Distribution of di-(2-ethylhexyl) phthalate in leaf cuticular waxes and leaf tissues of plants

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Abstract

Di-(2-ethylhexyl) phthalate (DEHP) as plasticizer is widely used in the modern plastic manufacturing industry, DEHP and its breakdown products have been identified as a global environmental contaminant. Vegetables and crops which are the energy sources of human beings are often exposed to DEHP, which enriched in humans through the food chain, resulting in many diseases. The content distribution of DEHP in leaf cuticular waxes and tissues of 14 plants including vegetables and crops, and in various parts of cells of 4 plants were investigated by Gas Chromatography-Mass Spectrometry (GC-MS). The results show the stronger the DEHP uptake ability of the plant the less ratio of DEHP in leaf cuticular wax occupying the total DEHP in the leaves of the plant. DEHP in atmosphere is adsorbed by leaf cuticular wax or stomata, then transfer to inner tissues through cell wall. Interestingly, we found that the leaf cuticular wax and cell wall of plants are possible barriers to uptake of DEHP for the plants possessing lower DEHP uptake ability. Our results will provide some information for further study on the mechanism of DEHP uptake by plants.

Introduction

Di-(2-ethylhexyl) phthalate (DEHP), which is the most commonly used plasticizer among the phthalate esters, has been widely used as an additive in the manufacture of plastics, varnishes, cosmetics and so on (Ghisari and Bonefeld-Jorgensen 2009; Lucas et al. 2012; Saeidnia and Abdollahi 2013). The general population is ubiquitously exposed to this chemical, which is posed a potential risk to human beings (Jobling et al. 1995; Tickner et al., 2001; Heudorf et al. 2007; Zarean et al. 2016). Health adverse impact of exposure to DEHP including effects on reproductive health, carcinogenesis, pregnancy outcome, and respiratory system (Zarean et al. 2016). The clinical manifestations are endocrine disorder, testicular feminization, ovarian dysplasia, neural lesion, hepatotoxicity, and cardiotoxicity (Rowdhwal and Chen 2018). DEHP and its breakdown products have been identified as a global environmental contaminant (Zarean et al. 2016; Zhang et al. 2019), therefore, this is a serious problem, which has aroused researchers' high attention.

DEHP possibly like polychlorinated biphenyl (PCB) accumulate in vegetation due to its contaminant affinity for lipids, and the accumulation is important in terms of fate of pollutants (Barber et al. 2003; Kania-Korwe and Lehmler 2016). Past efforts to understand the uptake mechanism of pollutants like DEHP by plants have been considerable, it was demonstrated that the major pathway is air-to-leaf (Wild et al. 2005a; Wild et al. 2005b; Wild et al. 2006). Vegetation can accumulate DEHP from the atmosphere may be by the means of deposition on the surface portion such as leaves, stems and bark (Smith and Jones 2000; Schuhmacher et al. 2006; Desalme et al. 2013), foliar uptake of DEHP may depend on physiological parameters of plants including specific area of leaves, surface hair, stomatal apparatus density, and cuticle structure (Howsam et al. 2000; Smith and Jones. 2000; Schuhmacher et al. 2006; Tao et al. 2006). Sequential extraction procedures can separate chemicals adsorbed on surfaces from those absorbed in plant tissues, Kaupp et al. (2000) and Wang et al. (2008) have been made to sequentially extract organic pollutants from leaf cuticles and inner leaf tissues, leading to insights on uptake processes. Laboratory exposure experiments under controlled conditions can provide information for a better understanding of foliar uptake of organic pollutants (Wild et al. 2005a; Wild et al. 2005b).

