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GENG, Xing-Min

Department of Landscape Plant, Nanjing Forestry University

YANG, Qiu-Yu

Department of Landscape Plant, Nanjing Forestry University

YUE, Yuan

Department of Landscape Plant, Nanjing Forestry University

OZAKI, Yukio

Laboratory of Horticultural Science, Department of Bioresource Sciences, Faculty of
Agriculture, Kyushu University

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Effects of High Temperature on Photosynthesis, Membrane Lipid Peroxidation and Osmotic Adjustment in Four *Rhododendron* Species

Xing-Min GENG^{1*}, Qiu-Yu YANG¹, Yuan YUE¹ and Yukio OZAKI

Laboratory of Horticultural Science, Department of Bioresource Sciences,
Faculty of Agriculture, Kyushu University, Fukuoka 819-0395, Japan

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In order to make clear the response mechanism of *Rhododendron* species to high temperature, two-year-old seedlings of *R. fortunei*, *R. ovatum*, *R. simsii* and *R. mariesii* were heat-stressed at 42°C / 30°C (day/night) for 24 h. Four *Rhododendron* species under heat stress showed significant differences in heat injury indices (HI), and *R. fortunei* was more sensitive to high temperature than the other three species. The lipid peroxidation of *R. fortunei* seedling was most serious indicated by malondialdehyde (MDA), which could be related to the significant decrease of superoxide dismutase (SOD) activity in *R. fortunei* seedlings. In the other three heat-resistant species, the content of soluble proteins and SOD activity increased under heat stress. The accumulation of proline and soluble sugars was found in greater heat-resistant species, which could alleviate osmotic stress of rhododendron seedlings under high temperature. Net photosynthesis (P_N) rates for four species were inhibited at different levels due to heat stress, but reduction proportion of P_N for *R. fortunei* was highest due to mainly nonstomatal factors.

Key words: compatible osmolytes, heat stress, photoinhibition, *Rhododendron*, superoxide dismutase

INTRODUCTION

In order to cope with heat stress, plants implement various mechanisms, including maintenance of membrane stability, scavenging of reactive oxygen species (ROS), accumulation and adjustment of compatible solutes, induction of differentially accumulated proteins or up-regulation of some protein. ROS production can be accelerated by heat stress (Larkindale and Knight, 2002) and other environmental stresses (Cruz de Carvalho, 2008). Excessive ROS causes oxidative stress, leading to lipid peroxidation, protein degradation, enzyme inactivation, and DNA damage (Apel and Hirt, 2004; Kai *et al.*, 2012). ROS toxicity can be alleviated by ROS-scavenging enzymes include both enzymatic antioxidants such as superoxide dismutase (SOD) and other non-enzymatic antioxidants. And keeping the steady-state level of ROS enables ROS to act as signaling molecules to control and regulate plant growth, development, contributes to the responses to the environmental stresses (Mittler *et al.*, 2004; Mittler, 2017).

Under the stresses, different plant species may accumulate a variety of osmolytes such as sugars and sugar alcohols (polyols), proline, and tertiary sulphonium compounds (Wahid *et al.*, 2007). Accumulation of proline may contribute to enhanced stress tolerance of plants (Kaushal *et al.*, 2011; Wilson *et al.*, 2014) by protecting some vital enzymes related to carbon and oxidative metabolism (Kaushal *et al.*, 2011). Supplementation with proline considerably reduced H_2O_2 production and showed decrease in oxidative injury coupled to elevated levels of antioxidants in sugarcane (Rasheed *et al.*, 2011). But proline that accumulated in plants subjected

to drought did not accumulate in *Arabidopsis* during a combination of drought and heat stress, sucrose replaces proline in plants as the major osmoprotectant (Rizhsky *et al.*, 2004). Similarly, accumulation of soluble sugars under heat stress has been reported in sugarcane, which entails great implications for heat tolerance (Wahid and Close, 2007). Sugars may protect the chloroplast membranes partially or completely (Santarius, 1973).

