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Low temperature-induced water-soaking of *Dendrobium* inflorescences: Relation with phospholipase D activity, thiobarbaturic-acid-staining membrane degradation products, and membrane fatty acid composition

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ABSTRACT

We compared the effects of cold storage (5 °C) on two *Dendrobium* cultivars. After various periods of storage the cut inflorescences were transferred to 25 °C. Water-soaking of the tepals was the main chilling-injury symptom. Cv. Earsakul showed higher CI sensitivity than cv. Khao Sanan. Phospholipase D (PLD) activity increased during 5 °C storage in both cultivars, but generally increased more in cv. Earsakul during the subsequent period at 25 °C. The level of lipid degradation products (including malondialdehyde) that react with thiobarbaturic acid was higher in cv. Earsakul than in cv. Khao Sanan. In contrast to expectation, the ratio between unsaturated and saturated fatty acids was higher, and remained higher during cold storage, in cv. Earsakul than in cv. Khao Sanan. The data suggest that water-soaking due to cold storage of *Dendrodium* inflorescences involves membrane damage, likely related to activation of PLD and accumulation of lipid degradation products. The level of unsaturated fatty acids apparently did not protect against CI. The data seem to question the general view that unsaturated fatty acids protect against CI.

1. Introduction

We previously studied the negative effect of low temperature (5 °C) storage and subsequent placement at room temperature (25 °C), in cut inflorescences of two *Dendrobium* cultivars. Chilling injury (CI) was mainly water-soaking in the floral buds and the tepals of open flowers. Water-soaking (tissue translucency, glassiness) is a visible result of CI, due to loss of the semi-permeable state of the plasma membrane. This leads to diffusion of cellular sap into the cell walls and into the intercellular air spaces (Hurr et al., 2010). In our previous work, cv. Princess inflorescences showed more CI than cv. Sakura. This difference was correlated with lower total antioxidant capacity in cv. Princess. We also determined electrolyte leakage, as well as the activity of lipoxygenase (LOX). Both were positively correlated with water-soaking (Phetsirikoon et al., 2012).

Here we extended this work, using two other cultivars. In the present experiments we determined phospholipase D (PLD) activity, the levels of thiobarbaturic acid-reactive lipid degradation compounds such as malondialdehyde (MDA), and the fatty acid composition of the membranes. We also determined electrolyte leakage and LOX activity to compare the present data with those published earlier.

The immediate effect of low temperature, in products showing chilling injury symptoms, seems a phase transition of membrane fatty acids, from liquid-crystalline to solid-gel phase. This process is initially reversible. The phase change apparently induces the activity of enzymes that degrade membrane lipids. This causes membrane disruption, leading to loss of semipermeability (Lyons, 1973; Marangoni et al., 1996; Nishida and Murata, 1996; Sevillano et al., 2009). Lipolytic enzymes involved in membrane lipid degradation include phospholipase D (PLD) and lipoxygenase (LOX). PLD is involved in the initiation of phospholipid degradation. LOX uses free linoleic and linolenic acid as substrates, resulting in the formation of fatty acid hydroperoxides and malondialdehyde (MDA) (Pinhero et al., 1998). Membrane degradation as a result of chilling injury has been estimated by the increase in the level of lipid degradation compounds such as MDA (Campos et al., 2003; Rui et al., 2010).

Several papers suggest that membranes are more resistant to the low temperature-induced phase transition when they contain relatively more unsaturated fatty acids. Resistance to chilling injury

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was also correlated with a higher ratio of unsaturated to saturated fatty acids (Marangoni et al., 1996; Cao et al., 2011). The difference in chilling sensitivity of loquat fruit from two cultivars was correlated with an initial higher ratio of unsaturated/saturated fatty acids (Cao et al., 2011). Additionally, during exposure to low temperatures the membrane fatty acid composition often changes, whereby levels of C16:0 and C18:0 decrease and those of unsaturated lipids increase (Whitaker, 1995; Wongsheree et al., 2009). These changes seem an adaptation of the cells to low temperature, whereby chilling injury becomes mitigated.

Three hypotheses have been tested in the present study: the degree of water-soaking induced by cold storage is correlated with (a) the activity of PLD, (b) the level of fatty acid degradation products such as MDA, as assessed by the thiobarbaturic acid (TBA) method, and/or (c) the composition of the membranes, in particular the level of unsaturated fatty acids and the ratio of unsaturated and saturated fatty acids.

2. Materials and methods

2.1. Plant material

Preliminary experiments used six cultivars of *Dendrobium* orchids (Earsakul, Khao Sanan, Khao Chaimongkol, Miss Teen, Juree, and Yunnan Blue). Cv. Khao Sanan and cv. Earsakul were used for further experimentation.