Vegetables and crops are the main sources which provide plant protein, dietary fiber, vitamins and energy to human beings. As the DEHP is widely used in modern industry, more and more DEHP is released into the environment, resulting in these vegetables and crops are often exposed to DEHP. Finally, DEHP through food chain enriched human body cause a variety of diseases. The present study investigates the partition of DEHP in cuticular waxes and in inner tissues of 14 plants including 11 kinds of vegetables (*B. hispida*, *C. sativus*, *C. moschata*, *M. charantia*, *L. cylindrica*, *B. parachinensis*, *D. carota*, *L. sativa*, *C. frutescens*, *I. aquatica*, and *L. esculentum*) and 3 kinds of crops (*G. max*, *T. aestivum*, and *Z. mays*), and compares the distribution of DEHP in various parts of cells of 4 plants (*B. hispida*, *C. moschata*, *B. parachinensis*, and *I. aquatica*) with different uptake ability of DEHP.

Materials And Methods

Solvents and reagents

All solvents and reagents used for this experiment were of analytical grade and purchased from Huadong Chemicals Co. Ltd. (Hangzhou, China) except DEHP and chromatographically chloroform for dissolving DEHP standard were purchased from sigma Beijing Division (Beijing, China).

Sampling

Eleven vegetable plants including *B. hispida* (wax gourd), *C. sativus* (cucumber), *C. moschata* (pumpkin), *B. parachinensis* (flowering cabbage), *M. charantia* (bitter melon), *Lactuca sativa* (lettuce), *C. frutescens* (hot pepper), *L. cylindrical* (towel gourd), *I. aquatica* (water spinach), *D. carota* (carrot) and *L. esculentum* (tomato) and three crops which are *G. max* (soy bean), *Z. mays* (maize) and *T. aestivum* (wheat) were utilized for experiment of foliar uptake of DEHP. Each plant was cultivated in a 30 cm-diameter-pot loaded 5 kg of soil (soil physical and chemical properties: pH (H₂O) = 6.1, organic matter 5.7%, cation-exchange capacity (CEC) 14.5 cmol·kg⁻¹, total N 129 mg·kg⁻¹, available P 16.7 mg·kg⁻¹, or changeable K 133mg·kg⁻¹) and fertilizer with 3.3g DEHP. Each plant of a species with three replications (three pots) was randomly placed in a glass greenhouse with a 23°C/18°C light/dark temperature regime, and 65% relative humidity under natural light. DEHP treatment was carried out after each seedling grew 2 weeks. Four initial leaves were fixed upper four 4 petri dishes originally loaded 1 mL of DEHP, and added 1 mL of DEHP every week for a supplement. One ml of DEHP was equably distributed in the surface of the dish by volatilizing 10 mL of acetone solution of DEHP. After 4 weeks of foliar uptake of DEHP, the leaves of each plant were harvested and dip into liquid nitrogen immediately, and stored - 80°C until DEHP determination in leaf cuticular waxes and leaf tissue, respectively.

Four plants *B. hispida*, *C. moschata*, *B. parachinensis* and *I. aquatica*, which stand for supper, strong, middle and weak foliar uptake of DEHP, were selected for study on distribution of DEHP in various cellular parts. The pot-cultivation and DEHP treatment of these 4 plants was the same as the above description.

Isolation of plant tissues

The isolation of leaf cuticular wax and leaf tissue followed a procedure modified from Riederer and Schonherr (1984). Fresh leaf samples weighing 5 g were extracted with 100 mL dichloromethane in 250-mL flask with gentle shaking for 2 min. The extract was filtered and used for measurement of DEHP in cuticular wax after clean-up. The extracted leaf samples were freeze-dried and pulverized to pass through a 40-mesh sieve. The dried samples were then extracted in a 50 mL vessel using 1:1 mixture of dichloromethane and acetone at room temperature for 24 h. The extraction solution was concentrated to near dryness in a vacuum rotary evaporator.