Net photosynthesis (P_N), in particular, is one of the most heat-sensitive processes governing plant growth (Bjorkman *et al.*, 1980). And P_N has been shown to correlate with heat tolerance of *Rhododendron* plants (Ranney *et al.*, 1995), a combination of stomatal and nonstomatal limitations on P_N at high temperatures resulted in difference in heat tolerance. Photoinhibition in evergreen leaves can be brought about by oxidative damage to PSII by ROS (Adams III *et al.*, 2004). However, ROS has been suggested that it does not result in photodamage directly, but inhibits the repair of photo-damaged PSII by suppressing the synthesis of PSII proteins in chloroplasts (Murata *et al.*, 2007; Takahashi and Murata, 2008).

The genus *Rhododendron* (± 1000 species) is divided into eight subgenera (Chamberlain *et al.*, 1996). Wild *Rhododendron* species are most distributed in high mountains, they favor cold environmental conditions, and high temperature has been the primary obstacle to cultivation and propagation. Therefore, the research on mechanism of heat tolerance and possible strategies for improving heat tolerance is imperative. In this present research, photoinhibition, the accumulation of osmolytes, the production and scavenging of ROS were investigated to screen the physiological indices, which may be related to heat tolerance of *Rhododendron* seedlings.

¹ Department of Landscape Plant, Nanjing Forestry University, Nanjing 210037, Jiangsu Province, People's Republic of China;

* Corresponding author (E-mail: xmgeng76@163.com)

MATERIALS AND METHODS

Plant materials and heat stress

Two-year-old seedlings of four *Rhododendron* species including *R. fortunei*, *R. ovatum*, *R. simsii* and *R. mariesii* were used as plant materials. According to the method of heat stress as reported in previously (Gu *et al.*, 2016), the seedlings were heat-stressed at 42°C/30°C (day/night) for 24 h and light intensity of 4000 LX/0LX (day/night). As a control, the other potted plants were placed at 28°C/18°C (day/night). After the heat stress experiments, the plants were moved back to the greenhouse of 23–33°C.

The plants were continuously observed every day, and the injury index was recorded on the first day after heat stress. The extent of heat injury was divided six grades, 0: no heat injury; 1: yellowing or scorching of 1–2 leaves; 2: yellowing or scorching of 2–4 leaves, one leaf wither; 3: 2 leaves wither; 4: more than 2 leaves wither; 5: the whole plant died. The heat injury index was calculated according to the following formula: Heat injury index = (\sum injury grades \times corresponding number of seedlings) / the highest grade \times total number of seedlings

Determination of other physiological indices

Leaves were collected from heat-stressed and control seedlings on the fifth day after heat stress, these samples were stored at –80°C for the determination of proline, MDA, soluble proteins and SOD activity. Proline was determined by the specific colorimetric method at 515 nm, which measures the red and stable product from reaction of proline and hydrindantin dehydrate under acidic condition. Malondialdehyde (MDA) was determined by the method of thiobarbituric acid (TBA) as previously described (Geng *et al.*, 2009). The content of soluble proteins was determined according to the method of Bradford (1976) using bovine serum albumin as standard.

Samples were prepared for SOD enzyme analyses by homogenizing 0.3 g of frozen samples in 5 ml of an ice-cold 50 mM sodium phosphate buffer (pH 7.8). The extract was centrifuged at 4°C for 20 min at 12500/g. The supernatant was then used for enzyme assays. Total activity of SOD in the extract was determined by measuring the inhibition in the photochemical reduction of nitroblue tetrazolium (NBT). One enzyme unit was defined as the amount of enzyme required to cause 50%

inhibition of the rate of NBT reduction measured at 560 nm.

Glucose was used to prepare a standard solution to make a calibration curve for determination of soluble sugar contents. 0.3 g of leaves was placed in 20 mL distilled water. After boiling for 30 min followed by cooling to room temperature and filtration with filter paper, the sample filtrates were collected. Dilute anthrone sulfuric acid reagent (5 mL) was mixed with each standard or sample solution (0.1 mL), immediately placed in a boiling water bath for 1 min, and then cooled to room temperature. The absorbance at 630 nm was measured.

