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Effects of heat stress on changes in physiology and anatomy in two cultivars of *Rhododendron*

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ABSTRACT

Rhododendron × *hybridum*, an ornamental plant, is usually heat sensitive. High temperatures cause an array of morphological, anatomical, physiological, biochemical changes in plants, which affect plant growth and development. Evaluating difference between two cultivars in *R.* may facilitate future understand the heat tolerance mechanisms. We conducted a comparative study on growing plants of *R.* 'Lan Yin' and *R.* 'Liu Qiu Hong' at 38/30 °C (day/night) and 25/17 °C (day/night) to elucidate the heat tolerance mechanism of *Rhododendron* in terms of morphology, physiology, anatomy and biology. After the plants were subjected to 38/30 °C (day/night) incubation for 6 d, the chlorophyll and carotenoid contents decreased, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) accompanying lesser increase in *R.* 'Lan Yin' revealed that the heat resistant cultivar was easier to adjust physiologically in heat disappearing. Morphologically, the xylem vessels, leaf ultrastructure and chloroplast of *R.* 'Lan Yin' is superior to *R.* 'Liu Qiu Hong', and *R.* 'Lan Yin' had a decreased stomatal opening and stomatal aperture under heat stress, confirming stomatal factors play a crucial role in heat tolerance in *R.* cultivars. The electrolyte leakage increased, superoxide dismutase (SOD) and peroxidase (POD) activities, osmotic adjustment solute contents (proline, total soluble protein, and sugar) decreased to scavenge reactive oxygenspecies (ROS) and maintain cell membrane stability in two cultivars, but *R.* 'Lan Yin' had a higher level relatively, which may suggests that CMT may use a reliable index for selecting heat-tolerant cultivars of *R.* In addition, there was evidence of plasmolysis and chloroplast damage in *R.* 'Liu Qiu Hong'.

1. Introduction

Rhododendron, a member of the Ericaceae, is primarily distributed in Asia and Southeast Asia. It includes some of the most popular ornamental plants worldwide and certain species have medicinal and/or food value (Dampc and Luczkiewicz, 2013). *Rhododendron* × *hybridum* has long been used in landscape plantings in Europe and North America, where it is valued for the vibrant colors and long blooming time of its flowers. However, owing to genetic factors, most of the current *Rhododendron* × *hybridum* cultivars are heat sensitive. The comprehensive evaluation on heat resistant of *Rhododendron* and further screening heat-resistant cultivar may facilitate future understand the heat tolerance mechanisms.

Global climate change may lead to elevated temperatures (Intergovernmental Panel on Climate Change, 2013). High temperatures may result in heat stress, which not only affects plant morphology and causes leaf etiolation and wilting but also alters the anatomy, physiology, photosynthetic capability, and genetic expression of plants (Chen et al., 2014). Furthermore, heat stress also changes primary and secondary plant metabolism (Macedo, 2012). Among the deleterious, the overgeneration and reactions of reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), superoxide radicals ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and so on, are common under heat stress and may damage chloroplasts and cells by attacking membrane lipids, DNA, and proteins (Chen et al., 2014). Conversely, plants have developed different physiological mechanisms at the transcriptomic, proteomic, and metabolomic levels to counteract ROS and adjust to or avoid prevailing oxidative damage (Dobra et al., 2015; Waqas et al., 2016). The factors that lead the photosynthesis under heat stress including the structural and functional disruptions of chloroplasts, degradation or fewer accumulations of photosynthetic pigments. Therefore, scavenging ROS, maintaining cell membrane stability and/or enhancing photosynthesis are effective way to harvest light and sustain normal growth. The accumulation of osmotic proline, total soluble sugars, and total soluble protein is helpful to protect the structure of enzymes and proteins and maintain cell membrane integrity in the way of low-molecular weight chaperones (Huve et al., 2006; Hameed et al., 2012; Manaa et al., 2014). In addition, plants have developed complex antioxidative defense systems consisting of an enzymatic system and a nonenzymatic system to counteract the injurious effects of ROS (Xu et al., 2006).