Fresh leaves of *B. hispida*, *C. moschata*, *B. parachinensis* and *I. aquatica* were harvested. Fresh leaf samples weighing 10 g were extracted with 200 mL dichloromethane in 500-mL flask with gentle shaking for 2 min. DEHP in the extract was determined as DEHP in leaf cuticular wax. The extracted leaf samples were homogenized with 250 mL of cold extraction medium composed of 0.3 M sucrose, 25 mM tetrasodium pyrophosphate, 10 mM KH₂PO₄, 2 mM EDTA, 1% (w/v) and 20 mM ascorbate (pH 7.5) at 4°C. The cell fragments isolation according the method of Honda et al. (1969), we modification as follows: the homogenate was sieved through a four-layer nylon cloth and then centrifuged for 10 min at 400×*g*, the precipitate was cell wall fraction. The supernatant was centrifuged for 20 min at 2,000×*g* resulting chloroplast fraction and a supernatant which was centrifuged for 20 min at 50,000×*g* giving mitochondrial fraction and a supernatant which was further centrifuged for 40 min at 100,000×*g* yielding nucleoprotein fraction and soluble cytoplasm fraction. The 5 fractions were freeze-dried, and the residues were subjected to DEHP extraction. The extraction was the same as the above procedure of the extraction of the extracted leaf samples.

Clean-up of extraction samples and determination of DEHP

The concentrated extracts were loaded on a combined column of silica gel and alumina. A glass chromatographic column (25 cm × 1 cm, length × I.D.), was packed with 3 cm alumina plus 10 cm silica, followed by 2 cm anhydrous sodium sulfate. Twenty mL of CHCl₂ were used for elution. The collected fraction was blown down to 0.5 mL under a gentle stream of nitrogen. The cleaned-up samples were analyzed by GC-MS (Sablayrolles et al., 2005) with a Hewlett-Packard 5890/5971 GC-MSD (Agilent Technologies, Palo Alto, CA, USA) equipped with an HP-5 trace analysis column (30 m, 0.32 mm i.d., 0.25 µm film thickness). The GC oven temperature was held at 150 °C for 3 min, and then a temperature program was performed: 20 °C min⁻¹ to 300 °C and invariableness at 300 °C for 3 min. The temperatures of the injector and transfer line were 250 and 280 °C, respectively. Helium was the carrier gas at a linear flow rate of 20.7 cm·min⁻¹. The full scan electron impact data is obtained as follows: solvent delay 5 min, electron impact energy 70 eV, source temperature 200 °C, emission current 150 µA, scan rate 4 scan/s, detector voltage 350V. The amounts of DEHP were calculated from a calibration curves $y = 10^{-6} 5x + 0.2638$ (concentration range 1–50 µg·mL⁻¹, $R^2 = 0.9930$) and $y = 10^{-6} 4x + 3.4439$ (concentration range 50–500 µg·mL⁻¹, $R^2 = 0.9973$). All samples were extracted and analyzed in triplicate.

Quality control

Standard DEHP was used to check the recoveries by doing through the entire procedure. Two procedural blanks were run with every set of extractions by going through the same extraction and cleanup procedures without leaf samples. Recoveries of DEHP were determined by spiking leaf samples of 14 plants with standards at both higher and lower concentrations. The recoveries of the measured DEHP varied from 81.1–108.2%.

Leaf stomatal characteristics

Optical microscope (Olympus, model STM6-LM, Japan) eyepiece 8×, objective lens 40× under field observation and measurement, 50 stomatal sizes were randomly measured and recorded. Stomatal sizes were mainly used to measure the length and width of guard cells, and the average value was calculated and the length-width ratio was calculated.

Statistical analysis

Statistical analysis was performed using ANOVA Duncan's Multiple Range Test to compare all groups. All statistical analyses were performed using SAS 9.4 (SAS Institute Inc., 2018). A probabilistic $p < 0.01$ value was considered significant. Experimental results of DEHP were expressed as means ± SD from the values of three replications of each potted plant.

