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Effect of benzyl butyl phthalate on physiology and proteome characterization of water celery (*Ipomoea aquatica* Forsk.)

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ABSTRACT

This study examined the effect of benzyl butyl phthalate (BBP), a phthalate ester (PAE) and an endocrine disruptor, on water celery, *Ipomoea aquatica* Forsk., one of the most popular leaf vegetables in Taiwan. After 28 days of cultivation, treatment with 100 mg L^{-1} BBP retarded plant growth and decreased biomass and number of mature leaves and caused the accumulation of proline in leaves of water celery, but the concentrations of chlorophyll a and b in the leaves remained constant. 2-D gel electrophoresis and matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis of the proteome of leaf tissue revealed five protein spots with up- and down-regulated expression. The predicted protein XP_001417439 was down-regulated, which explained inhibition of plant growth, and the proteina XP_001417040, calreticulin, GAI-like protein 1, and (-)-linalool synthase were up-regulated, which indicates interference with the cell cycle and protein synthesis, as well as dwarfism of water celery. BBP is a stressor on the growth of water celery, and proteome analysis revealed the up- and down-regulation of genes involved in plant growth with BBP treatment.

1. Introduction

Phthalate esters (PAEs) are a diverse group of compounds that are broadly used in a wide array of industrial applications (Peakall, 1975). They are characterized by their low solubility in water and high octanol-to-water partition coefficients. Regulatory oversight of the manufacture, transport, use, and disposal of PAEs has produced a large number of data on the properties, environmental fate, exposure, and toxicity of these compounds (Barnabé et al., 2008). Such data are critical for the development of safe and acceptable production practices, effluent discharge limits, and human exposure limits. Because of their high production and low degradation rates, PAEs are commonly found in wastewater, sewage sludge, and aquatic environments adjacent to or downstream of industrial sites (Gledhill et al., 1980; Peijnenburg and Struijs, 2006; Xu et al., 2007, 2008). The U.S. Environmental Protection Agency and some of its international counterparts have classified the most common PAEs as priority pollutants and as endocrine-disrupting compounds.

Benzyl butyl phthalate (BBP; Fig. 1), also called *n*-butyl benzyl phthalate, is an ester of phthalic acid, benzyl alcohol, and *n*-butanol. It is known under trade names such as Palatinol BB, Unimoll BB, and Sicol 160. It is largely used as a plasticizer for polyvinyl chloride. Although BBP is commonly used as a plasticizer for vinyl foams often used as floor tiles or in traffic cones, food conveyor belts, and artificial leather, it is considered a toxicant (CICAD, 2009). Adverse health effects and estrogenic properties have been reported both in vivo and in vitro (Chatterjee and Karlovsky, 2010). However, the distribution of BBP has also been reported. According to Yuan et al. (2002), six sediment samples from rivers in Taiwan contained an average of 0.2 μg g^{-1} of BBP. At a lime disposal area of a refinery in Regina, BBP concentrations of 0.15 and 0.55 μg g^{-1} in soil were reported. In soil in the neighborhood of three phthalate-emitting plants in Germany from 1986 to 1989, the highest concentration in an individual sample was 100 μg L^{-1}; the highest mean value for a single site was 30 μg kg^{-1} (Müller and Kördel, 1993). As a result, high doses of BBP amendment (10–100 mg L^{-1}) were included in the research.

Like all other PAEs, BBP can be degraded by many factors in the environment. Some microorganisms utilize BBP as their sole carbon and energy source; for example, 10 mg L^{-1} of BBP disappeared within 6 days under a shake culture condition (Xua et al., 2007).

PAEs can cause diverse effects on different plants, such as disturbance of carotenoid synthesis or chlorophyll formation, irregular grana formation, crumbled or white leaves, and necrosis (Hemming et al., 1981). Thus, exposure to BBP can be a stressor for plants. However, under stress, plants have evolved a high capacity to synthesize and accumulate nontoxic solutes such as malondialdehyde or proline, sucrose, polyols, trehalose, and

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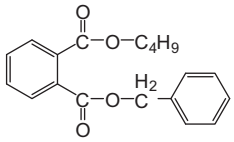

Fig. 1. Chemical structure of BBP.

quaternary ammonium compounds (QACs) (Rhodes and Hanson, 1993) as part of an overall mechanism to increase cellular osmotic adjustment, detoxification of reactive oxygen species, protection of membrane integrity, and stabilization of enzymes/proteins (Yancey et al., 1982; Bohnert and Jensen, 1996; Ashraf and Foolad, 2007). Therefore, proline accumulation is one of the widespread phenomena in stressed plants.

