24 h, then tissues color change extended to leaf vein, and the appearance looked very like early frost injury, which absolutely suggest interior injury of plant (Fornasiero 2001). Chilling could increase the percentage of leaves that withered and the levels of H$_2$O$_2$, O$_2^{\cdot-}$, and MDA in cucumber leaves under low light (Liu et al. 2009a), which is similar with the appearance of rape seedling leaves in this experiment. However, the alternation caused by target DEHP was still acceptable for seedlings no matter judging by the leaves or by sensitive parameters. Soil allowable concentration threshold should be proposed to DnBP based on more investigated data of its ecotoxicity effects, especially in China, where the contamination of DnBP in soil is more and more frequently discovered.

Conclusions

DnBP appeared to show higher phytotoxicity than DEHP in our testing of the target plants. Higher sensitivity of the roots was also observed. Although neither of the target pollutants led to severe inhibition of seed germination, effects on initial growth parameters, especially of the roots, were observed. Antioxidant enzyme responses and elevation of Pro, FAA, and TSS at 72 h and photos at 14 days confirm this result, and 5 mg kg^{-1} can be recommended as the allowable concentration of DnBP in soil. APXase and Pro in the roots of

rape are recommended and proved as biological indicators because of their sensitivity when subjected to DnBP and DEHP. Other plant species recommended such as wheat with higher sensitivity should be included in further investigations of the phytotoxicity of PAE compounds to confirm the results using rape and refine the recommended allowable limits of soil PAEs.

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