Results

DEHP in leaf cuticular waxes and in leaf tissue

The determination results of DEHP in leaf cuticular waxes and in leaf tissues of fourteen vegetables were showed in Table 1. Based on a statistical difference ($p < 0.01$) of DEHP in leaf cuticular waxes, the 14 plants could be classified into 3 groups. A highest content, $19.0 \pm 2.95 \mu\text{g}\cdot\text{g}^{-1}$ fresh mass (FM) was found in the leaves of *B. hispida*; higher contents, 7.88 ± 1.12 and $7.95 \pm 0.87 \mu\text{g}\cdot\text{g}^{-1}$ FM in the leaves of *C. sativus* and *C. moschata*; and lower contents, 1.49 ± 0.30 to $3.52 \pm 0.84 \mu\text{g}\cdot\text{g}^{-1}$ FM in the leaves of *L. esculentum*, *Z. mays*, *L. cylindrical*, *I. aquatica*, *C. frutescens*, *T. aestivum*, *L. sativa*, *D. carota*, *M. charantia*, *G. max* and *B. parachinensis*.

Table 1
DEHP content in leaf tissues and in leaf cuticular wax

Plants	DEHP in leaf cuticular wax ($\mu\text{g g}^{-1}$ /fresh leaves)	DEHP in leaf tissues ($\mu\text{g g}^{-1}$ /fresh leaves)
<i>B. hispida</i>	19.0 $\pm$ 2.95 a	27.9 $\pm$ 3.10 a
<i>C. sativus</i>	7.88 $\pm$ 1.12 b	8.12 $\pm$ 1.17 b
<i>C. moschata</i>	7.95 $\pm$ 0.87 b	7.15 $\pm$ 0.92 b
<i>B. parachinensis</i>	3.52 $\pm$ 0.84 c	2.32 $\pm$ 0.49 c
<i>G. max</i>	2.93 $\pm$ 0.51 c	1.84 $\pm$ 0.17 c
<i>M. charantia</i>	2.88 $\pm$ 0.68 c	1.80 $\pm$ 0.38 c
<i>D. carota</i>	2.28 $\pm$ 0.44 c	1.61 $\pm$ 0.26 c
<i>L. sativa</i>	1.77 $\pm$ 0.23 c	0.74 $\pm$ 0.14 d
<i>T. aestivum</i>	1.58 $\pm$ 0.22 c	0.68 $\pm$ 0.17 d
<i>C. frutescens</i>	1.73 $\pm$ 0.31 c	0.62 $\pm$ 0.19 d
<i>I. aquatica</i>	1.85 $\pm$ 0.36 c	0.62 $\pm$ 0.21 d
<i>L. cylindrica</i>	1.65 $\pm$ 0.19 c	0.52 $\pm$ 0.14 d
<i>Z. mays</i>	1.65 $\pm$ 0.14 c	0.48 $\pm$ 0.09 d
<i>L. esculentum</i>	1.49 $\pm$ 0.30 c	0.40 $\pm$ 0.10 d
Note: A significant difference ($p < 0.01$) between the sections labeled with different letter (a, b, c or d)		

The situation of DEHP in leaf tissues was very similar to that of DEHP in leaf cuticular waxes though the plants should be classified into 4 groups according to a statistical difference ($p < 0.01$) of DEHP in leaf tissues. The percentage of DEHP in leaf cuticular waxes occupying DEHP in whole leaves of the 14 plants was listed in Table 2. For *B. hispida*, the plant with super DEHP uptake ability, the ratio was about 40%; for the plants *C. sativus* and *C. moschata* with strong uptake ability of DEHP, the ratios were about 50%; for plants *B. parachinensis*, *G. max*, *M. charantia* and *D. carota* with middle uptake ability of DEHP, the ratios were about 60%; while for the other plants with weak uptake ability of DEHP, the ratios were more than 70%.

