Effects of Salicylic Acid and Calcium Chloride on Heat Tolerance in *Rhododendron* ‘Fen Zhen Zhu’

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ADDITIONAL INDEX WORDS. injurious level, withered leaf, chlorophyll content, antioxidant enzymes, malondialdehyde, soluble protein

ABSTRACT. *Rhododendrons* (*Rhododendron*) are ornamental plants that exhibit poor thermotolerance. Salicylic acid (SA) and Ca^{2+} regulate the physiological and biochemical mechanisms in plants adapted to adverse environmental conditions. This study investigated the role of SA and CaCl_2 in managing heat tolerance of *Rhododendron* ‘Fen Zhen Zhu’. Plants of the triennial *Rhododendron* ‘Fen Zhen Zhu’ were pretreated with SA and CaCl_2 , alone and combined. Following this pretreatment, the plants were subjected to 38/30 °C (day/night) incubation for 6 days, and then allowed to recover for 20 days under 25/17 °C (day/night) in a chamber. Changes in morphology were observed and recorded. Data were collected on plant chlorophyll content, malondialdehyde (MDA) content, H_2O_2 level, antioxidant enzyme activity, and total soluble protein content. The results revealed that the plant growth was considerably affected by heat stress, the leaves became brown and withered, and the plant defoliated. Under heat stress, chlorophyll content and total soluble protein levels decreased. Peroxidase (POD) activity and superoxide dismutase (SOD) also decreased, whereas the H_2O_2 and MDA content increased. Individual or combined application of SA and CaCl_2 had a positive effect on plant growth, chlorophyll content, total soluble protein levels, and enzymatic antioxidant activity under heat stress. In general, the effect of the combined application of SA and CaCl_2 was superior to individual application. In addition, treatment with high CaCl_2 concentrations effectively alleviated the decrease in chlorophyll content. However, at low SA and CaCl_2 concentrations, SOD and POD activity and total soluble protein accumulation increased whereas MDA and H_2O_2 levels decreased. These results suggest that SA and CaCl_2 may interact to alleviate heat stress.

Global climate change may not only increase CO_2 concentration, but also sustain elevated temperatures (Intergovernmental Panel on Climate Change, 2013; Lavania et al., 2015). As a result, the distribution pattern of plant communities will be negatively affected and the scenario where the vegetation in different landscapes is drastically affected over a period of time might become a reality (Grover et al., 2013). Although some ornamental plants have the capacity to evolve and cope with temperature fluctuations, this capacity might not keep pace with global warming, reduced ornamental value, and economic losses (Tao et al., 2015). Heat stress not only affects the phenotype of a plant, causing leaf etiolation and wilting, but it alters the anatomy, physiology, and photosynthesis capability of plants as well, even at the genetic level (Chen et al., 2014; Mishkind et al., 2009; Perez et al., 2009). Heat stress induces overproduction of reactive oxygen species (ROS) in plant tissues, thus seriously affecting the balance between the generation and degradation of ROS. Furthermore, plants respond quickly to various abiotic stresses by regulating complex physiological, biochemical, and molecular mechanisms for acclimation and adaptation to stress (Chandel et al., 2013; Chen et al., 2014). Antioxidants act as ROS scavengers to mitigate oxidative injury and to maintain metabolic functions; these include antioxidant enzymes and nonenzymatic low molecular weight antioxidant molecules (Semida and Rady, 2014a, 2014b).

Heat tolerance can be improved by genetic selection as well as with the use of exogenous regulators, which aid the adaptation of physiological response in plants. SA and Ca^{2+} are recognized as signal molecules known for their roles in plant adaptation to changing environments (Janda and Ruellan, 2015; Laanemets et al., 2013). SA, a small phenolic compound, plays a significant role in regulating physiological processes in plants involved in response to various abiotic stress such as drought (Nazar et al., 2015), cold (Sibozaa et al., 2014), heat (Boatwright and Karolina, 2013), intense light (Zhao et al., 2011), and salinity (Agami, 2013; Manaa et al., 2014; Rady and Mohamed, 2015). Application of SA enhances the photosynthetic rate and maintains the stability of the cell membrane through regulating enzymatic activity (Ghanta et al., 2014), which eventually alleviated oxidative stress (Wasti et al., 2012), but the underlying mechanism remains unclear (Wang et al., 2014). This effect of SA on oxidative stress response was observed in a variety of plants: *Solanum lycopersicum* (Manaa et al., 2014; Wasti et al., 2012), *Arabidopsis thaliana* (Borsani et al., 2001), *Vigna radiata* (Singh et al., 2014), and *Triticum aestivum* (Kumar et al., 2015; Li et al., 2013; Zhao et al., 2011). In different *T. aestivum* cultivars, variations in the expression levels of heat stress-associated proteins have been documented (Kumar et al., 2012). In contrast, there are limited studies on heat tolerance of flowers, especially the heat tolerance of *Rhododendron*.

