**Carboxylesterase-involved metabolism of di-*n*-butyl phthalate in
pumpkin (*Cucurbita moschata*) seedlings**

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Abstract

Uptake and accumulation by plants is a significant pathway in the migration and transformation of phthalate esters (PAEs) in the environment. However, limited information is available on the mechanisms of PAE metabolism in plants. Here, we investigated the metabolism of di-*n*-butyl phthalate (DnBP), one of the most frequently detected PAEs, in pumpkin (*Cucurbita moschata*) seedlings via a series of hydroponic experiments with an initial concentration of 10 mg L⁻¹. DnBP hydrolysis occurred primarily in the root, and two of its metabolites, mono-*n*-butyl phthalate (MnBP) and phthalic acid (PA), were detected in all plant tissues. The MnBP concentration was an order of magnitude higher than that of PA in shoots, which indicated MnBP was more readily transported to the shoot than was PA because of the former's dual hydrophilic and lipophilic characteristics. More than 80% of MnBP and PA were located in the cell water-soluble component except that 96% of MnBP was distributed into the two solid cellular fractions (i.e., cell wall and organelles) at 96 h. A 13-20% and 29-54% increase of carboxylesterase (CXE) activity shown in time-dependent and concentration-dependent experiments, respectively, indicated the involvement of CXEs in plant metabolism of DnBP. The level of CXE activity in root subcellular fractions was in the order: the cell water-soluble component (88-94%) >> cell wall (3-7%) > cell organelles (3-4%), suggesting that the cell water-soluble component is the dominant locus of CXE activity and also the domain of CXE-catalyzed hydrolysis of DnBP. The addition of triphenyl phosphate, a CXE inhibitor, led to 43-56% inhibition of CXE activity and 16-25% increase of DnBP content, which demonstrated the involvement of CXEs in plant metabolism of DnBP. This study contributes to our understanding of enzymatic mechanisms of PAE transformation in plants.

Keywords: Di-*n*-butyl phthalate (DnBP); Carboxylesterases; Metabolism; Pumpkin (*Cucurbita moschata*); Subcellular distribution

Capsule: Hydrolysis of di-*n*-butyl phthalate takes place mainly in the cell water-soluble component and mono-*n*-butyl phthalate and phthalic acid are the primary metabolites.

1. Introduction

Phthalate esters (PAEs), one of the most common types of plastics additives, are commonly used in the manufacture of industrial, consumer and agricultural products. Approximately 6 million tons of PAEs are produced and consumed globally each year, and the consumption of PAEs in China was about 2.2 million tons in 2011, most of which was used in the production of plastic film (He et al., 2015; Niu et al., 2014; Wang et al., 2013a). The large-scale consumption and wide application of plastic products (eg., plastic film or polytunnels) has led to the detection of high levels of PAEs in various environmental matrices worldwide (Niu et al., 2014; Tran et al., 2015). Di-*n*-butyl phthalate

(DnBP), listed as a priority pollutant by both the United States Environmental Protection Agency (USEPA) and the European Union, is one of the most commonly detected PAEs in soils (Niu et al., 2014) or sediments (Sun et al., 2013). DnBP is also classified as an endocrine disruptive chemical (EDCs) due to its adverse effects on animal and human beings (Fennell et al., 2004; Gaspar et al., 2014). Therefore, this compound has attracted considerable attention from both the public and scientists.

The fate of environmental chemicals in plants is of great concern due to the important role of plants in biotransformation of xenobiotics, especially organic compounds (Bártíková et al., 2015; Bartrons et al., 2016; Wu et al., 2015). Much research has been conducted to investigate the phytotoxicity of PAE to crops (Zhang et al., 2015a; Zhang et al., 2015b; Zhao et al., 2016), as well as the potential for phytoremediating PAEs contamination (Cai et al., 2008; Zhao et al., 2015). Unfortunately, few studies have focused on the metabolism of PAEs in plants. Monoalkyl phthalate esters (MPEs) were found to be the primary metabolite of PAEs in soils and sediments due to degradation by microbes (Wang and Chi, 2012). The formation of corresponding MPEs from PAEs in edible plants has recently been demonstrated in both *in vivo* and *in vitro* experiments (Sun et al., 2015). However, detailed information about the metabolic pathway(s) of PAEs is not yet available. Moreover, knowledge of the translocation and distribution of PAE metabolites at the subcellular level, which might provide new insights into the metabolic process, is lacking. In addition, some toxicology tests have shown that metabolism of PAEs does not usually lead to detoxification of themselves. MnBP and/or its further metabolites may be responsible, at least in part, for the production of developmental toxic effects of DnBP (Ema et al., 1995). Likewise, the toxic effects of orally ingested DEHP are more likely to be due to the properties of the corresponding monoester rather than the intact DEHP (Ito et al., 2005). Therefore, understanding the fate of PAEs and their metabolites in plants will contribute to more proper risk assessments of PAE pollution in agriculture.