Inflorescences were obtained from a commercial grower in Ratchaburi province, Thailand and were brought to the laboratory with the stem ends in distilled water, within 2 h after harvest. The inflorescences had 5–7 open flowers and 5–7 flower buds. Flowers were selected for uniformity, whereby infected ones were discarded. Peduncles of individual inflorescences were cut in air, 12 cm below the lowermost open flower. Stem ends of individual inflorescences were inserted into plastic centrifuge tubes containing 12 mL of distilled water. The inflorescences were placed into cardboard boxes and stored at 5 or 15 °C (85 ± 5% RH) for 8 d, and were transferred to room temperature (25 °C) at 2 d intervals during the cold storage period. When brought to 25 °C, the inflorescences were individually placed in new centrifuge tubes containing 12 mL of distilled water. Water-soaking (WS) was monitored both during storage and the subsequent period at room temperature. The tissues of entire floral buds and open flowers were frozen in liquid N_2 and stored at −70 °C for further experimentation.

2.2. Water-soaking index

Water-soaking (WS) was visually detectable as translucent tissue. Its degree was assessed using the scale described by Phetsirikoon et al. (2012): (1) no symptoms; (2) slight (symptoms visible in 1–25% of flower buds and open flowers on the inflorescence); (3) moderate (symptoms visible in 26–50% of flower buds and open flowers on the inflorescence); (4) moderate to severe (symptoms visible in 51–75% of flower buds and open flowers on the inflorescence); (5) severe (symptoms visible in >75% of flower buds and open flowers on the inflorescence). The results were expressed as the WS index, which was calculated for each individual inflorescence:

$$\text{Water} - \text{soaking index} = \frac{\sum(\text{Classification level} \times \text{Number of buds and open flowers at that level})}{(\text{Total number of buds and open flowers})}$$

2.3. Electrolyte leakage (EL)

EL was measured as described by Phetsirikoon et al. (2012). Tepal segments (0.5 cm^2) were excised with a razor blade, washed in deionized water, after which 0.3 g FW was placed in 15 mL of 0.3 M mannitol, using a capped polypropylene tube (50 mL). The tubes were shaken at 100 rpm for 1 h. The electric conductivity of the solution was measured using a Consort model C831 meter (Turnhout, Belgium). The solution was then incubated in boiling water (100 °C) for 10 min and allowed to cool to 25 °C before the maximum conductivity of the tissue was determined. Data were expressed as the percentage of maximum conductivity.

2.4. Lipoxygenase (LOX) activity

All chemicals used for enzyme analysis were purchased from Sigma–Aldrich, St. Louis, MO, USA.

The method used was described in Phetsirikoon et al. (2012). Lipoxygenase was extracted from 0.5 g of frozen floral tissues mixed with 5 mL of cold 100 mM phosphate buffer (pH 8.0) containing 2% PVPP (w/v), using a homogenizer. After filtering through 4 layers of cotton cloth and centrifuging at 9170 × g for 30 min at 4 °C, the supernatant was assayed using the method of Wang et al. (2004) with modification. The reaction mixture contained 2.4 mL of a 100 mM phosphate buffer (pH 6.8), 0.1 mL of a 10 mM sodium linoleic acid solution, and 0.5 mL crude enzyme extract. One unit of activity was defined as an increase in absorbance at 234 nm of 0.1 per min. Protein content in enzyme extract was determined using the standard Bradford method, with bovine serum albumin as a standard.

2.5. Phospholipase D (PLD) activity

PLD activity was determined using a modification of the method described by Mao et al. (2004). Flower tissue was ground in liquid N_2, using a mortar and pestle. One gram of homogenate was mixed with 10 mL of 50 mM Tris–HCl (pH 8.0) containing 10 mM KCl, 200 mM sucrose, and 0.5 mM phenylmethylsulfonylfluoride (PMSF) and 2% PVPP (w/v). After filtering and centrifuging at 9970 × *g* for 30 min at 4 °C, the supernatant was assayed, using D-nitrophenylphosphorylcholine (NPPC) as the substrate. Reaction mixtures consisted of 0.9 mL of 50 mM Ca-acetate, pH 5.6, containing 27.4 mM NPPC. This was mixed with 0.1 mL (0.4 units) of acid phosphatase dissolved in 50 mM Ca-acetate, (pH 5.6), and 0.3 mL crude enzyme extract. The reaction took place at 37 °C for 60 min, after which it was ended by adding 50 mM NaOH, whereby the final NaOH concentration in the reaction mixture was 3.6 mM. The D-nitrophenol content was then determined by the absorbance at 400 nm. One unit of PLD was defined as the amount of enzyme that resulted in the formation of 1 nmol D-nitrophenol h^{-1}. Protein content in the enzyme extract was determined using the standard Bradford method, with bovine serum albumin as a standard.

2.6. Thiobarbituric acid (TBA)-reactive compounds

Thiobarbituric acid (TBA)-reactive compound levels, which include MDA, were assayed according to Hodge et al. (1999), with slight modification. Floral tissues were homogenized in 5% trichloroacetic acid (TCA) 1:10 (w/v), and centrifuged at 10,000 × *g* for 20 min. 2 mL of supernatant was placed in a test tube containing equal volumes of (i) a solution of 20% (w/v) TCA and (ii) a solution containing 20% (w/v) TCA plus 0.65% TBA. This was mixed vigorously. The mixture was heated at 100 °C in a water bath for 20 min, cooled immediately in an ice bath for 10 min, and centrifuged at 4000 × g for 10 min. Absorbance at 440 nm, 532 nm, and 600 nm