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Growth inhibition in Chinese cabbage (*Brassica rapa* var. *chinensis*) growth exposed to di-*n*-butyl phthalate

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ABSTRACT

The toxicity and effects of di-*n*-butyl phthalate (DBP), an endocrine disruptor, on the growth of Chinese cabbage (*Brassica rapa* var. *chinensis*) were studied. Etiolation occurred on leaves of Chinese cabbage plant treated with 50 mg/L of DBP for 42 d. DBP even below 1 mg/L had a significant effect on the concentration of chlorophyll in Chinese cabbage and the biomass showed a severe decrease under treatment with more than 30 mg/L of DBP. At a concentration below 1 mg/L of DBP, no significant difference in accumulation was found, but treatments with concentration exceeding 10, 30, 50 and 100 mg/L all resulted in significant accumulation of DBP. Six protein spots extracted from leaf tissue of DBP-treated Chinese cabbage displaying a differential expression are shown in 2-DE maps. According to proteome level studies, three protein spots were found to increase and were identified, respectively, as acyl-[acyl-carrier-protein] desaturase (acyl-ACP desaturase), root phototropism protein 3 (RPT3) and ferredoxin-nitrite reductase (Fd-NiR). The other three protein spots were found to decrease and were identified respectively as dihydroflavonol-4-reductase (DFR), aminoacyl-tRNA synthetase (aaRS) and ATP synthase subunit beta. The key finding is that the other closely related plant, Bok choy (*Brassica rapa* subsp. *chinensis*), the subspecies of Chinese cabbage, respond differently to the same chemicals.

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1. Introduction

The effects of endocrine-disrupting compounds (EDCs) on organisms have been studied for years. Among them, some phthalate esters (PAEs) constitute a group of chemical compounds that are widely used in different industrial activities [1]. As in plasticizers, PAEs showed low water solubility and high octanol/water partition coefficients but are not covalently bound to the plastics [2]. PAEs are commonly found in atmosphere, rainwater, freshwater, sediments and soils [3–5]. They are also considered to be moderately to highly toxic toward aquatic organisms [6], soil microbial communities [7], and insects [8]. Therefore, the Environmental Protection Agency of United States, the European Union and other international organizations have classified PAEs as priority environmental pollutants and as EDCs.

Di-*n*-butyl phthalate (DBP) is one of the most popular PAEs and is used mainly as a solvent for dyes and a plasticizer for plastics [9]. DBP has a half-life of 22 years in aqueous solution [10] and were often detected in environmental samples [5,11]. In recent years, accumulation of DBP in aquatic environments and agricultural soils has been dramatically increased as a result of large quantity of wastewater, compost, municipal and industrial sewage sludge disposed to the environment [12–15]. Meanwhile, PVC plastic products containing DBP are widely used in agriculture as mulch bags and contaminate agricultural fields. DBP released into the environment can be taken up by crops or vegetables, thus posing a latent risk. The chemical structure of DBP is presented in Fig. 1.

Reports on the effects of DBP on plants are scarce in the literature. The effect of DBP on the synthesis of carotene during seedling growth has been reported [16,17]. Subsequently, the relative sensitivity of DBP to radish (*Raphanus sativus*) and cabbage (*Brassica oleracea*) were also reported [18,19]. These two species were also used as test plants in later research. In other studies, DBP but not di-(2-ethylhexyl) phthalate (DEHP) causes decrease in vitamin C and capsaicin content in capsicum fruit (*Capsicum annum*) [20]. In addition, the effects of DBP on plant growth as well as color changes and crinkle in leaf of six plant species have been studied using the fumigation method [21]. Although the results showed that DBP can be taken up and have adverse effects on plants, the studies were carried out with the addition of DBP in its vapor phase [16,21]. In view of this, we used hydroponic methods to cultivate Bok choy (*Brassica rapa* subsp. *chinensis*) in our previous study with DBP addition to the

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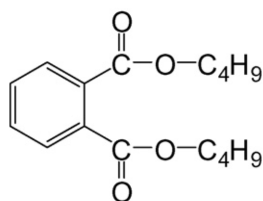

Fig. 1. Chemical structure of DBP.

water for cultivation. DBP was absorbed throughout the plant from root to leaf [28]. The differential expression of proteins between normal and DBP-treated Bok choy were investigated. The changes in morphology, chlorophyll concentration, and proteome pattern were also observed in our former study. The present research was designed to study Chinese cabbage (*Brassica rapa* var. *chinensis*), a vegetable with similar look-and-feel but different in species according to plant classification, to understand the difference between Bok choy and Chinese cabbage. Both of them are the most popular leaf vegetables in Taiwan, commonly used in Chinese cuisine and have been cultivated for over 6000 years in East Asia.