Table 2
The percentage of DEHP in leaf cuticular wax occupying DEHP in whole leaves

Plants	DEHP in whole leaves ^A (μg in 1 g of fresh leaves)	Uptake ability	Ratio of DEHP in leaf cuticular wax to DEHP in whole leaves ^B (%)
<i>B. hispida</i>	46.9	super	40.5
<i>C. sativus</i>	16.0	strong	49.3
<i>C. moschata</i>	15.1	strong	52.6
<i>B. parachinensis</i>	5.84	middle	60.3
<i>G. max</i>	4.77	middle	61.4
<i>M. charantia</i>	4.68	middle	61.5
<i>D. carota</i>	3.89	middle	58.6
<i>L. sativa</i>	2.51	weak	70.5
<i>T. aestivum</i>	2.26	weak	69.9
<i>C. frutescens</i>	2.35	weak	73.6
<i>I. aquatica</i>	2.47	weak	74.9
<i>L. cylindrical</i>	2.17	weak	76.0
<i>Zea mays</i>	2.13	weak	77.5
<i>L. esculentum</i>	1.89	weak	78.8
Note: ^A sum of the mean of DEHP in leaf cuticular wax and the mean of DEHP in leaf tissue; ^B calculated with the means of DEHP in the leaf cuticular wax and in whole leaves			

DEHP in the various cellar parts of leaves

On the basis of the above results of DEHP in leaf cuticular waxes and in leaf tissues of fourteen plants and a consideration of easily obtaining enough leaf samples, *B. hispida*, *B. parachinensis*, *C. moschata* and *I. aquatica* were selected as representative plants with larger difference of foliar uptake of DEHP for the study of DEHP in the various cellar parts of leaves. Table 3 exhibited the distribution of DEHP in leaf cuticular waxes and in the various cellar parts of leaves of the four plants. Verifiably, we can find the ratio of DEHP in leaf cuticular waxes to in whole leaves of the 4 plants were very close to the previous values. DEHP in different cell components mainly distributed in cell wall, 79.4–51.0% for *B. hispida*, *C. moschata*, *B. pekinensis* and *I. aquatica*. For each plant, the amount of DEHP in mitochondrial (2.8–9.8%) and nucleoprotein (2.6–5.9%) were less than in chloroplast (5.1–15.7%) (Table 3), which indicate chloroplast possibly possess more fat-soluble components to fix DEHP than mitochondria and nucleoprotein. There was a larger amount in the soluble components (10.1–17.6%) (Table 3) which exist a variety of carriers of DEHP for transfer in cells.

Table 3

Distribution of DEHP in leaf cuticular wax and in the various cellular parts of leaves in four representative plants

Plants	Distribution of DEHP in 10 g fresh leaves											
	Total	Cuticular wax	Cell wall		Chloroplast		Mitochondria		Nucleoprotein		Soluble cytoplasm	
	(μg)	(μg)	(μg)	(%) ^A	(μg)	(%) ^A	(μg)	(%) ^A	(μg)	(%) ^A	(μg)	(%) ^A
<i>B. hispida</i>	408.1	161.0	196.1	79.4	12.5	5.1	7.0	2.8	6.5	2.6	25.0	10.1
<i>C. moschata</i>	146.8	77.5	50.6	73.0	5.3	7.6	3.2	4.6	2.7	3.9	7.5	10.8
<i>B. parachinensis</i>	56.2	34.5	13.8	57.7	2.4	10.0	1.5	6.3	1.2	5.0	2.8	11.7
<i>I. aquatica</i>	23.5	18.4	2.6	51.0	0.8	15.7	0.5	9.8	0.3	5.9	0.9	17.6
Note: ^A percentage of DEHP in cellular part occupied DEHP in inner tissue												

The relationship between DEHP accumulation and stomatal characteristics

The results showed that the stomatal length, stomatal width and stomatal device aspect ratio were not significantly correlated with DEHP accumulation, but positively correlated with stomatal density. The stomatal density of plants with strong DEHP accumulation was significantly higher than that of plants with weak DEHP accumulation (Table 4), indicating that DEHP could enter into plants through stoma.