Photosynthesis measurement

P_n , stomatal conductance (Gs), intercellular CO₂ concentration (Ci) and transpiration rate (Tr) were measured with LI-6400 (Licor company, America). The values reported were the mean of two measurements per plant taken on marked leaves of three different plants. The measurements were taken during the first day and fifth day after heat stress between 10:00 AM and 11:00 PM.

Statistical analyses were carried out by using SPSS17.0 software. The Duncan's multiple range test was applied to test the significance in differences among treatments ($P > 0.05$).

RESULTS

Morphological symptoms of heat injury

To compare heat tolerance of four *R.* species and observe the differences of morphological symptoms in responses to heat stress, the rhododendron seedlings were exposed to high temperature of 42°C/30°C (day/night) for 24 h. The results showed phenotypic appearances of four *R.* species under heat stress were distinguishable (Fig. 1). After heat stress, *R. fortunei* seedlings exhibited significant heat injury symptoms related to chlorosis of leaf apex, leaf rolling up. *R. simsii* leaves slightly wilted after heat stress, there were a few middle leaves appearing etiolated symptom, but did not aggravate in the next few days. Some filemot spots appeared on the lower old leaves of *R. ovatum*, central functional leaf margin turned red, and new leaves curled. Heat injury symptoms on *R. mariesii* seedlings occurred mainly in the newly folded leaf buds of tip stem, the leaf apex scorched and central functional leaves exhibited red block.

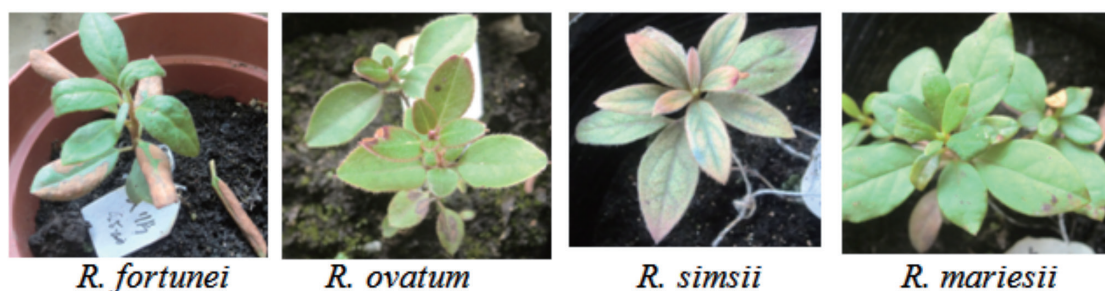


Fig. 1. Heat injury symptoms of four *Rhododendron* species on the first day after heat stress.

Four species under test showed significant difference in heat injury indices (HII) in the stress environment (Table 1). HII of *R. fortunei* seedlings was the highest, followed by *R. mariesii* seedlings. HII of *R. ovatum* and *R. simsii* seedlings was lower, 0.25 and 0.275 respectively.

Table 1. Effects of heat stress on the injury index of four *Rhododendron* species

	<i>R. fortunei</i>	<i>R. ovatum</i>	<i>R. simsii</i>	<i>R. mariesii</i>
Heat stress	0.5	0.25	0.275	0.325
Control	0.2	0.075	0.075	0.225

Lipid peroxidation of cell membrane and change of SOD activity

SOD enzymes catalyze the dismutation of superoxide into oxygen and hydrogen peroxide, provide the first line of defense against ROS in various subcellular compartments. To estimate the oxidative damage of four *Rhododendron* seedlings induced by excessive ROS under heat stress, the content of MDA, an important bio-marker of oxidative stress-induced damage (Asada, 1998), was determined. The results showed that MDA content in *R. fortunei* seedlings increased, no significant changes in MDA content in *R. ovatum*, *R. simsii* and *R. mariesii* leaves were observed (Fig. 2). The result indicated that *R. fortunei* suffered from more serious oxidative damage than other three species. It may be related to the change of SOD activity under heat stress. In our study, we found that heat stress resulted in the decrease of SOD activity in *R. fortunei* seedlings, but increased in *R. mariesii* seedlings. Data analyses showed the promoted effects were significant only in *R. mariesii* seedlings (Fig. 3).