2. Materials and methods

2.1. Chemicals

Di-*n*-butyl phthalate (DBP) (98.7% purity, CAS: 84-74-2) used in experiments was purchased from Riedel-deHaën Co, Germany. Acetone and *n*-hexane (HPLC-grade) solvents were purchased from E. Merck, Germany. The other chemicals were obtained from Sigma Chemical Co, USA. Stock solution of DBP was dissolved in acetone at a concentration of 100 g/L.

2.2. Cultivation of plant

Chinese cabbage (*B. rapa* var. *chinensis*) and its subspecies Bok choy (*B. rapa* subsp. *chinensis*) are the most popular leaf vegetables in Taiwan. In this study the seeds of Chinese cabbage were surface-sterilized for 1 min in 2% NaOCl and rinsed thoroughly with ddH_2O. The seeds were germinated between two sheets of moist filter papers on petri dishes in the dark at 24 °C for 60 h. After germination and growth for 8 d, four Chinese cabbage seedlings were planted in a 3-L pot containing 2.5 l hydroponic solution. The seedlings were fixed by a styrofoam board. The composition of hydroponic solution was adapted from Hoagland's solution [22], which consisted of (in g/L): Ca $(NO_3)_2 \cdot 4H_2O$ (0.1), KNO_3 (0.08), $MgSO_4 \cdot 7H_2O$ (0.05), $NH_4H_2PO_4$ (0.02), Fe-EDTA (3), H_3BO_3 (3), Cu-EDTA (0.01), Zn-EDTA (0.03), Mn-EDTA (0.4) and $NaMoO_4 \cdot 2H_2O$ (0.003). The pH of the hydroponic solution was adjusted to 6.5, and DBP was amended to the solution to become the concentrations of 0, 10, 30, 50 or 100 mg/L. An acetone control (0.5 mL) was also used. Seedlings were cultured under 25 ± 1 °C during daytime for 16 h and under 20 ± 1 °C at night for 8 h. All experiments were conducted in triplicate. The experiment was carried out in the phytotron of the College of Agriculture, National Taiwan University, Taiwan. The plants were planted during a period from May 31st to July 11th, 2004, with a relative humidity variation from 70% to 95%. The appearance and morphology of plants, and leaf damage including etiolation and necrosis were viewed and recorded. After treatment in DBP-amended hydroponic solution for 0, 7, 14, 21, 28, 35 and 42 d, the plant leaves were collected, the accumulations of DBP were analyzed and chlorophyll concentration were determined. The proteomic analysis was processed at 35th d.

2.3. Determination of chlorophyll a and b

Chlorophyll a and b were measured by spectrophotometry using the method adapted from Wintermans and de Mots [23]. One gram of fresh leaves was immersed in 2 mL of sodium phosphate buffer (pH 6.8) and then homogenized. After shaking with buffer (40 μL) sample was taken and extracted with 960 μL absolute ethanol in the dark. The extraction was carried out for 30 min and then centrifuged at $1000 \times g$ for 10 min. The absorption of the extracts was measured at 665 and 649 nm with a spectrophotometer and chlorophyll concentration (mg/L FW) was calculated using the relation: $6.1 \times A_{665\,\mathrm{nm}} + 20.04 \times A_{649\,\mathrm{nm}}$. All experiments were conducted in triplicate and identified using *t*-tests.

2.4. Analysis of DBP

The amounts of DBP in leaf, stem and root samples separately and in the whole plant of Chinese cabbage were analyzed following the methods of Chang et al. [24] and Yin et al. [20] with slight modification. Plant leaves were rinsed three times with deionized water, then air dried at 70 °C, ground in a mortar and sieved (<2 mm in size). Each 1.00-g sample was added to bottles containing 2 mL of *n*-hexane and shaken in a rotating shaker at 160 rpm for 2 h. Residual DBP was extracted twice. The extracts were combined and analyzed on a Hewlett-Packard 6890 gas chromatograph coupled with a mass-selective detector and DB-5 capillary column (film thickness, 0.25 μm; inner diameter, 0.25 mm; length, 30 m). The injector temperature was set at 25 °C. Helium was used as the carrier gas at a flow rate of 0.8 mL/min. Oven temperature was set initially at 50 °C for 4 min, then increased at a rate of 6 °C/min to 160 °C and held for 1 min, increased at a rate of 10 °C/min to 280 °C and held for 1 min. It was then increased at 10 °C/min to 300 °C. The mass-selective detector was programmed to scan over a mass range of 50–400 *m*/*z* units at 4.1 scan/s. The recovery percentage of DBP was 96.5% and the method detection limit (MDL) was 80 μg/L. External calibration procedure was employed to find out the identity and quantity of each analytic peak in the sample chromatogram. Differences in internal DBP concentration between normal and DBP-treated Chinese cabbage were identified using *t*-tests.