Metabolism of xenobiotics, especially that of pesticides, involves a three-phase process that has been well illustrated by Kvesitadze et al. (2009), Sandermann (1994), and Van Eerd et al. (2003). In phase I, an organic xenobiotic acquires hydrophilic functional groups through enzymatic reaction such as oxidation, reduction or hydrolysis, resulting in the formation of a more water-soluble, polar, and reactive intermediate. After phase I, the metabolite can conjugate with endogenous cell compounds (phase II), and then transfer into vacuoles or compartmentalize in cell wall (phase III). The hydrolysis of ester bonds of xenobiotics in plants, which is usually carried out by esterases, has been extensively reported (Cummins et al., 2001; Cummins and Edwards, 2004; Van Eerd et al., 2003). Carboxylesterases (CXEs, EC 3.1.1.1) are specific esterases that show hydrolyzing activity toward carboxylic esters (Haslam et al., 2001), and are involved in many functional roles in plants including detoxification of xenobiotics (Gershater and Edwards, 2007). PAEs, derived from the esterification of phthalic acid (PA) and two alcohol molecules, belong to the carboxylic esters. In light of the chemical structure of PAEs and previous research on their metabolites, it is likely that CXEs play a significant

role in the metabolism of PAEs in plants, especially in phase I hydrolysis. However, information about the importance of plant CXEs to PAE metabolism is limited, and little is known about CXE activity in the subcellular fractions of PAE-exposed plants, which might reflect the localization and mechanisms of the enzymes involved in PAE detoxification.

In this research, we investigated the metabolism of DnBP, as a representative PAE, in pumpkin (*Cucurbita moschata*) seedlings. The subcellular distribution of DnBP metabolites and CXE activity during the metabolic process was determined. To confirm the involvement of CXEs in the hydrolysis of DnBP, an inhibitor experiment was conducted. Pumpkin was chosen due to its frequent use in studies on the uptake and metabolism of xenobiotics (Lin et al., 2015; Yu et al., 2013).

2. Materials and methods

2.1. Chemicals

Standards of di-*n*-butyl phthalate (DnBP), mono-*n*-butyl phthalate (MnBP) and phthalic acid (PA) were purchased from AccuStandard (New Haven, USA), Aladdin Industrial Corporation (Shanghai, China) and Dr. Ehrenstorfer GmbH (Augsburg, Germany), respectively. The surrogate standard dibenzyl phthalate and internal standard benzyl benzoate for DnBP detection were supplied by Chem Service (West Chester, USA). The surrogate standards MnBP-*d*4 and PA-*d*4 were obtained from C/D/N Isotopes (Pointe-Claire, Canada) and Toronto Research Chemicals (Toronto, Canada), respectively. All organic solvents were GC or HPLC grade (CNW Technologies GmbH, Düsseldorf, Germany). The inhibitors used in the CXEs experiments was triphenyl phosphate (TPP), which was purchased from Macklin Biochemical (Shanghai, China). Detailed information about the chemicals and other reagents used in this study is shown in Supplementary Materials M1.

2.2. Plant preparation

Pumpkin (*Cucurbita moschata*) seeds were purchased from the Guangdong Academy of Agriculture Sciences (Guangzhou, China). After sterilization in 10% (v/v) NaClO solution, seeds were germinated on autoclaved moistened gauze for 2 d at 30 °C in the dark. The pumpkin seedlings were used for exposure experiment after growing in 1/4, 1/2 and full-strength Hoagland nutrient solutions (filter-sterilized) for 4, 6 and 3 d, respectively. Information on Hoagland solution is provided in Supplementary Materials M1. Before exposure, the pumpkin seedlings were immersed in autoclaved Milli-Q water for 24 h and then used in the subsequent hydroponic experiments (Zhan et al., 2010).