Several studies have shown that Ca^{2+} is of pivotal importance for various regulatory mechanisms in plants as a secondary messenger, controlling a variety of cellular processes such as stomatal movements (Hubbard et al., 2012; Laanemets et al., 2013), antioxidant enzyme activity, and lipid peroxidation under multiple stresses (Khan et al.,

Received for publication 2 Feb. 2016. Accepted for publication 6 May 2016. We thank the International Science and Technology Cooperation Foundation (A213021501), the National Natural Science Foundation of China (k305021110), and the Shaanxi Natural Science Foundation (2012JQ3008).

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2010). Thus, exogenous application of Ca^{2+} has become an important research topic due to concerns about the effects of climate change on plants. Cytosolic Ca^{2+} content increases under heat stress, which might alleviate heat injury and initiate cellular repair (Bamberg et al., 1998; Gong et al., 1998). The activities of antioxidant enzymes in plants pretreated with CaCl_2 either increase or do not decrease to the same extent as in the control group (Tan et al., 2011). It has been shown that Ca^{2+} treatment prevents solute leakage from the cytoplasm, stabilizing cell membrane surfaces, and regulating hormone metabolism in plants (Hirschi, 2004). However, the effect of Ca^{2+} treatment on thermotolerance of *Rhododendron* is poorly studied.

Individual exogenous application of SA and Ca^{2+} in mitigating heat stress has been studied extensively (Manaa et al., 2014). Combination application of SA and Ca^{2+} improved growth, photosynthetic pigment concentration, and mineral nutrition in *S. lycopersicum* during salt stress (Manaa et al., 2014). Studies on SA and Ca^{2+} , individual or combined, for *T. aestivum* under salt stress revealed that the combined application proved more effective in reducing oxidative stress (Al-Wahaibi et al., 2012). This research suggests that SA and Ca^{2+} interact to reduce stress. Other studies also support this conclusion (Guo et al., 2015; Xu et al., 2008).

Rhododendron is one of the most popular ornamental plants worldwide, with both medicinal and edible value (Dampc and Luczkiewicz, 2013). At present, genetic variation (Li et al., 2015; Zhao et al., 2012), flowering-related gene manipulation (Cheon et al., 2012), selective breeding of some species (Shen et al., 2015), and the identification of the major chemical constituents in leaf were the main subjects in studies on *Rhododendron* (Lou et al., 2015). Influenced by genetic factors, this genus is widely distributed at high altitudes, in cold and moist areas. Thus, heat intolerance is a major constraint in *Rhododendron* cultivation and landscape applications. *Rhododendron* $\times$ *hybridum* was obtained by hybridization over several generations: *Rhododendron simsii*, *Rhododendron phoeniceum*, *Rhododendron indicum*, and *Rhododendron capitatum*. *Rhododendron* 'Fen Zhen Zhu' is a compact plant, with pink flowers, and an early, long flowering period, from January to March. Despite its high ornamental value, it has poor thermal resistance. A limited number of studies have investigated heat resistance of *Rhododendron*, and the influence of SA and Ca^{2+} pretreatment on heat tolerance in ornamental plants, including *Rhododendron*, has rarely been reported. Because of its poor thermal resistance and difficulty in growing it in gardens, commercial *Rhododendron* is currently cultivated in greenhouses, thus requiring more energy, manpower, and resources. For these reasons, the influence of SA and CaCl_2 treatments, applied alone and in combination, on *Rhododendron* 'Fen Zhen Zhu' was assessed by observing changes in physiology and morphology of plants exposed to high temperatures. The results will provide theoretical basis and some practical recommendations for improving thermotolerance in *Rhododendron*, which can be used to develop management practices for its cultivation in garden and to reduce energy consumption and enhance cultivation in summer. In addition, the effect of SA and CaCl_2 application in *Rhododendron* will provide basis for further studies on molecular mechanisms responsible for the interaction of the two compounds.