2.5. Proteome analysis of plant

2.5.1. Protein extraction

Leaves from cabbage were washed with deionized water and ground to a fine powder in liquid nitrogen. Leaf sample solution was prepared by taking 1.0 g of ground tissue, being added to 10 mL of Tris–HCl solution (50 mM Tris–HCl, 1 mM EDTA, 20 mM dithiothreitol (DTT) and 0.01% protease inhibitor; pH 8.0) and kept for 30 min. Then, a 0.2 mL of TCA/acetone solution (10% (w/v) trichloroacetic acid (TCA) and 0.1% DTT in ice-cold acetone) were added to a 1.5-mL plastic tube containing 0.8 mL of leaf sample solution. Followed by incubation at −20 °C for 1 h, the sample solution was centrifuged in a centrifuge (12,000 rpm) for 10 min and the supernatant was discarded. The pellet was washed for another two times. The pellet was vacuum-dried for 1 h in a desiccator and then suspended in 400 μL of buffer (6 M urea, 4% CHAPS, 2 M Thiourea, 40 mM DTT). In the course of time, bovine serum albumin (BSA) was used as standard and the protein concentration was determined using 2-D Quant Kit (GE Healthcare, 80-6483-56). D-Tube Dialyzer Midi (Merck, 71506-3CN) and 2-D Clean-Up Kit (GE Healthcare, 80-6484-5) were used for desalting.

2.5.2. Two-dimensional gel electrophoresis

After being dialyzed and desalted, the protein solution was loaded onto immobilized pH gradient (IPG) gel strips (pH 3–10,

13 cm in length, GE Healthcare). The IPG strips were rehydrated overnight before use in a rehydration buffer solution (8 M urea, 0.5% Triton X-100, 2% IPG buffer, 65 mM DTT and 0.0002% bromophenol blue). The first dimension, isoelectric focusing (IEF) was carried out using the IPG phor system (GE Healthcare) at 18 °C with 8 kV for a total of 45 kVh. After IEF, the IPG strips were put into the equilibration solution A (0.375 M Tris–HCl, 6 M urea, 2% SDS, 20% glycerol, 130 mM DTT, pH 8.8) and then into the equilibration solution B (6 M urea, 2% SDS, 0.375 M Tris–HCl, 20% glycerol, 135 mM iodoacetamide, pH 8.8), separately, with gentle agitation for 15 min at room temperature. It was then attached with 0.5% agarose to the top of a 12.5% SDS-polyacrylamide gel. The two-dimensional step was carried out at 45 mA *per* gel for 5 h with Hoefer SE 600 Ruby (GE Healthcare) until the bromophenol blue reached the bottom of the gel. All experiments were conducted in duplicate by different plants in the same treatment. Staining was carried out by following the method of Hochstrasser et al. [25] with slight modifications. The gels were first fixed in 300 mL of 11.5% TCA and 4.5% sulfosalicylic acid, followed by fixing in a mixture of 300 mL of 40% ethanol and 10% acetic acid. The gels were then washed with water for 10 min, incubated in the sensitizer (a mixture of 0.5 M sodium acetate and 0.125% glutaraldehyde, 250 mL) for 20 min and washed twice for 10 min with water. Then the gels were incubated in 300 mL of silver solution (24 mM $AgNO_3$, 9 mM NaOH, and 0.14% NH_3), followed by washing in 500 mL of water for 1 min. The gels were then dipped in a mixture of citric acid (760 μM) and 0.0037% formaldehyde (300 mL) solution. The silver stain reaction was stopped by adding 300 mL of solution containing 30% ethanol and 7% acetic acid. After staining, the images of developed gel were obtained by digital scan. Protein spots were automatically detected and analyzed using Image-Master software (Amersham Biosciences).

2.5.3. Digestion of in-gel protein

The in-gel protein was digested following the method by Hellman et al. [26]. Each spot of interest in the silver-stained gel was sliced into 1-mm cubes and washed three times with 50% (v/v) acetonitrile (ACN) in 25 mM ammonium bicarbonate buffer (pH 8.0) for 15 min at room temperature. In-gel protein digestion was preformed using porcine trypsin (Promega, USA). The gel pieces were soaked in 100% ACN for 5 min, dried in a lyophilizer for 30 min and rehydrated in 25 mM ammonium bicarbonate buffer (pH 8.0) containing 35 μL of 10 mg/L trypsin until the gel pieces were fully immersed. After incubating for 20 h at 37 °C, the remaining trypsin solution was transferred into a new microtube. The gel pieces were resuspended with 50% ACN in 5.0% trifluoroacetic acid (TFA) for 60 min and then concentrated to dryness.