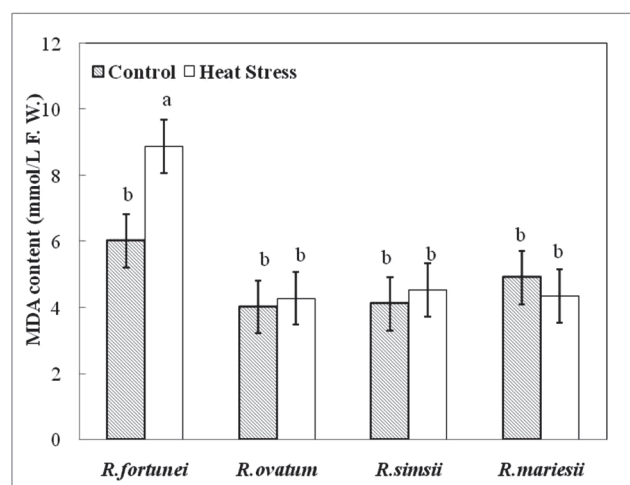


Fig. 2. Changes of MDA content of four *Rhododendron* species under heat stress. The values presented are the means \pm standard deviations (SD) of three replicates ($n = 3$). The Duncan's multiple range test was applied to compare significant differences between the control and heat stressed seedlings of the four *Rhododendron* species. Letters a and b indicate significant difference at the $p < 5\%$ level.

Accumulation of proline, soluble sugars and proteins

Proline is known to occur widely in higher plants and normally accumulates in large quantities in response to environmental stresses (Kishor *et al.*, 2005). In the four *Rhododendron* species, proline content in *R. simsii* seedlings was higher than other three species (Fig. 4). Upon exposure to heat stress, leaf proline content showed a significant increase in *R. ovatum* (70.5%), *R. fortunei* (52.4%), *R. simsii* (42.2%) whereas a non-significant increase (8.6%) was observed in heat-stressed *R. mariesii* seedlings compared to the control group.

The level of soluble sugars in *R. fortunei* was lower than the level in *R. mariesii* in control (Fig. 5). After

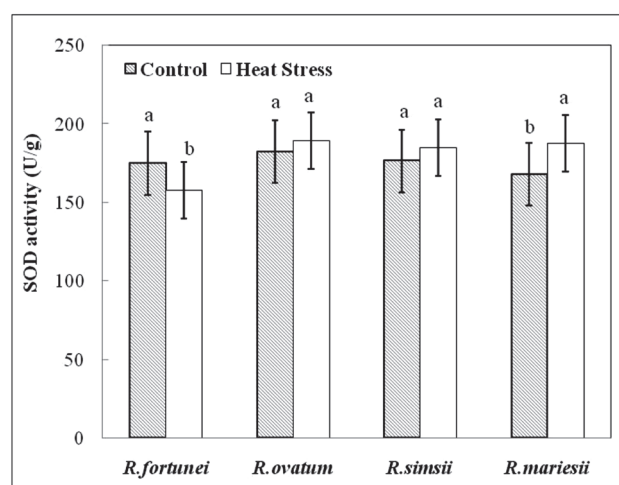


Fig. 3. Changes of SOD activity of four *Rhododendron* species under heat stress. The values presented are the means \pm standard deviations (SD) of three replicates ($n = 3$). The Duncan's multiple range test was applied to compare significant differences between the control and heat stressed seedlings of the four *Rhododendron* species. Letters a and b indicate significant difference at the $p < 5\%$ level.