2.3. Greenhouse hydroponic experiments

The experimental design is shown in Fig. S1. Briefly, in the DnBP exposure group, six uniform pumpkin seedlings were transferred into one beaker with their roots immersed in 500 mL Hoagland

nutrient solution (pH 6.0) containing 10 mg L⁻¹ DnBP (added in 250 µL of methanol). According to Chapin et al. (1993), no adverse effect on plant root growth is observed when the concentration of methanol is less than 0.1% (v/v) in nutrient solution. The seedlings were fixed by a styrofoam board and the beakers were wrapped with aluminum foil to avoid photodegradation of DnBP. All glassware was covered with aluminum foil and sterilized by baking at 160 °C for at least 4 h before use, and the Hoagland solution was filter-sterilized (0.2 µm cellulose acetate filter, Sartorius Stedim Biotech, Germany) (Hou et al., 2012). The whole pumpkin seedlings were rinsed by autoclaved Milli-Q water before planting into the beakers. The gap between beaker and styrofoam board was sealed with parafilm. During the exposure period of 12, 24, 48, 72, or 96 h, seedlings were grown under controlled conditions (photoperiod 16 h light/8 h dark; day/night temperature 25/20 °C; relative humidity 60%) unless otherwise stated. One beaker acted as a exposure sample and six samples were conducted at each exposure duration. After exposure, three beakers were sampled and analyzed for DnBP and its metabolites, and the rest were used for subcellular fractionation.

Three control groups were conducted simultaneously with the exposure group. In the untreated group, which was used to monitor possible cross contamination. 250 µL of methanol was added to the solution without DnBP. The unplanted group, used to determine any abiotic DnBP loss over a time period of 96 h, contained the same amount of DnBP and methanol as did the exposure group but no plants. In the microbes and exudates group, pumpkin seedlings were grown in Hoagland solution without DnBP for 96 h, after which the plants were removed and the solution, containing microbes and root exudates from the seedlings, was spiked with 10 mg L⁻¹ DnBP and 250 µL of methanol and exposed for an additional 96 h.

2.4. Subcellular fractionation

Subcellular fractions of plant cells were obtained following the methods of previous studies (Gao et al., 2013; Ling et al., 2012; Wang et al., 2013b) with modifications. Fresh roots and leaves were homogenized in pre-cooled extraction buffer [50 mmol L⁻¹ 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 500 mmol L⁻¹ sucrose, 1.0 mmol L⁻¹ DL-Dithiothreitol (DTT), 5.0 mmol L⁻¹ ascorbic acid, 1.0% (w/v) polyvinylpyrrolidone cross-linked (PVPP), pH 7.5] with a chilled mortar and pestle. The homogenate was passed through a 60-µm sieve and the liquid was squeezed from the residue. The residue was washed with extraction buffer 3 times, and the liquid was centrifuged at 500 × g for 5 min to obtain a cell debris pellet. The residue and the cell debris pellet were combined and referred to as the cell wall fraction. Then the supernatant was centrifuged at 10,000 × g for 30 min to obtain the cell organelles fraction. The remaining supernatant was a cell water-soluble fraction. The extraction procedures were performed at 4 °C. More information is available in Supplementary Materials M1.

2.5. Analysis of DnBP and its metabolites in plant samples

After harvest, pumpkin roots and shoots were washed several times using Milli-Q water and then rinsed with methanol and cyclohexane, respectively, to remove DnBP adsorbed on the surface, followed by wiping with pretreated tissue paper (Dueck et al., 2003; Jiao et al., 2007; Zhan et al., 2010). DnBP was extracted from roots, shoots and corresponding cell fractions following a modified ultrasonication procedure of Ma et al. (2013) followed by a cleanup procedures using silica gel and alumina oxide column (Zeng et al., 2008). To determined the concentrations of MnBP and PA, the procedures of sample ultrasonic-assisted extraction (Sun et al., 2015) and cleanup using Bond Elut C18 cartridges were conducted. DnBP was measured by a gas chromatograph (Thermo-Ultra Trace GC-DSQ) equipped with mass spectroscopy detector with a quadrupole analyzer. Analyses of MnBP and PA were performed by a Thermo TSQ Quantum Ultra LC-MS/MS system. Detailed procedures of the extraction, cleanup, and analysis of DnBP and its metabolites as well as information about quality assurance/quality control are provided in Supplementary Materials M1.

2.6. Carboxylesterase activity in pumpkin seedlings

In time-dependent experiments, uniform pumpkin seedlings were transferred to 500 mL Hoagland solution (pH 6.0) with 10 mg L⁻¹ DnBP. After 12, 24, 48, 72, or 96 h of exposure, roots and leaves of pumpkin seedlings were sampled and carboxylesterase (CXE) activity was determined. In concentration-dependent experiments, pumpkin seedlings were exposed to Hoagland solution with various concentrations of DnBP (0, 1, 2, 4, 6, 8, and 10 mg L⁻¹). After 96 h of exposure, CXE activity in pumpkin roots and leaves was analyzed. Each treatment was conducted with three replicates.