2.5.4. Proteomic mass spectrometry analyses

After in-gel protein digestion, lyophilized samples were premixed with 1:1 matrix solution (5 g/L CHCA in 50% acetonitrile, 0.1% (v/v) TFA and 2% (w/v) ammonium citrate) and spotted onto the 96-well format MALDI sample stage. Peptide mass fingerprinting (PMF) and MSMS ion search were carried out using a dedicated MALDI-TOF-MS (matrix assisted laser desorption ionization time of flight mass spectrometry, Micromass, Manchester, UK). The PMF data are a list of peptide mass values from an enzymatic digestion of a protein and the MSMS ion search data are the identified from raw MSMS data from one or more peptides. Samples were analyzed in the reflector mode at an accelerating voltage of 20 kV, 70% grid voltage, 0% guide wire voltage, a 100-ns delay and a low mass gate of 500 Da. The PMF and individual MSMS ion search data were saved as Mascot-searchable .txt file and .pkl files for independent searches against Swiss-Prot or NCBI database using the Mascot search engine (http://www.matrixscience.com/) [27]. Moreover,

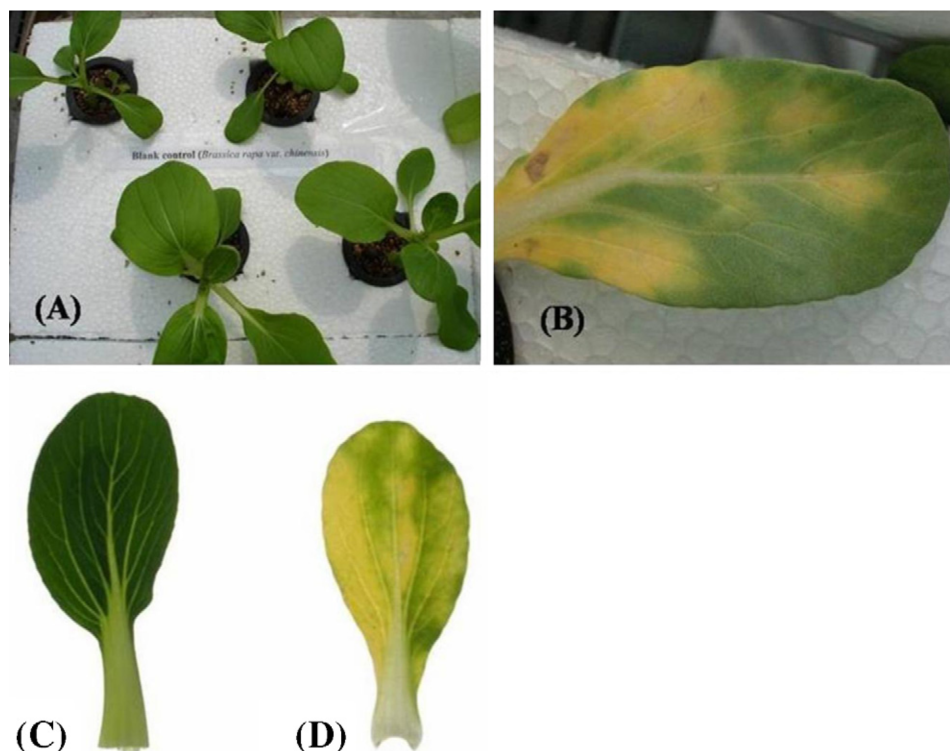

Fig. 2. Effect of DBP-treatment on Chinese cabbage leaf after 35 (A and C) and 42 (B and D) d. (A) Control (or normal) at 35 d, (B) 50 mg/L of DBP treated for 35 d, (C) control (or normal) at 42 d and (D) 50 mg/L of DBP treated for 42 d.