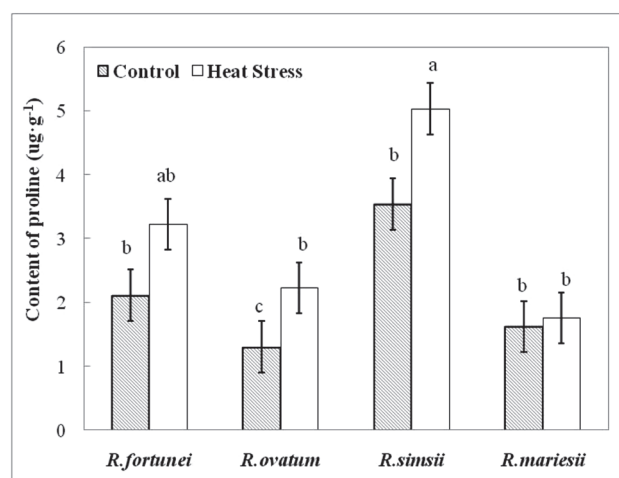


Fig. 4. Changes of proline content of four *Rhododendron* species under heat stress. The values presented are the means \pm standard deviations (SD) of three replicates ($n = 3$). The Duncan's multiple range test was applied to compare significant differences between the control and heat stressed seedlings of the four *Rhododendron* species. Letters a and b indicate significant difference at the $p < 5\%$ level.

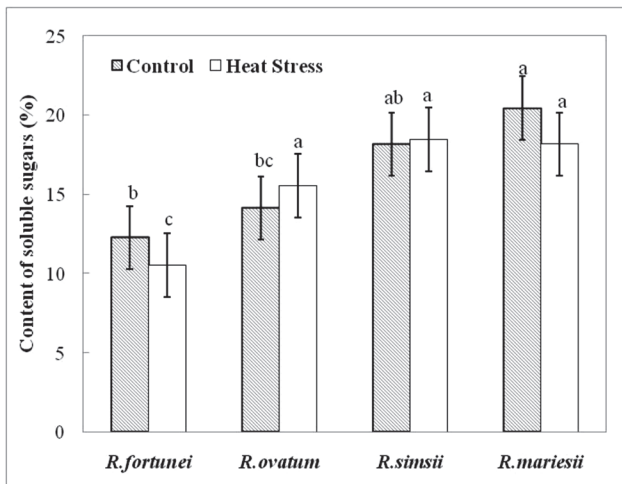


Fig. 5. Changes of content of soluble sugar in four *Rhododendron* species under heat stress. The values presented are the means \pm standard deviations (SD) of three replicates ($n = 3$). The Duncan's multiple range test was applied to compare significant differences between the control and heat stressed seedlings of the four *Rhododendron* species. Letters a and b indicate significant difference at the $p < 5\%$ level.

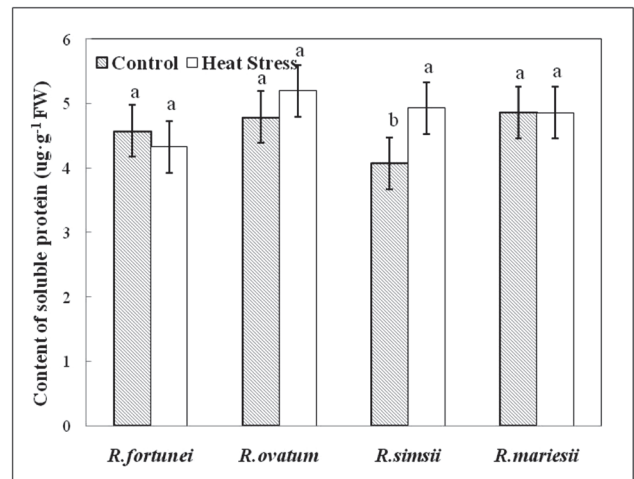


Fig. 6. Changes in content of soluble protein in four *Rhododendron* species under heat stress. The values presented are the means \pm standard deviations (SD) of three replicates ($n = 3$). The Duncan's multiple range test was applied to compare significant differences between the control and heat stressed seedlings of the four *Rhododendron* species. Letters a and b indicate significant difference at the $p < 5\%$ level.