DnBP metabolism in pumpkin as influenced by a CXE inhibitor was investigated. Pumpkin seedlings were transferred to 500 mL solution (pH 6.0) with 10 mg L⁻¹ DnBP and the CXE inhibitor triphenyl phosphate (TPP; 1 or 10 μM, in 250 μL acetone) for 72 h of exposure (Yamato et al., 2005). The same amount of acetone was added to the solution of control without TPP. Each treatment was conducted with three replicates.

Fresh tissue (0.2 g) was homogenized with 5 mL of phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1% (w/v) polyvinylpyrrolidone in an ice bath. Homogenate was then centrifuged at 15,000 × g for 15 min at 4 °C (Qiu et al., 2008). The supernatant was used for enzyme determination. CXE activity was determined by a modified colorimetric method of van Asperen (1962) and Yang et al. (2010). The determination methods for CXE activity are provided in Supplementary Materials M1.

2.7. Statistical analysis

All data were calculated using Microsoft Excel 2007. Every data point in the figures and table is an average value. The standard deviation (SD) obtained from replicate samples using SPSS Statistics 17.0

is shown in the figures as an error bar. Data were analyzed for significant differences from the control treatment or between treatments using one-way analysis of variance, **post ANOVA multiple comparison test** or *t*-test at the 95% or 99% confidence level.

3. Results and discussion

3.1. Metabolism of DnBP in pumpkin seedlings

To investigate the metabolism of DnBP, pumpkin seedlings were exposed to 10 mg L⁻¹ DnBP. **After 96 h of exposure, the concentration of DnBP in root, stem and leaves of all six seedlings per beaker were determined (Fig. 1a). The DnBP concentration in the root reached a peak at 24 h and declined thereafter gradually to 223 mg kg⁻¹. The concentration of DnBP in the stem increased quickly during first 24 h and then continued to increase gradually until the end of the period. DnBP concentration in the leaves reached a maximum at 48 h. After that, it decreased from 67 to 55 mg kg⁻¹. The amount of DnBP in whole plant was shown in Fig.S2. The total amount of DnBP increased rapidly during the first 24 h, then continued to rise at a much slower rate for the next 48 h, reaching a peak (307 µg per beaker) at 72 h. From 72 h to 96 h, the amount of DnBP declined significantly to 290 µg per beaker (*p* < 0.05, Fig. S2). For each organ, similar trends of initial increase then decrease were observed during the 96 h exposure (Fig.S2). When pumpkin roots were exposed to DnBP, its uptake and accumulation were the dominant processes initially. As the concentration in the plant increased, distribution of DnBP to other tissues and potential metabolism occurred simultaneously, **which led to less rapid increases in the concentration of DnBP**. The decrease in plant DnBP content during the last stage of the experiment may have been due to volatilization from leaves or to metabolism of DnBP in the plant. The phytovolatilization of DnBP was negligible due to its relatively high *n*-octanol-water partition coefficient (*K*_{ow}) and low Henry's law constant (Staples et al., 1997). Therefore, it is likely that DnBP metabolism in the plant was the primary contributor to the observed decrease of DnBP content in pumpkin seedling.**

It has been reported that PAEs can be transformed to MPEs and PA by microbes through the hydrolysis of one or two ester bonds (Liang et al., 2008; Zhang et al., 2014). Recently, MPEs were also found as metabolites in some edible plants after *in vivo* and *in vitro* exposure to PAEs (Sun et al., 2015). However, the presence of other PAE metabolites, including PA, has not been previously reported in plant metabolism studies. In this study, both MnBP and PA were simultaneously detected in pumpkin seedlings (Fig. 1). In order to confirm that these metabolites were derived directly from internal plant metabolism of DnBP, several control treatments were conducted (Fig. S1). No detectable contents of MnBP and PA were found in the solution of the unplanted control, which means that abiotic loss of DnBP **was minimal** under the conditions used in this study. Potential photodegradation of DnBP was prevented by wrapping the exposure beakers with aluminum foil. In addition, MnBP and PA were not

observed in plants or solution of the untreated control, indicating that no cross contamination occurred between these treatments. In the microbes and exudates control, only small amounts of MnBP and PA were detected in solution with values of 26.2 and 2.06 $\mu\text{g L}^{-1}$, respectively. MnBP is a monoprotic acid with a pK_{a} value of 4.2 (Blair et al., 2009), and PA is a dibasic acid with pK_{a1} and pK_{a2} values of 2.9 and 5.4, respectively (Table S3) (Gagliardi et al., 2010). Thus they are both weak acids and existed mainly in the ionized form at the pH (6.0) used in this study. Uptake of anions by plants is generally low due to repulsion between the negatively charged anion and the cell membrane, which has a similar electrical potential (-71 to -174 mV) (Trapp, 2009). These results and considerations suggest that the presence of MnBP and PA was primarily due to metabolism of DnBP in pumpkin.