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An overall tendency exists to survive under heat stress by reducing cell size, enlarging the xylem vessel diameter, increasing stomatal density to benefit water transport and reducing transpiration (Banon et al., 2004; Chen et al., 2012). High temperature stress has a strong influence on cell ultrastructure, especially chloroplasts, which are often assessed for evidence of stress. Any heat-related damage to thylakoid membranes in chloroplasts is expected to result in chlorophyll loss (Vacha et al., 2007).

Research on abiotic stress of *Rhododendron* was studied earlier, which mainly focused on drought and cold stress (Anisko and Lindstrom, 1996; Lipp and Nilsen, 1997; Cordero and Nilsen, 2002). However, fewer studies about heat stress were reported (Ranney et al., 1995). Heat stress studies of many other plants have recently focused on physiological effects (Gupta et al., 2013). Little is known about how heat stress affects anatomical structures, such as stoma, mesophyll tissue, and epidermal cells, and ultrastructures, such as chloroplasts. The objective of this study was to evaluate difference between two cultivars in *R.* and discuss preliminarily the heat tolerance mechanisms, A comparative study was conducted between *R.* 'Liu Qiu Hong' and *R.* 'Lan Yin' The relationship between heat tolerance and photosynthetic pigments reduction, cell membrane thermostability (CMT), ROS scavenging ability, anatomy morphology and ultrastructure of leaf was investigated.

2. Materials and methods

2.1. Plant materials

The cuttings of two *Rhododendron* cultivars, *R.* 'Lan Yin' (moderate heat-tolerant), *R.* 'Liu Qiu Hong' (heat-sensitive) were grown in plastic pots (12 cm high, diameter 16 cm at the top and 10 cm at the bottom). The culture substrate was a 1:1 (v:v) mixture of peat and pine needle mulch. Forty-eight pots of each cultivar were transferred to a growth chamber (day/night: 25/17 °C, 80% relative humidity, photoperiod of 14 h light/10 h dark, and light irradiance of 150 $\mu mol \cdot m^2 \cdot s^{-1}$) for 1 week. A tray was placed under the pots to retain water.

2.2. Plant physiology response to high temperature

After moderate temperature pre-culture (25/17 °C), twenty-four pots were exposed to a temperature of 38/30 °C (day/night) for 6 days, and another twenty-four pots were maintained at 25/17 °C (day/night) throughout as control. The plants were managed regularly and watered once a day to keep 75% of the growth substrate moisture content during the experiment. Leaf samples were collected after 6 days to investigate the changes of anatomic structure and ultrastructure. Leaf was sampled for physiological analysis at 0, 2, 4 and 6 days after the onset of 38/30 °C treatment.

2.2.1. Measurement of chlorophyll

Chlorophyll content was measured according to the method of Lichtenthaler (1987). The pigments were extracted from fresh leaves by macerating in 80% acetone. Absorption of the chlorophyll and carotenoids present in the extract was determined with a spectrophotometer (UV-2450, Shimadzu, Tokyo, Japan). The total content was calculated using the following equations:

$$C_a = 12.25A_{663} - 2.79A_{645}$$

$$C_b = 21.50A_{645} - 5.10A_{663}$$

$$C_{a+b} = 7.15A_{663} + 18.71A_{645}$$

$$C_{t+c} = [1000A_{470} - 1.82C_a - 85.02C_b]/198$$

where C_a = chlorophyll *a*; C_b = chlorophyll *b*; C_{a+b} = total chlorophyll; C_{t+c} = carotenoid; A_λ = absorbance at λ (nm).