Table 1
DBP accumulation (μg/g) in Chinese cabbage by treating with various amounts of DBP until 35 d, and accumulation in different plant parts after 42 d incubation

Sampling days	DBP treatment (mg/L)					
	0	1	10	30	50	100
0	n.d.[a]	n.d.	n.d.	n.d.	n.d.	n.d.
7	n.d.	n.d.	0.06 ± 0.04	0.21 ± 0.13	0.52 ± 0.26	0.63 ± 0.24
14	n.d.	n.d.	0.14 ± 0.06	0.44 ± 0.21	1.02 ± 0.53	1.32 ± 0.73
21	n.d.	n.d.	0.22 ± 0.14	0.73 ± 0.32	1.53 ± 0.68	1.96 ± 0.69
28	n.d.	n.d.	0.24 ± 0.12	0.82 ± 0.36	1.86 ± 0.59	2.39 ± 0.94
35	n.d.	n.d.	0.24 ± 0.09	0.89 ± 0.44	1.94 ± 0.72	2.73 ± 0.87
42 Root	n.d.	n.d.	0.14 ± 0.06	0.26 ± 0.12	0.38 ± 0.18	0.64 ± 0.22
42 Stem	n.d.	n.d.	0.16 ± 0.06	0.22 ± 0.14	0.54 ± 0.26	0.95 ± 0.46
42 Leaf	n.d.	n.d.	n.d.	0.48 ± 0.28	1.19 ± 0.66	1.44 ± 0.88

[a] n.d. = not detectable (below the detection limit of 0.1 mg/L in triplicate).

references of each protein can be gained in the ExPASy proteomics server (http://tw.expasy.org/) using their accession number.

3. Results and discussion

3.1. Effect of DBP on Chinese cabbage growth

The morphological changes between normal and DBP-treated Chinese cabbage are shown in Fig. 2. Fig. 2A shows the normal growth (no DBP added) of Chinese cabbage and Fig. 2B shows that the surface of leaf turned partially yellow after exposure to 50 mg/L of DBP for 35 d. After exposure of 42 d in the absence of DBP treatment, the leaf still looked green (Fig. 2C), but etiolation appeared on the whole leaf of Chinese cabbage (Fig. 2D). These results show that the leaves of Chinese cabbage turning yellow are related to DBP and the length of exposure. In our previous study, DBP treatment caused all leaves of Bok choy (*B. rapa* subsp. *chinensis*) to turn white with the occurrence of chlorosis and necrosis [28]. These results revealed that DBP exposure caused different injuries on different plants. Dueck et al. [21] and Hemming et al. [16] also reported several outer injuries on the plants studies due to DBP treatment in its vapor phase or in closed glass containers.

Table 1 shows DBP accumulation in Chinese cabbage by treatment with various amounts of DBP for 35 d in the hydroponic solution. At a DBP concentration below 1 mg/L, no significant difference in accumulation was found, but treatment with DBP of concentrations of 10, 30, 50 and 100 mg/L all resulted in significant accumulation (paired *t*-test; $p < 0.05$). On the other hand, DBP accumulation in different parts of Chinese cabbage plant after treatment for 42 d were determined and shown in Table 1. As can be seen, DBP was taken up by the root, translocated to the whole plant and finally gathered in the leaf. These data were similar to the results of Bok choy [28]. However, the accumulation of DBP in Chinese cabbage was less than that in Bok choy. This result showed that the absorption and effect of DBP in Chinese cabbage was found to be slightly less than that in Bok choy [28].

Further, the chlorophyll concentration in leaf decreased with increase in DBP treatment (Table 2). The result showed that treatment with DBP of less than 1 mg/L had a significant effect on chlorophyll concentration in Chinese cabbage (paired *t*-test; $p < 0.05$). The biomass, chlorophyll concentration and DBP accumulation in Chinese cabbage after being treated with DBP of various levels for 42 d are given in Table 3. As can be seen, the biomass of the whole plant and chlorophyll in leaves were significantly decreased with increasing DBP treatment when compared with the control. The biomass showed a severe decrease with DBP treatment exceeding 30 mg/L. The DBP accumulated in Chinese cabbage increased with the amount of treatment and exposure time. An average amount of DBP accumulation was found to be 3.03 μg/g in the whole plant being treated with 100 mg/L of DBP for 42 d. In our previous work, the percentage of chlorophyll decreased and DBP accumulation in Bok choy was significant higher than that in Chinese cabbage. However, the decrease in biomass of Chinese cabbage was higher than that of Bok choy. Furthermore, transmission electron microscopic (TEM) images revealed changes in chloroplast structures in the white leaves of Bok choy but not in the yellow leaves of Chinese cabbage [28]. Other studies on *Raphanus* and *Browallia* [16], capsicum fruit [20] and *Brassica campestris* [21] showed that only DBP can be absorbed and gathered in plants, but other PAEs compounds cannot be taken up by plants.