The concentrations of MnBP and PA in different pumpkin organs during the exposure period are shown in Fig. 1. MnBP concentration in the root increased rapidly during first 24 h followed by a slight decline at 48 h, after which it rose sharply to 83.8 mg kg^{-1} at 72 h before dropping again at the end of the exposure period. The concentration of MnBP in the stem increased quickly during the initial 12 h then continued to ascend gradually, for the most part, until the end of the period. MnBP concentration in the leaves increased slowly over the entire exposure period with a maximum of 6.29 mg kg^{-1} at 96 h (Fig. 1b). For PA, the concentration in the root showed a similar pattern to that of MnBP, with comparatively rapid increases during the first 24 h and from 48 h to 96 h. However, PA concentrations in stem and leaves both exhibited a slowly increasing trend over the entire 96 h (Fig. 1c). PA concentrations were always an order of magnitude lower than those of MnBP in corresponding organs of pumpkin, suggesting that PA was derived primarily from metabolism of MnBP.

It is interesting to note the obvious differences in the concentration profiles of MnBP and PA in pumpkin seedlings. Generally, the concentrations of MnBP in each organ were (in descending order): stem > root >> leaf. However, a different pattern was observed for PA concentrations, which were in the order root >> stem $\approx$ leaf (Fig. 1). The translocation factor (TF) values of MnBP were also higher than those of PA during the entire 96 h (Table S4). The derived TFs of MnBP were higher than 1 (except at 72 h), whereas the TF values of PA were consistently lower than 1, indicating a higher tendency for MnBP to be translocated to the shoot. It has been reported that anionic pharmaceuticals with intermediate polarity ($\log D_{\text{ow}}$, the pH-dependent octanol-water partition coefficient, between 0.5 and 3) can be easily transported to plant shoots (Tanoue et al., 2012). PA is relatively more hydrophilic ($\log D_{\text{ow}}$ 0.03) than MnBP ($\log D_{\text{ow}}$ 1.03) (Table S3), suggesting that MnBP can more readily pass through lipid bilayers to enter the xylem and then be transported to the shoot. In addition, slow transfer across cell membranes due to electrical repulsion, sequestration and compartmentalization within root cells may also contribute to the poor translocation of PA to the shoot.

3.2. Distribution of DnBP metabolites in subcellular fractions of pumpkin

The subcellular distribution of DnBP metabolites (i.e., MnBP and PA) in different organs of pumpkin versus time were investigated (Fig. 2). Each organ of pumpkin was fractionated into three

subcellular fractions: the cell wall, cell organelles, and the cell water-soluble component. The concentrations of MnBP and PA in the cell wall and organelles were calculated as milligram MnBP or PA per kilogram cell wall or organelle on a dry weight basis. MnBP concentrations in root cell walls and organelles rose in the first 48 h, then slightly decreased over the next 24 h, followed by a sharp increase in the last stage of exposure (Fig. 2a). In contrast, MnBP concentrations in the cell walls and organelles of stem and leaves exhibited a smoothly ascending trend. Generally, concentrations of MnBP were higher in cell organelles than in the cell wall for all pumpkin organs. The trends of PA concentrations in these two subcellular fractions were similar to those of MnBP (Fig. 2b). However, PA concentrations were far lower than those of MnBP, and this difference became more obvious in the root and stem in the later period of exposure.

The proportion of a metabolite (P_M) present in a particular subcellular fraction of pumpkin was calculated by the following equation (Gao et al., 2013):