Materials and Methods

PLANT MATERIAL, GROWTH CONDITIONS, AND TREATMENTS. *Rhododendron* 'Fen Zhen Zhu', collected from a greenhouse in the city of Dandong, were grown in plastic pots (12 cm high, diameter 16 cm at the top, and 10 cm at the bottom) in a greenhouse in Yangling, Shaanxi province, China. The culture substrate was a 1:1 (v:v) mixture of peat and pine needle mulch. In July 2015, plants of similar stature, height ≈ 28 –30 cm and crown 18–21 cm in diameter, were selected and transferred to a growth chamber [25/17 °C (day/night), 80% relative humidity, photoperiod of 14 h light/10 h dark, and light irradiance of 150 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$] for 1 week. A tray was placed under the pots to retain water.

After 7 d, the leaves were sprayed daily until saturation with different concentrations of SA (0.5, 1.0, and 2.0 mM) and CaCl_2 (3.5, 7.0, and 10.5 mM) solutions alone and in combination, between 0800 and 0900 HR, for 3 d (consecutive). Plants sprayed with water were used as a control. In combined treatments, SA spray was applied first, followed by CaCl_2 spray, and left to dry (Wang, 2014). Different treatments are listed in Table 1. After 3 d, all plants were exposed to a temperature of 38/30 °C (day/night) for 6 d, and then allowed to recover at a temperature of 25/17 °C (day/night) until new leaves appeared. The plants were managed regularly and watered frequently to keep the growth substrate moist during the experiment. After 6 d of heat stress, and 20 d of recovery period, leaf samples were collected for physiological, biochemical, and phenotypical analysis.

MORPHOLOGICAL ANALYSIS. Throughout the experiment, the morphology of the plants was observed and rated with Roman numbers I–V based on the level of heat-induced injury (Fig. 1; Table 2).

LEAF CHLOROPHYLL INDEX. The pigments were extracted from fresh leaves of experimental plants using 80% acetone as described by Lichtenthaler (1987). Absorption of the chlorophyll and carotenoids present in the extract was determined with a spectrophotometer (ultraviolet-2450; Shimadzu, Tokyo,

Table 1. Different treatments of salicylic acid (SA) and/or CaCl_2 applied to the leaves of *Rhododendron* 'Fen Zhen Zhu' for improving heat resistance.

Treatment no.	Treatments	
	SA (mM)	CaCl_2 (mM)
1	0.0	0.0
2	0.5	0.0
3	1.0	0.0
4	2.0	0.0
5	0.0	3.5
6	0.0	7.0
7	0.0	10.5
8	0.5	3.5
9	0.5	7.0
10	0.5	10.5
11	1.0	3.5
12	1.0	7.0
13	1.0	10.5
14	2.0	3.5
15	2.0	7.0
16	2.0	10.5

Fig. 1. Injurious levels of heat stress, salicylic acid (SA) and/or CaCl_2 application on morphological appearance of *Rhododendron* 'Fen Zhen Zhu' after 0 and 6 d of high temperature (38°C) and 20 d recovery of optimum temperature (25°C): (I) normal growth; (II) 20% of the leaves became brown and withered; (III) 40% of the leaves became brown, withered, and defoliated; (IV) 60% of the leaves became brown and withered; (V) 80% of the leaves became brown, withered, and defoliated.

Table 2. Different damage level of heat stress, salicylic acid (SA) and/or CaCl_2 spray concentration on morphological appearance of *Rhododendron* 'Fen Zhen Zhu' after 0 and 6 d of high temperature (38°C) and 20 d recovery of optimum temperature (25°C).