Table 3
Biomass, chlorophyll concentration and DBP residue in Chinese cabbage leaf by treating with various amounts of DBP under hydrophonic solution for 42 d

DBP treatment (mg/L)	Biomass (g, dry wt)[a]	Chlorophyll concentration (mg/g, fresh wt)	DBP accumulated in whole plant (μg/g)
0	4.59 ± 0.51	0.2693 ± 0.048	n.d.[b]
1	4.01 ± 0.44	0.2518 ± 0.056	n.d.[b]
10	3.34 ± 0.36	0.1906 ± 0.032	0.32 ± 0.08
30	2.59 ± 0.28	0.1632 ± 0.046	0.96 ± 0.24
50	1.42 ± 0.24	0.1607 ± 0.038	2.11 ± 0.56
100	1.12 ± 0.28	0.1534 ± 0.064	3.03 ± 0.74

[a] Average of three plants.
[b] n.d. = not detectable (all three replicates were below the detection limit of 0.1 μg/g in triplicate).

Table 2
Chlorophyll concentration (mg/g, fresh wt) in Chinese cabbage leaf after DBP treatment for different durations

Days	DBP treatment (mg/L)					
	0	1	10	30	50	100
0	0.2372 ± 0.032	0.2319 ± 0.028	0.2391 ± 0.028	0.2334 ± 0.025	0.2278 ± 0.028	0.2312 ± 0.025
7	0.2412 ± 0.035	0.2347 ± 0.034	0.2112 ± 0.026	0.2012 ± 0.027	0.1936 ± 0.024	0.1827 ± 0.028
14	0.2436 ± 0.045	0.2366 ± 0.042	0.1963 ± 0.034	0.1868 ± 0.032	0.1724 ± 0.015	0.1726 ± 0.025
21	0.2442 ± 0.038	0.2420 ± 0.042	0.1812 ± 0.032	0.1747 ± 0.038	0.1767 ± 0.028	0.1564 ± 0.032
28	0.2452 ± 0.032	0.2428 ± 0.044	0.1724 ± 0.026	0.1652 ± 0.022	0.1554 ± 0.037	0.1582 ± 0.046
35	0.2448 ± 0.028	0.2436 ± 0.042	0.1712 ± 0.024	0.1592 ± 0.026	0.1506 ± 0.025	0.1493 ± 0.034

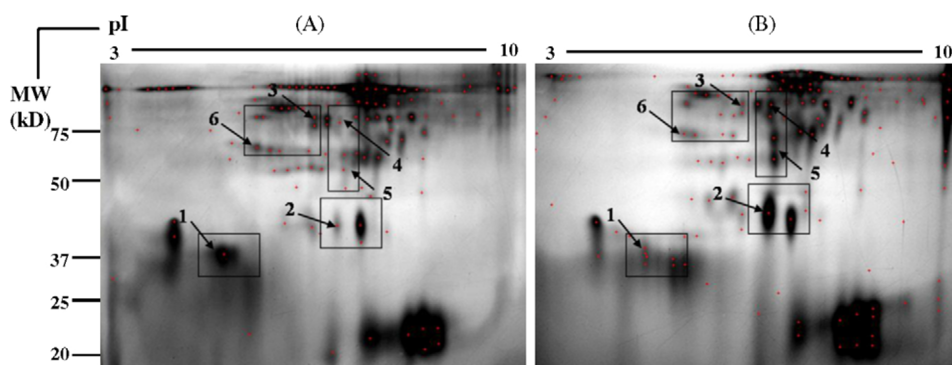

Fig. 3. 2-DE photographs of Chinese cabbage leaf sample after growth for 35 d. (A) Control and (B) 50 mg/L DBP treated. Each arrow shows the proteins that are reproducible in expression. The name, estimated molecular weight and p*I* values of proteins are listed in Table 4.