$$P_M (\%) = M_{\text{cell}}/M_{\text{plant}} \times 100 \quad (1)$$

where M_{cell} is the amount (μg) of metabolite (i.e., MnBP or PA) in a certain cell fraction of root, stem or leaf, and M_{plant} is the total amount (μg) of MnBP or PA in the corresponding organ. The proportions of MnBP and PA in the cell wall, the organelles, and the cell water-soluble component in pumpkin roots, stems, and leaves versus time are shown in Fig. 3. In general, most of the MnBP and PA were distributed into the cell water-soluble component in each organ of pumpkin throughout the exposure period, except for MnBP in the root. In roots, the proportion of MnBP in the cell water-soluble fraction was 78.0% at 12 h and then gradually decreased to 3.93% at 96 h. In contrast, the proportion of MnBP rose from 2.46% to 31.0% and from 19.6% to 65.1% in the cell wall and organelles, respectively (Fig. 3a). Distribution of PA in the root subcellular solid fractions (i.e., cell wall and organelles) showed a similar but slower increasing trend (Fig. 3b). These results are consistent with the trends of MnBP and PA concentrations in the root cell wall and organelles (Fig. 2). The increase in the proportions of MnBP and PA in the root subcellular solid fraction may be a result of partition from the water-soluble fraction and/or local degradation of DnBP in these fractions. Gao et al. (2013) reported that anthracene, a representative polycyclic aromatic hydrocarbon, was degraded in tall fescue, and that its more hydrophilic metabolites anthrone and anthraquinone were initially distributed primarily into the cell soluble fraction, whereas after 16 days the dominant storage compartments were the cell wall and organelles. In the current study, negligible amounts of pure cell membrane could not be separated from other cell fractions by the centrifugation and it was merged into the cell water-soluble component. Hence, this fraction can be regarded as an intracellular buffer containing cell solution and other water-soluble substances (Kang et al., 2010), and MnBP and PA may be preferentially located in the cell water-soluble fraction due to their being more hydrophilic than their parent compound DnBP (see Table S3). Although the proportions of MnBP and PA in the cell water-soluble component of all organs (except that of MnBP in the root) were shown to slightly decrease or to fluctuate slightly over the entire 96 h of the experiment (Fig. 3), the absolute quantities of MnBP and PA in this fraction were 136-177%

and 58-294% higher, respectively, at 96 h than at 12 h (Table S5). Moreover, transportation to the shoot and hydrolysis of MnBP to form PA contributed to the large decrease of MnBP in the root cell water-soluble component.

3.3. Carboxylesterase activity in pumpkin and its subcellular fractions

The activity of carboxylesterases (CXEs) in pumpkin seedlings was analyzed during 96 h of 10 mg L⁻¹ DnBP exposure. As shown in Fig. 4a, CXE activity gradually increased throughout the exposure period in both the control and the DnBP treated groups. Little to no difference in CXE activity was observed between the two groups in the first 24 h. However, CXE activity in pumpkin seedlings treated with DnBP was significantly higher than that of the control from 48 h to 96 h ($p < 0.01$). This phenomenon was consistent with trends in the quantity of DnBP in whole pumpkin seedlings and in the concentrations of MnBP and PA in the root, as shown in Fig. S2 and 1, respectively. In the leaves, however, CXE activity did not differ significantly between the DnBP and control treatments (Fig. S3). A concentration-dependent experiment was also conducted. After 96 h of cultivation in solutions with 0 to 10 mg L⁻¹ DnBP, CXE activity in pumpkin roots ranged from 0.0298 to 0.0458 U mg protein⁻¹ (Fig. 4b). No significant change in CXE activity was observed when the concentration of DnBP in solution was ≤ 2 mg L⁻¹, but DnBP concentrations ≥ 4 mg L⁻¹ significantly stimulated CXE activity in pumpkin roots. CXE activity in the leaves was not affected by exposure of the plant to 0-10 mg L⁻¹ DnBP (Fig. S4).

Bioactivation of xenobiotics (e.g., herbicides) by CXEs has been widely reported. With the action of CXEs, herbicides applied in the inactive carboxylic ester form are degraded in the plant, releasing the phytotoxic entity, which generally is the respective carboxylic acid (Cummins and Edwards, 2004; Haslam et al., 2001). Moreover, it has long been known that CXEs are often up-regulated in plants in response to biotic stress such as fungal disease (Muarlidharan et al., 1996). In the current study, the appearance in the plant of MnBP and PA suggests that hydrolysis of ester bonds is the first step in the metabolism of DnBP, and that CXEs are most likely involved in this reaction. Therefore, a significant increase in CXE activity should, in principle, result from plant exposure to DnBP. Although MnBP and PA were detected in pumpkin before 48 h, no significant change in CXE activity was observed in this period, which may have been due to low sensitivity to DnBP stress of the pumpkin used in this study. However, based on the high de-esterification activity of CXEs (Gershater and Edwards, 2007), we believe that hydrolysis of DnBP by CXEs began when DnBP entered the root cell. A significant increase in CXE activity was not found until the DnBP concentration was high enough to reach the “threshold concentration”, which varies for different compounds and plant species (Ling et al., 2012). The difference between the patterns of CXE activity observed in roots versus leaves indicates that metabolism of DnBP by CXEs occurred primarily in pumpkin roots.