Table 4

Stomatal density,length, breadth and ratio of stomatal length to breadth in four representative plants

Plants	Stomatal density (mm ²)	Stomatal length	Stomatal breadth	Ratio of stomatal length to breadth
		(μm)	(μm)	
<i>B. hispida</i>	262.56 $\pm$ 13.572a	23.98 $\pm$ 3.438c	17.94 $\pm$ 1.841b	1.35bc
<i>C. moschata</i>	218.47 $\pm$ 7.889b	23.41 $\pm$ 9.117c	15.88 $\pm$ 2.531c	1.48b
<i>B. parachinensis</i>	139.03 $\pm$ 12.357c	33.20 $\pm$ 7.684b	16.84 $\pm$ 0.842bc	1.99a
<i>I. aquatica</i>	77.91 $\pm$ 4.4173d	39.22 $\pm$ 5.559a	20.15 $\pm$ 1.252a	1.45b
Note: A significant difference ($p < 0.01$) between the sections labeled with different letter (a, b, c or d)				

Discussion

Generally, it is considered that the cuticular wax may be one of the major limiting factors in the uptake of chemicals into leaves (Barber J. L. et al., 2004; Schreiber, L. et al., 2005; Smith K. E. C. et al., 2000). For example, the surface of the cuticle is believed to be the location of the barrier to uptake of persistent organic pollutants (POPs) in the leaf (Riederer and Schonherr, 1984), and the composition of cuticles that controls cuticular permeability (Kerler and Schonherr, 1988). Wax composition of plants, which is therefore probably the key factor regulating uptake of POPs into plants (Beattie and Marcell, 2002). In present study, DEHP accumulation in leaf cuticular wax is higher than in tissues from 12 of the 14 species of plants, except for *B. hispida* and *C. sativus* (Table 1). This result suggests that *B. hispida* and *C. sativus* have a strong ability to transport DEHP across the cell wall, and perhaps stoma may also be involved in the absorption of DHEP

(Table 4). We speculated that stomatal density of the nether epidermis of *B. hispida* and *C. sativus* may be higher than that of other 12 plants. *B. hispida* has a strong ability to absorb DEHP (Table 2), and the total DEHP content is much higher than other 13 plants (Table 1), therefore, it is a potential candidate for DEHP phytoremediation.

A comparison between species showed a larger proportion of DEHP in cell wall for the plant with higher foliar uptake ability of DEHP (Table 3). Therefore, a saturated effect of foliar uptake of DEHP likely occurs in chloroplast, mitochondrial, nucleoprotein and the soluble cytoplasm. A composite analysis of DEHP in leaf cuticular wax and in different cell components reveals the way that DEHP in atmosphere is adsorbed by leaf cuticular wax, then transfer to inner tissue through cell wall. It also can be concluded that cell wall is an effective transport barrier of DEHP into other inner tissues since a large amount of DEHP retains in cell wall.

A distinguishing point of this investigation indicated that the stronger the uptake ability of the plant the less ratio of DEHP in leaf cuticular waxes occupying the total DEHP in the leaves of the plant (Table 3). Inferred from this, the leaf cuticular wax is a barrier to uptake of DEHP for the plants possessing lower uptake ability of DEHP. The effect of surface of the cuticle that bars uptake of DEHP is possibly similar to PCBs (Barber et al. 2003), or POPs with low polarity and high fat-solubility (Barber et al. 2004;). Therefore, advanced research of composition and constructure of leaf cuticular waxes to the barrier of DEHP may help us to understand uptake of POPs of plants, which is a severe environmental problem. Based on our present research soluble cytoplasm accumulated more DEHP than chloroplast, mitochondrial and nucleoprotein due to its high fat-soluble components i.e. plasma membrane and tonoplast. High contents of DEHP in cuticular waxes and cell wall provides novel information for us to understand the mechanism of DEHP accumulation in plant leaves.

Declarations

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Authors'contributions

LKX, SGM, ZM, LY, CZW and WYL participated in the design of experiments, collected the data and drafted the manuscript. ZM and WYL helped write the manuscript. LKX, SGM and WYL coordinated the research and helped to finalize the manuscript. All authors read and approved the final manuscript.

Declaration of interest statement

No conflict of interest.

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