3.2. Proteomic analysis

After separating with two-dimensional gel electrophoresis (2-DE) followed by staining with silver, the 2-DE images of Chinese cabbage leaf proteome are shown in Fig. 3. There are six protein spots revealing significant alterations. Three proteins (spots 2, 4 and 5) were found to increase in amount and other three proteins (spots 1, 3 and 6) either decreased or disappeared. Alternate difference between control (Fig. 3A) and DBP treatment (Fig. 3B) at spots 1–6 are shown in Fig. 4. The differentially expressed proteins were identified by MALDI-TOF MS and PMF analyses and listed in Table 4. Among the changes in proteome, acyl-[acyl-carrier-protein] desaturase (acyl-ACP desaturase, spot 2), root phototropism protein 3 (RPT3, spot 4) and ferredoxin-nitrite reductase (Fd-NiR, spot 5) increased in amounts in contrast to the control treated with 50 mg/L of DBP, however, the other three proteins (spots 1, 3 and 6 in Fig. 3) were identified respectively as dihydroflavonol-4-reductase (DFR, spot 1), aminoacyl-tRNA synthetase (aaRS, spot 3) and ATP synthase subunit beta (spot 6) decreased. Acyl-[acyl-carrier-protein] desaturase (spot 2) is involved in the biosynthesis of fatty acids. This desaturase was the primer in biosynthesis of fatty acids and catalyzes the principal changeover of saturated fatty acids to unsaturated fatty acids in the synthesis of vegetable oils. In this procedure, acyl-ACP desaturase introduces the first double bond into C18 fatty acids in higher plants, and is therefore involved in controlling the extent of unsaturation of membrane lipids [29–31]. Root phototropism protein 3 (RPT3, spot 4) is a signal transducer of the phototropic response and photo-induced movements in plants [32,33]. Plant growth is strongly dependent on the environment, and plants regulate their growth and development in response to many different environmental stimuli. One of these regulatory mechanisms is phototropism. Phototropism allows plants to change their growth direction in response to the location of light source. Then plants are able to sense and respond to the changes in quality, quantity, and direction of light through the action of photoreceptors. These changes are associated with signal-response systems which involve phototropism proteins. Ferredoxin-nitrite reductase (Fd-NiR, spot 5) is essential for nitrate assimilation in plants and catalyzes the six-electron reduction of nitrite to ammonia, using reduced ferrodoxin (Fd) as the electron donor. Fd-NiR contains siroheme and [4Fe-4S] cluster as described by Lancaster et al. [34]. Low plant Fd-NiR shows significant sequence homology with Fd-sulphite reductase (SiR) and NADPH-sulphite reductase, and the regions form the [4Fe-4S] cluster and the siroheme are, therefore, well conserved. Plant SiR and NiR can catalyze the multielectron to decrease reactions of both sulfite and nitrite. However, each enzyme has a K_m for its preferred substrate about two orders in size lower than that of the other substrate [35].

Our results showed that acyl-ACP desaturase, RPT3 and Fd-NiR were increased in the protein pattern of leaf tissue on DBP-treated Chinese cabbage. These three proteins are responsible for biosynthesis of fatty acids, signal transduction of the phototropic response, and nitrate assimilation in plant cells. According to this result, DBP seems to be capable of inducing increase in some physiological reactions in the Chinese cabbage cell. Therefore, these proteins may be used as indicators of DBP-stress on Chinese cabbage growth. Another illustration of the point is the effects of diethyl phthalate (DEP) in radish [36]. It was found that DEP, but not di-(2-ethlyhexyl) phthalate (DEHP), caused retardation of growth in radish. Moreover, DEP elicited the synthesis of many proteins

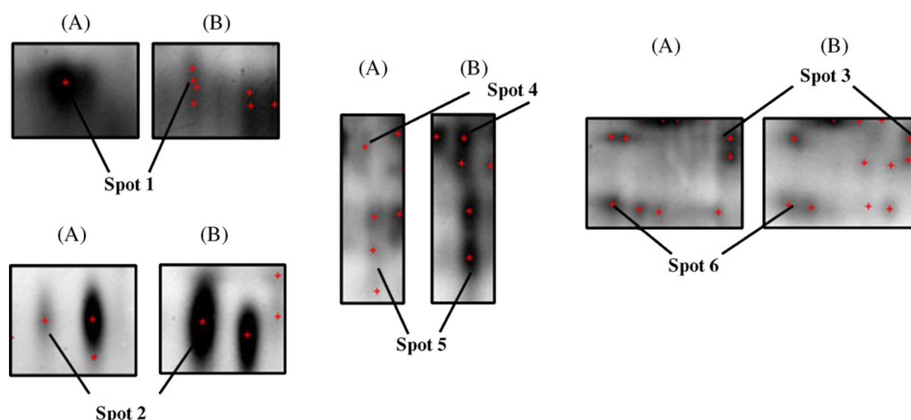

Fig. 4. Comparison of control (A) and DBP-treatment (B) on each protein spot dissipation and increase showed in Fig. 3.

Table 4
Proteomic characterization of polypeptide differences in expression between control and DBP treatment of Chinese cabbage

Spots	Protein name	Accession number	Estimated MW (kD)	Estimated pI	Amounts[a]
1	Dihydroflavonol-4-reductase	P51102	42.7	5.43	–
2	Acyl-[acyl-carrier-protein] desaturase	P28645	45.6	5.94	+
3	Aminoacyl-tRNA synthetase	O23627	81.9	6.59	–
4	Root phototropism protein 3	Q9FMF5	81.7	7.85	+
5	Ferredoxin-nitrite reductase	P05314	66.4	6.51	+
6	ATP synthase subunit beta	Q9MTG8	53.7	5.68	–

[a] + = Increase and − = decrease.

and certain heat shock proteins (HSPs) can be used as an indicator of DEP-stress in radish.