We used 2-D gel electrophoresis (2-DE) to identify physiological and proteomic changes in water celery (*Ipomoea aquatica* Forsk.) with BBP treatment. We measured chlorophyll a and b concentrations, as well as proline concentration, to determine whether the BBP stress triggered proline accumulation.

2. Materials and methods

Any study in this research involving humans or experimental animals was conducted in accordance with national and institutional guidelines for the protection of human subjects and animal welfare.

2.1. Chemicals and reagents

Benzyl butyl phthalate (BBP) (98% purity, CAS number [85-68-7]), purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), was dissolved in acetone to establish a 100,000 mg L^{-1} stock solution. The chemical structure of BBP is presented in Fig. 1. Glassware was meticulously cleaned to reduce any background contamination of phthalates. All deionized water-washed glassware was placed in a 180 °C oven overnight. After cooling, the glassware was rinsed twice with acetone and air-dried for use.

2.2. Plant culture

Water celery (*I. aquatica* Forsk.) is a popular leaf vegetable in Taiwan. After germination and growth for eight days, seedlings were placed in 3-L pots (four seedlings per pot) containing a 2.5-L water culture solution modified from Hoagland's solution (Hoagland and Arnon, 1950). The water culture solution consisted of (in g L^{-1}): $Ca(NO_3)_2 \cdot 4H_2O$ (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 value of the water culture solution was adjusted to 6.5 and the electric conductivity (EC value) to 2 (mmho cm^{-1}, 25 °C). Then 0.1% v/v of Tween 80 was added and mixed thoroughly to ensure that BBP could be dispersed homogeneously in the solution. BBP was added to the water culture solution at 0 (control), 10, 30, 50, and 100 mg L^{-1} when the seedling was transplanted to the hydroponic system. All experiments were performed in triplicates.

The experiment was carried out in the phytotron of National Taiwan University, Taipei, Taiwan. Seedling growth conditions were 11 h at 25 ± 1 °C during the day and 13 h at 20 ± 1 °C during the night. Relative humidity ranged from 70 to 95%. During plant growth, the type and morphological features of leaf damage, such as chlorosis and necrosis, were recorded. After 28 days, regarded as the growing period of water celery, the plants were harvested. One set of triplicates were weighed and dried at 70 °C overnight for dry weight measurement. The leaves of other samples were excised to determine proline and chlorophyll concentrations and for proteomic analysis.

2.3. Proline concentration

Proline concentration was measured by ninhydrin chromogenic methods with modification (Bates et al., 1973). An amount of 0.1 g fresh leaves was homogenized in 1 ml of sulfosalicylic acid (3%, w/v); 0.7 ml of homogenized solution was put into a 1.5-ml Eppendorf tube. After being centrifuged at 10,000 g for 20 min, 0.5 ml of supernatant was transferred to a glass tube. The glass tube was put in a 100 °C water bath for 60 min after the addition of 1 ml chromogenic solution (0.25 g ninhydrin in 10 ml acetic acid). The glass tube was put into an ice bath to end the reaction above. An amount of 2 ml toluene was then added to the tube, which was then vortexed for 15 s and left alone for the next 10 min. The absorption of the toluene layer was measured at 520 nm with a spectrophotometer. A standard curve was made by detecting 0–0.04 mg mL^{-1} of L-proline in the toluene. All experiments were conducted in triplicates.

2.4. Chlorophyll concentration

The chlorophyll (a+b) concentration was measured by spectrophotometry (Wintermans and Mots, 1965) with modification. An amount of 1 g fresh leaves was homogenized in 2 ml sodium phosphate buffer (pH 6.8). Chlorophyll was then extracted immediately with ethanol and placed in the dark for 30 min. The chlorophyll solution was centrifuged at 1000 *g* for 10 min and chlorophyll (a+b) concentration was calculated by the following equation:

$$\text{Chlorophyll (a+b) concentration } (\mu g\,\text{Chl ml}^{-1}\,\text{FW}) = 6.1 \times A_{665\,nm} + 20.04 \times A_{649\,nm}.$$

All experiments were performed in triplicates.