Table 2. Photosynthetic indices of *Rhododendron* leaves under heat stress

	Treatment	<i>R. fortunei</i>	<i>R. ovatum</i>	<i>R. simsii</i>	<i>R. mariesii</i>
P_N ($\mu\text{mol}/\text{m}^2 \cdot \text{s}^{-1}$)	Control	2.216 \pm 0.15 ^z	3.460 \pm 0.16	3.578 \pm 0.1	3.394 \pm 0.12
	HS	0.853 \pm 0.05	2.827 \pm 0.2	2.162 \pm 0.14	2.489 \pm 0.14
C_i ($\text{mmol}/\text{m}^2 \cdot \text{s}^{-1}$)	Control	318.9 \pm 10.05	283.445 \pm 17.86	249.679 \pm 11.13	256.91 \pm 14.87
	HS	359.67 \pm 12.37	296.852 \pm 18.87	262.251 \pm 16.83	272.438 \pm 9.35
G_s ($\mu\text{mol}/\text{m}^2 \cdot \text{s}^{-1}$)	Control	0.059 \pm 0.005	0.055 \pm 0.003	0.048 \pm 0.003	0.047a \pm 0.007
	HS	0.063 \pm 0.002	0.049 \pm 0.006	0.033 \pm 0.006	0.035 \pm 0.007
T_r ($\text{mmol}/\text{m}^2 \cdot \text{s}^{-1}$)	Control	1.577 \pm 0.14	2.017 \pm 0.04	1.993 \pm 0.05	1.711 \pm 0.13
	HS	1.579 \pm 0.1	1.652 \pm 0.09	1.341 \pm 0.07	1.286 \pm 0.14

^z the means \pm standard deviation

heat stress the decrease of soluble sugars in *R. fortunei* was observed. Heat stress did not affect the content of soluble sugars in *R. simsii* seedlings, but *R. ovatum* accumulated more soluble sugars than the responding control plants.

As shown in Fig. 6, the content of soluble proteins in *R. simsii* leaves was the lowest in control temperature, but only *R. simsii* leaves after heat stress appeared the significant increase in the content of soluble proteins. The content in *R. fortunei* and *R. mariesii* leaves decreased by 5.42% and 0.13%, respectively.

Effects of heat stress on photosynthesis indices

Changes of photosynthetic indices including P_N , G_s , C_i and T_r were measured 1 d (Table 2) and 5 d (data not shown) after heat stress. One day after heat stress P_N rates for *R. simsii*, *R. ovatum*, *R. mariesii* and *R. fortunei* were 3.6, 3.5, 3.4 and 2.2 $\mu\text{mol}/\text{m}^2 \cdot \text{s}^{-1}$ respectively at control temperature. P_N rates for four species were decreased at different levels due to heat stress, but

reduction proportion of P_N for *R. fortunei* was 61.5%, and most significantly, the other three species including *R. simsii*, *R. ovatum* and *R. mariesii* decreased by 39.6%, 18.3% and 26.7% respectively.

High temperature decreased G_s of *R. simsii*, *R. ovatum* and *R. mariesii*, T_r of these three species was inhibited. Although G_s of *R. fortunei* increased by 7.34% 1 d after heat stress, however, its transpiration rate did not change compared to control plants. Heat stress increased internal leaf CO_2 concentrations of four species, and the increase in *R. fortunei* was most significant, its increasing rate was 12.79%.

DISCUSSION

According to heat injury indexes and symptom of four *R.* species (Table 1 and Fig. 1), *R. fortunei* is the most susceptible for heat stress, *R. simsii* and *R. ovatum* have higher heat tolerance than *R. mariesii*. MDA level in *R. simsii*, *R. ovatum* and *R. mariesii* seedlings

did not change under heat stress, however, significantly decreased in *R. fortunei* seedlings due to heat stress (Fig. 2), which indicated high temperature resulted in the increase of ROS level (Nagesh and Devaraj, 2008; Kumar *et al.*, 2011) and serious oxidative damage of cell membrane in *R. fortunei* seedlings (Moller *et al.*, 2007). Heat-resistant *Rhododendron* species keep more stable anatomical structure under heat stress than heat-sensitive species (Gu *et al.*, 2016; Shen *et al.*, 2017).