2.2.2. Measurement of electrolyte, H_2O_2, MDA

0.1 g samples were thoroughly washed in double distilled water, and thereafter placed in 25 ml double distilled at 25 °C for 30 min, the electrical conductivity was measured by conductivity bridge (DDSJ-308A, Shanghai, China) after 2 h (EC_1). Subsequently, the same tubes contained samples were placed on boiling water (100 °C) for 20 min, cooled to 25 °C and their electrical conductivity was recorded (EC_2). The electrolyte leakage was calculated as (EC_1 / EC_2) × 100% and determined as per the protocol of Palta et al. (1977).

Lipid peroxidation levels were assessed using malondialdehyde (MDA) according to the method described by Madhava Rao and Sresty (2000). Fresh leaves weighing 0.1 g were homogenized using a pre-chilled mortar and pestle with 10 mL 5% (w/v) trichloroacetic acid (TCA), and centrifuged at 12000 × *g* for 20 min at 4 °C. The supernatant (2 mL) was added to a tube containing 2 mL 0.67% (w/v) thiobarbituric acid. This tube was heated in water bath 100 °C for 30 min, then rapidly cooled to 4 °C in an ice-bath and followed centrifuged at 10000 × *g* for 10 min at 4 °C. The absorbance of the supernatant was measured at 532 nm and 600 nm. The MDA content was calculated using the extinction coefficient of 155 $mM^{-1} \cdot cm^{-1}$.

The hydrogen peroxide (H_2O_2) content in leaves was measured according to the manufacturer's instructions of the Trizol reagent with H_2O_2 (Jiancheng, Nanjing, China). The products were stored at 4 °C. Samples (0.1 g) of fresh leaves were weighed, Absorbance of the mixture was recorded at 405 nm with a spectrophotometer (UV-2450, Shimadzu, Tokyo, Japan) and double-distilled water as a control.

2.2.3. Measurement of proline, total soluble sugar and protein

Fresh leaf samples weighing 0.1 g were homogenized in 5 ml of 3% sulfosalicylic acid and centrifuged at 4000 × *g* for 10 min at 4 °C (Bates et al., 1973). Supernatant (2 ml) was reacted with equal volume of acid ninhydrin and glacial acetic acid for 30 min in a test tube placed in a water bath at 100 °C, then cooled in an ice-bath and added 5 ml toluene. Absorbance of the toluene layer was measured at 520 nm with a spectrophotometer (UV-2450, Shimadzu, Tokyo, Japan) and the standard curve was used calculation of proline quality.

Total soluble sugars were measured according to the method of Irigoyen et al. (1992) with slight modifications. Samples (0.1 g) of fresh leaves were homogenized in 25 ml double distilled water. The extract was centrifuged at 3500 × *g* for 10 min. Supernatant (2 ml) was used, and 0.5 ml anthrone reagent and 5 ml concentrated sulfuric acid were added to it, and then placed in a boiling water bath for 1 min. After cooling, absorbance of the mixture was recorded at 625 nm using a UV-2450 spectrophotometer (Shimadzu, Tokyo, Japan).

The total soluble protein content in leaves was measured according to the manufacturer's instructions of the Trizol reagent with coomassie brilliant blue (Jiancheng, Nanjing, China). The products were stored at 4 °C. Fresh leaf samples weighing 0.1 g were homogenized in saline. And absorbance of the extract was measured at 595 nm using a UV-2450 spectrophotometer (Shimadzu, Tokyo, Japan).

2.2.4. Analysis of enzymatic antioxidant system

Superoxide dismutase (SOD) activity was measured in accordance with Giannopolitis and Ries (1977). The 3 mL reaction solution included 13 mM methionine, 75 nM ethylenediaminetetraacetic acid, 50 μM NBT, 1.3 μM riboflavin, 50 μM potassium phosphate buffer (pH 7.8), and 50 μL enzyme extract, which was irradiated under a light irradiance of 50 $\mu mol \cdot m^2 \cdot s^{-1}$ for 20 min, and absorbance was measured at 560 nm, non-irradiated reaction solution was used as a blank. Peroxidase (POD) activity was analyzed as described by Chance and Maehly (1955), with slight modifications and absorbance was measured at 470 nm.