Dihydroflavonol-4-reductase (DFR, spot 1) is a catalyst for converting dihydroflavonols to flavan-3,4-diols (flavanol). Flavanol is one of the flavonoid forerunners, which are the chief secondary metabolites in higher plants because flavanol and other flavonoids are responsible for the color of the flower. DFR is an up-regulated enzyme during floral color development [37–39]. Aminoacyl-tRNA synthetase (aaRS, spot 3) being an enzyme that ligates an amino acid to tRNA was first discovered by Hoagland et al. [22]. When tRNAs are charged with their cognate amino acids in the nucleus before being exported to the cytoplasm, the universal genetic code is determined. Although the same 20 amino acids are found in the organisms in all taxonomic domains, tRNAs are more highly distinguished and have undergone charges throughout evolution. These changes alter the contacts of synthetase-tRNA leading to adaptations [40]. Protein aaRS involved in a broad repertoire of roles that not only impact protein synthesis, but also extend to several other critical cellular activities [41]. Specific aaRSs play important roles in cellular fidelity, tRNA processing, RNA splicing, RNA trafficking, apoptosis and transcriptional and translational control. In recent years, aaRS is reported to signal a transcription termination factor that may interact with the 3′-end of pre-mRNA to promote 3′-end formation. "Structure and Function of Aminoacyl-tRNA Synthetases" that highlighted the diversity of the aaRSs' role within the cell was presented in a European Molecular Biology Organization (EMBO) workshop [42]. Protein ATP synthase subunit beta (spot 6) is one of the subunits of chloroplast ATP synthetase involved in the synthesis of adenosine triphosphate (ATP). ATP synthetase is present in chloroplasts (CF_0F_1-ATP synthetase) and is integrated into thylakoid membrane in plants; the CF_1-part sticks in stroma where dark reactions of photosynthesis and ATP synthesis occurs. The catalytic mechanisms of the chloroplast ATP synthase are almost the same as those of the mitochondrial enzyme. The ATP synthetase subunit beta contains a catalytic site for ATP synthesis during photooxidative phosphorylation in the chloroplast [43]. However, in chloroplasts the protonmotive force is created not by the respiratory electron transport chain, but by primary photosynthetic proteins.

The results in this study revealed that some damages or disorder of metabolism inside the Chinese cabbage cells may partially be attributed to the change in some proteins of the cell. A decrease in DFR suggested that DBP might affect flavonoid biosynthesis and floral color development. This effect might be the cause of Chinese cabbage leaf turning yellow. A decrease in aaRS suggested that DBP might affect the aminoacylation of tRNA and other transcriptional or translational roles; this effect might cause some disorders of regular metabolism or development in the Chinese cabbage cell. A decrease in ATP synthase subunit beta (spot 6) suggested that ATP synthesis in the Chinese cabbage cell might be affected by DBP, thus restricting growth and development. According to these results, DBP may induce some physiological reactions and restrict the growth and development of Chinese cabbage plant. In comparison with our previous study, inhibition of growth and development by DBP was also found in Bok choy; however, leaf tissue injuries, organelle structures and proteomic patterns in these two plants are all different [28].

4. Conclusions

Effects of DBP on the morphological and proteome-level changes in Chinese cabbage were noted. The results showed that the leaves of Chinese cabbage turned yellow and displayed etiolation with decrease in biomass and concentration of chlorophyll.

Increase and accumulation of DBP in different parts of plant implied that DBP not only translocated from roots to the whole plant but also had a significant dose-response relationship on delivery from hydroponic culture. In the proteomic analysis, six protein spots derived from the normal control and the DBP-treated Chinese cabbage plants showed reproducible differences in the expression in 2-DE. Three proteins appeared or increased while the other three proteins decreased or disappeared during growth in hydroponic culture with DBP added. Three spots of increasing protein were responsible for biosynthesis of fatty acids, signal transduction of phototropic response and nitrate assimilation in plant cells, respectively. The other three spots that disappear or show decrease in protein with DBP treatment were responsible for biosynthesis of flavonoids and floral color development in the cell, aminoacylation of tRNA in the cell, and synthesis of ATP in the cell to restrict growth and development in Chinese cabbage, respectively. In conclusion, DBP seems to be able to induce physiological reactions or metabolism disorder in the Chinese cabbage cell. We might say that DBP limits the growth and development of Chinese cabbage; however, the outer injuries, proteomic patterns, pathogenic mechanisms are totally different from those found in Bok choy (*B. rapa* subsp. *chinensis*) [28].

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