The amount of CXE activity in each cell fraction is shown in Fig. 5a. CXE activity in root cell walls, cell organelles, and the cell water-soluble component ranged from 0.0199 to 0.0306, 0.0100 to 0.0145,

and 0.0396 to 0.0757 U mg protein⁻¹, respectively. The amounts of CXE activity in these three cell fractions were (in descending order): the cell water-soluble component > cell wall > cell organelles. There was a trend of increased CXE activity in the cell water-soluble component with increasing concentration of DnBP in the nutrient solution, especially up to 4 mg L⁻¹. The proportion of CXE activity (P_{CXEs}) occurring in a particular subcellular fraction of pumpkin roots was calculated according to the following equation (Ling et al., 2012):

$$P_{CXEs} (\%) = C_{cell}/C_{root} \times 100 \quad (2)$$

where C_{cell} (U mg protein⁻¹) is the amount of CXE activity in a certain cell fraction, and C_{root} (U mg protein⁻¹) is the total amount of CXE activity in the root. As shown in Fig. 5b, approximately 90% of root CXE activity occurred in the cell water-soluble component, while the remaining 10% was distributed between the cell wall and organelles. Regardless of the DnBP level in solution, the proportions of CXE activity occurring in the subcellular fractions of pumpkin root were (in descending order): the cell water-soluble component >> the cell wall > cell organelles.

It has been demonstrated that CXEs located in the extracellular apoplast are involved in the metabolism of foliar-applied herbicides in plants (Haslam et al., 2001). However, little information is available on CXE activity in the intracellular fractions of plants under xenobiotics exposure. The present study revealed that 90% of CXE activity occurred in the cell water-soluble component, suggesting that CXEs are present primarily in this fraction. Given that the quantity and proportion of CXE activity as well as the quantities of MnBP and PA were all highest in the cell water-soluble component, compared to the other cell fractions, it can reasonably be postulated that this fraction was the primary domain for DnBP metabolism in the pumpkin cell.

The CXE inhibitor TPP was used to confirm the involvement of CXEs in the metabolism of DnBP in pumpkin. There was no significant difference among biomass of each organ (root, stem and leaf) of pumpkin seedlings per beaker under different concentration of TPP treatments (Fig. S5), suggesting that TPP addition caused no obvious harm to the plant. Fig. S6 shows the effect of TPP on the quantities of DnBP and its metabolites in whole pumpkin seedlings. The quantities of DnBP in the 1 μM and 10 μM TPP treated groups were 25% and 16% higher, respectively, than that in the control (0 μM TPP). In addition, TPP treatment significantly reduced the quantity of MnBP in pumpkin and the reduction was greater at the higher concentration of TPP (Fig. S6). Moreover, TPP treatment resulted in much lower CXE activity in roots (Fig. S7). In spite of the fact that the quantity of PA in the TPP-treated groups was unchanged or even increased instead of declining in relation to the control, it is probable that the much larger increases in the quantity of DnBP in TPP-treated pumpkin seedlings resulted from the inhibitory effect of TPP on CXE activity, leading to a reduction of DnBP metabolism. Therefore, these results strongly demonstrated that CXEs were involved in plant metabolism of DnBP.

3.4. The fate of DnBP and its metabolites in pumpkin seedling

After a 96-h exposure period, 6.93% of the initial DnBP in solution has been taken up by pumpkin

seedlings, where it existed in both un-hydrolyzed and hydrolyzed forms (Table S6). Researchers studying several other plant species have reported that the contribution of plant uptake to the total dissipation of PAEs from soil or solution was negligible (mostly less than 1%) (Cai et al., 2008; Sun et al., 2015). However, up to date, no similar experimental design of concentration level, culture method and exposure duration, and consequently comparable results, could be found in the literature. Therefore, although pumpkin appears to have a relatively high uptake ability for DnBP than other species reported in the literature, more valid data are certainly needed to support this assumption. Roots routinely contain the highest concentrations of DnBP and its metabolites; however, because the shoot comprised most of the total plant biomass, 66.4-91.8% of these compounds were present in the shoots (Table S6). Similar results for *p,p'*-DDE uptake by pumpkin were reported by White (2002). The relatively high PAE accumulating ability of this pumpkin cultivar indicates its potential for use in a plant-based remediation strategy of PAE contaminated soil or water.