2.5. Determination of BBP concentration

To determine the remaining BBP in the culture medium, 1-ml samples were obtained after the medium was stirred on days 0, 2, 5, 7, 9, 11, 14, and 16. The medium was analyzed using HPLC (HP 1100 Series, Hewlett-Packard, USA) equipped with a C-18 reverse phase column (Purospher STAR RP-18, E. Merck). The mobile phase consisted of acetonitrile and water (70: 30; v/v), with a flow rate of 1.0 ml min^{-1}. BBP was detected with a UV–vis detector at 225 nm and 10.2 min the retention time.

2.6. Protein extraction

The leaves of plants were washed with deionized water and ground in liquid nitrogen to a fine powder. Approximately 1.0 g of the ground tissue was added to 10 ml Tris–HCl solution (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 20 mM dithiothreitol [DTT], 0.01% protease inhibitor) and set aside for 30 min. A solution of 200 μl consisting of 10% (w/v) trichloroacetic acid (TCA) and 0.1% DTT in ice-cold acetone was added to 800 μl of sample solution before being incubated at −20 °C for 1 h. The sample solution was centrifuged at 12,000 rpm for 10 min. The pellet was resuspended in 1 ml of 0.1% DTT in ice-cold acetone and incubated at −20 °C for 30 min. The centrifuge and suspension procedures were repeated another two times. The pellet was vacuum-dried for 1 h and then suspended in 400 μl lysis buffer (6 M urea, 4% CHAPS, 2 M thiourea, 40 mM DTT). Protein concentration was determined in each sample by the Bradford method (Bradford, 1976).

2.7. Two-dimensional gel electrophoresis (2-DE)

Protein samples from control or after 100 mg L^{-1} of BBP treatment underwent separation by isoelectric points and mass. First, the protein solution was loaded onto IPG gel strips (pH 3–10, 13 cm long; Amersham Biosciences, Uppsala, Sweden). The IPG strips were rehydrated overnight prior to 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). First-dimension separation, IEF, involved use of the IPGphor system (Amersham Biosciences) at 18 °C at 8000 V. After IEF, the IPG strips were equilibrated in solution A (0.375 M Tris–HCl, pH 8.8, 6 M urea, 2% SDS, 20% glycerol, and 130 mM DTT) with gentle agitation at room temperature twice for 15 min each. The strips were equilibrated in solution B (6 M urea, 2% SDS, 0.375 M Tris–HCl, pH 8.8, 20% glycerol, and 135 mM iodoacetamide) with gentle agitation at room temperature twice for 15 min each. The gels were attached with 0.5% agarose to the top of a SDS–polyacrylamide gel. 2-DE involved use of a Hoefer SE 600 Ruby system (Amersham Biosciences) at 45 mA per gel for 5 h until the bromophenol blue reached the bottom of the gel. With slight modifications, the staining process followed the method of Hochstrasser et al. (1988). The gels were fixed in 300 ml of 11.5% TCA and 4.5% sulfosalicylic acid, and then were fixed in 300 ml of 40% ethanol and 10% acetic acid. Then the gels were washed in 300 ml of water for 10 min and incubated in 250 ml of sensitizer (0.5 M sodium acetate and 0.125% glutaraldehyde) for 20 min, followed by two washes of 10 min each in 300 ml of water. The gels were incubated in 300 ml of silver solution (24 mM $AgNO_3$, 9 mM NaOH, and 0.14% NH_3), followed by 1 min of washing in 500 ml of water. The gels were developed in 300 ml of 760 μM citric acid and 0.0037% formaldehyde. The silver reaction was stopped in 300 ml of 30% ethanol and 7% acetic acid. Sixteen spots with distinct expression levels of two treatments were sliced and digested according to the following procedures.

2.8. In-gel protein digestion and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

The in-gel protein digestion protocol for MALDI-TOF-MS was as previously described (Hellman et al., 1995). Proteomic mass spectrometry was performed by the Core Facilities for Proteomics Research, Academia Sinica, Taiwan. Lyophilized