Level no.	Morphological appearance
I	Normal growth
II	20% of the leaves became brown and withered
III	40% of the leaves became brown, withered, and defoliated
IV	60% of the leaves became brown and withered
V	80% of the leaves became brown, withered, and defoliated

Japan). The total content was calculated using the following equations:

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

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

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

$$C_{x+c} = \frac{1000A_{470} - 1.82C_a - 85.02C_b}{198}$$

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

ROS AND OSMOREGULATORY SYSTEM. Lipid peroxidation levels were assessed using MDA according to the method described by Madhava Rao and Sresty (2000). Fresh leaves weighing 0.1 g were homogenized using a prechilled mortar and pestle with 10 mL 5% (w/v) trichloroacetic acid, and centrifuged at $12,000 g_n$ 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 to 100°C for 30 min, then rapidly cooled to 4°C in an ice bath, and then centrifuged at $10,000 g_n$ for 10 min at 4°C . The absorbance of the supernatant was measured at 532 and 600 nm. The MDA content was calculated using the extinction coefficient of $155 \text{ mm}^{-1} \cdot \text{cm}^{-1}$. The H_2O_2 and soluble protein content in leaves were measured according to the manufacturer's instructions of the Trizol reagent with H_2O_2 and Coomassie brilliant blue (Jiancheng, Nanjing, China). The products were stored at 4°C .

ENZYMATIC ANTIOXIDANT SYSTEM.

A crude enzyme was extracted from the homogenate of 0.1 g fresh leaf tissue in a 50 mM potassium phosphate buffer (pH 7.8) with a pre-cooled mortar and pestle. The homogenate was centrifuged at $12,000 g_n$ for 20 min at 4°C . The supernatant was used to determine enzymatic activity. According to the methods described by Giannopolitis and Ries (1977), SOD activity was determined by measuring its ability to inhibit the photoreduction of 50% nitro blue tetrazolium (NBT). 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. The reaction solution was irradiated under a light irradiance of $50 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ for 20 min, and the supernatant absorbance was measured at 560 nm; nonirradiated reaction solution was used as a blank. One unit of SOD activity was defined as the amount of enzyme that caused a 50% inhibition of NBT photoreduction. POD activity was analyzed as described by Chance and Maehly (1955), with slight modifications, the supernatant absorbance was measured at 470 nm. The assay reaction solution contained 0.2 M potassium phosphate buffer (pH 6.0), 30% (v/v) H_2O_2 , 100% guaiacol solution, and 50 μL enzyme extract for a total of 3 mL. One unit of POD activity was defined as the amount of enzyme that oxidized micromoles of guaiacol radical per minute.

STATISTICAL ANALYSIS. The data analysis was performed using statistical software (SPSS version 22.0; IBM Corp., Armonk, NY), and Duncan's multiple range test was used to compare treatments when the analysis of variance showed a significant difference between means at $P \leq 0.05$; a probability value less than 0.05 was regarded as statistically significant (Al-Whaibi et al., 2012). Means and standard errors were calculated from three replicates.

Results

PLANT GROWTH. After 6 d of exposure to heat stress, the plants sprayed with SA and CaCl_2 alone or combined, showed some changes in morphology: the leaves turned brown, withered, and the plants defoliated with different levels of injury (Table 3), depending on the treatments. Thus, the plants treated with exogenous SA and CaCl_2 , alone or in combination, were markedly protected from heat-induced growth inhibition. In contrast, the control group had the highest degree of injury among all the treatments, with 80% of the leaves withering and defoliating. The control group also exhibited the largest damage rate, 25%, compared with its growth rate under optimal temperature. For all the treatments, the results revealed that the damage rate in plants treated with a combination of SA and CaCl_2 was lower than that in plants treated with SA and CaCl_2 alone. Treatment groups 8, 9, and 11 improved the most; the damage rate of these plants was only 4.2%.

PIGMENT CONTENT. As presented in Fig. 2, chlorophyll *a*, total chlorophyll, and carotenoid content degraded in most of

Table 3. Injurious level and damage rate of heat stress, salicylic acid (SA) and/or CaCl₂ spray concentration on morphological appearance of *Rhododendron* 'Fen Zhen Zhu' after 0 and 6 d of high temperature [38 °C (0 d and 6 d)] and 20 d recovery of optimum temperature [25 °C (R20 d)].