In the present study, the stepwise hydrolysis of DnBP began with the involvement of CXEs, leading to the generation of MnBP and PA. Although in a previous study we reported that DnBP taken up by pumpkin roots was present mainly (> 80%) in the cell wall and organelles (Lin et al., 2015), in this study the metabolites of DnBP were located primarily in the cell water-soluble component (Fig. 3), which also was the dominant location of CXE activity (Fig. 5). The hydrolysis reactions might preferentially occur in this fraction due to its higher proportion and activity of CXEs (Fig. 5). Over the course of exposure period, the proportions of MnBP and PA in the root cell wall and organelles gradually increased (Fig. 3), which may have resulted from the transfer of these metabolites from the cell water-soluble component. In addition, the observation from our previous work that DnBP concentrations in the root cell wall and organelles decreased over time indicates the existence of local degradation in these solid fractions as well (Lin et al., 2015). It should be noted that conjugation may occur after hydrolysis of DnBP (Kvesitadze et al., 2009); however, the attempt to separate and determine of the potential conjugated metabolites were failed due to the lack of commercial standards. The failure to detect conjugated metabolites may underestimate the degree of plant metabolism of PAEs (Sun et al., 2015). Further research using radioactive isotope labeling should be conducted to explore the possibility of the existence of unidentified conjugated metabolites (Yue et al., 2012) and to understand the specific pathway of PAE metabolism in plants. Besides, considering the possible metabolites might be present in low levels, a relatively high exposure concentration (10 mg L⁻¹) appeared to be suitable for the metabolism study. Although the use of concentration of 10 mg L⁻¹ could mimic the actual condition that plant might encounter (Gao et al., 2016; Zhao et al., 2015; Zhao et al., 2016), multiple doses instead of only one concentration are recommended in future study, which could provide more detailed and comprehensive information about the metabolism of xenobiotics.

4. Conclusions

This is the first study to investigate plant metabolism of DnBP and the subcellular distribution of its metabolites. The metabolism of DnBP by pumpkin was demonstrated in a hydroponic experiment by the observation of a decrease in the amount of DnBP in the plant as well as by the appearance of its metabolites MnBP and PA. Hydrolysis of DnBP took place primarily in the root, and the metabolites were then transferred to the shoot. The higher TF value of MnBP compared to that of PA suggested that the difference in the extents to which MnBP and PA were translocated to the shoot might have been due to their different properties (e.g., $\log D_{ow}$). These two metabolites were located primarily in the cell water-soluble component but were gradually partitioned into the two solid cellular fractions studied (the cell wall and organelles). The response of CXEs to plant exposure to DnBP was also investigated, including at the intracellular level. A relatively high level of DnBP ($\geq 4 \text{ mg L}^{-1}$) in the nutrient solution stimulated CXE activity in pumpkin roots but not in leaves, confirming that the pumpkin root was the primary site of DnBP hydrolysis. The level of CXE activity and the proportion of CXEs present in root subcellular fractions were (in descending order): the cell water-soluble component $\gg$ cell wall $>$ cell organelles, suggesting that the cell water-soluble component is the dominant locus of CXE activity and also the domain of CXE-catalyzed hydrolysis of DnBP. The involvement of CXEs in the metabolism of DnBP was confirmed by addition of the CXE inhibitor TPP, which reduced the degradation of DnBP in pumpkin by depressing CXE activity. This study provides new insight into the transformation and distribution of PAEs and their metabolites in plants.

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Fig. 1. Concentrations of DnBP (a), MnBP (b) and PA (c) in root, stem, and leaf of pumpkin seedlings as a function of time.

Fig. 2. Concentrations of MnBP (a) and PA (b) in root cell wall (solid square), root cell organelles (hollow square), stem cell wall (solid circle), stem cell organelles (hollow circle), leaves cell wall (solid triangle) and leaves cell organelles (hollow triangle) of pumpkin seedlings as a function of time. The insert graphs show, at an expanded scale, only the concentrations of MnBP and PA in root cell wall, stem cell wall and leaves cell wall and organelles.

Fig. 3. Proportions of MnBP and PA distributed into three subcellular fractions (cell wall, cell organelles, and the cell water-soluble component) of roots, stems, and leaves of pumpkin seedlings as a function of time. (a) MnBP in roots, (b) PA in roots, (c) MnBP in stems, (d) PA in stems, (e) MnBP in leaves, (f) PA in leaves.

Fig. 4. Carboxylesterase activity in pumpkin roots as a function of time (a) and of DnBP concentration in solution after 96 h of exposure (b). Single and double asterisks above the columns indicate the significant differences from the control treatment at the 95% and 99% confidence levels, respectively. Bars with the same letter are not significantly different ($p > 0.05$).

Fig. 5. Amount (a) and proportion (b) of Carboxylesterase activity in subcellular fractions of pumpkin roots as a function of DnBP concentration in solution after 96 h of exposure.