After heat stress, the increase of SOD activity of three heat-tolerant species was observed, only SOD activity of *R. fortunei* seedlings decreased (Fig. 3). SOD functions as the first defense against ROS, whose activity contributes to the scavenging of the excessive ROS and increase the stress tolerance. The decrease of SOD activity in *R. fortunei* seedlings should be directly related to the accumulation of MDA and serious cell membrane damage of *R. fortunei* seedlings. The important role of antioxidant enzymes in response of rhododendron seedlings to high temperature have also been reported by Shen *et al.* (2017). So genetic transformation approach by adjusting ROS scavenging systems of rhododendrons can enhance oxidative stress tolerance. Similar researches have been reported in other plants. Overexpression of Cu/Zn SOD and APX induced thermotolerance to 42°C in transgenic potato plants (Kim *et al.*, 2010). Overexpression of SOD and glutathione reductase (GR), were reported to result in an increased resistance to drought, ozone, low temperature, and high light stress (McKersie *et al.*, 2000; Panchuk *et al.*, 2002; Van Camp *et al.*, 1996).

In essence, accumulation and adjustment of compatible solutes are an important adaptation toward heat-stress tolerance by plants. In this study, proline level increased in the four *Rhododendron* species after heat stress (Fig. 4), and the increase was more significant in heat-tolerant species. Accumulation of proline under stress in many plant species has been correlated with stress tolerance (Kaushal *et al.*, 2011; Wilson *et al.*, 2014), and its concentration has been shown to be generally higher in stress-tolerance plants than in stress-sensitive plants (Ashraf and Foolad, 2007; Yuan *et al.*, 2011; Chen *et al.*, 2012). But in the present study, the further data analysis indicated there was not significant correlation between proline accumulation and heat tolerance of rhododendron seedlings, maybe because heat-sensitive species such as *R. fortunei* accumulated more proline than *R. ovatum* and *R. mariesii* in control and stress temperature (data analysis not shown). Lv *et al.* (2011) indicated that proline accumulation under heat stress decreases the thermotolerance, probably by increasing ROS production via the Pro/P5C cycle and inhibition of ABA and ethylene biosynthesis.

The accumulation of soluble sugars induced by heat stress was investigated only in heat-tolerant *Rhododendron* species including *R. simsii* and *R. ovatum* (Fig. 5), their content in *R. fortunei* seedlings decreased obviously after heat stress. And the data analysis showed that heat tolerance of *Rhododendron* seedlings was closely related to the level of soluble sug-

ars. Similarly, accumulation of soluble sugars under heat stress has been reported in sugarcane, which entails great implications for heat tolerance (Wahid and Close 2007). High temperature caused the increase of soluble leaf protein in heat-tolerant *Rhododendron* species (Fig. 6). This increase in total soluble proteins under heat stress may be the induction of stress proteins such as dehydrin proteins (DHNs) and heat shock proteins (HSPs). Further, as suggested by Wahid and Close (2007), most of stress-induced proteins are soluble in water and therefore contributes to stress tolerance presumably via hydration of cellular structures.

P_N was inhibited in the four *R.* species (Table 2). Ranney *et al.* (1995) suggested that the decrease of P_N was due to the combination of stomatal and nonstomatal factors. In the present study, heat stress at 42°C/30°C (day/night) for 24 h resulted in the decrease of G_s and the increase of C_i in *R. mariesii*, *R. simsii* and *R. ovatum* seedlings, which indicated the decline in photosynthesis under heat stress appeared to mainly result from nonstomatal factors. G_s and P_N are inhibited by moderate heat stress in many plant species due to the decreases in the activation state of rubisco (Crafts-Brander and Salvucci, 2002; Morales *et al.*, 2003). Heat stress resulted in the damage of cell membrane of *R. fortunei* seriously (Fig. 2), which may be main reason of the decrease of P_N . It has been reported that the damage of the structural organization of thylakoids may be main reason of the decrease of P_N under stresses (Karim *et al.*, 1997 and Vani *et al.*, 2001).

AUTHOR CONTRIBUTIONS

Xing-Ming GENG carried out substantial contribution to the concept and design on this paper. Qiu-Yu YANG and Yuan YUE carried out analysis and interpretation of the data. Yukio OZAKI verified the data. All authors contributed in editing the manuscript and approved the final version.

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