Treatment no. ^z	Level of performance at different times			Damage rate R20 d (%)
	0 d	6 d	R20 d	
1	I ^y	IV–V	IV	25.0
2	I	II	II	12.5
3	I	II–III	II	16.7
4	I	III	II–III	20.8
5	I	II–III	II	16.7
6	I	II–III	III	20.8
7	I	IV	III	20.8
8	I	II	I	4.2
9	I	II	I–II	4.2
10	I	II	I–II	8.3
11	I	II	I–II	4.2
12	I	II	I–II	8.3
13	I	II–III	II	8.3
14	I	II–III	II	12.5
15	I	II–III	II	16.7
16	I	III	II–III	16.7

^zTreatments described in Table 1.

^yDifferent injurious level: I = normal growth; II = 20% of the leaves became brown and withered; III = 40% of the leaves became brown, withered, and defoliated; IV = 60% of the leaves became brown and withered; V = 80% of the leaves became brown, withered, and defoliated.

the treatments. The total pigment content before the stress treatment was higher than after the stress period, although there was a slight rebound in the content in some treatments after the recovery period. Chlorophyll *b* content decreased initially and then increased (Fig. 2B). The pigment content in all plants treated with exogenous application of SA and CaCl₂, both alone and combined, was significantly greater compared with that in the control ($P \leq 0.05$), whatever the treatment, except for chlorophyll *b* in treatment 10 before the exposure to heat stress. Particularly, plants treated with SA and CaCl₂ alone showed an enhanced chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoid content before the heat treatment compared with plants treated with both SA and CaCl₂. The single treatments with either SA or CaCl₂ decreased pigment loss triggered by the exposure to heat stress, especially in plants exposed to treatment 6 in which chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoid contents decreased by 30.0%, 30.5%, 30.1%, and 39.9% respectively. The smallest variation in pigment range was observed in plants treated with the combination of SA and CaCl₂: 4.7% in treatment 15 for chlorophyll *a*, 4.6% in treatment 10 for chlorophyll *b*, 2.0% in treatment 13 for total chlorophyll, and 3.5% in treatment 15 for carotenoid. Thus, combined application of SA and CaCl₂ was more efficient in relieving the adverse effects of heat stress on pigments. However, during the restoration stage and compared with the pigment content in plants grown under optimal temperature, the largest decline in pigment content occurred in treatment 3 for chlorophyll *a* (by 6.3%) and total chlorophylls (by 4.9%), in treatment 13 for chlorophyll *b* (by 5.3%), and in treatment 15 for carotenoids (by 18.5%). The

smallest decline in pigment content was observed in treatment 6 for chlorophyll *a* (by –10.1%), total chlorophylls (by 10.7%), and carotenoids (by –29.9%) and in treatment 14 for chlorophyll *b* (by 24.8%), when compared with the pigment content in plants not exposed to heat stress. Therefore, a simple application of CaCl₂ effectively protected chlorophyll *a*, total chlorophylls, and carotenoid content. According to the significance analysis, treatments with SA and CaCl₂ alone or in combination significantly enhanced the pigment content under heat stress at all time periods ($P \leq 0.05$). There were significant differences between the treatments ($P \leq 0.05$): treatment 6 resulted in the highest pigment content before heat stress treatment, whereas the plants in treatment 15 were better in morphology after the heat stress treatment and 20 d of recovery.

ROS SYSTEM. As shown in Table 4, heat stress significantly increased the content of MDA [by 22.7% ($P \leq 0.05$)], which is the final product in membrane lipid peroxidation, compared with the control plants in treatment 1. However, although the MDA content decreased after 20 d of recovery, it was 6.6% higher compared with its initial level before heat stress. In addition, the MDA content in leaves was significantly higher compared with that in other treatments, whereas treatment 8 showed the largest decline in MDA content, by 26.9% to 0.092 $\mu\text{M}\cdot\text{g}^{-1}$ after 6 d of heat stress. The MDA content in treatment 8 was still lower compared with that of other treatments after 20 d of recovery. According to the significance analysis, a combined SA and CaCl₂ application was superior to their individual application, although the significance of difference was poor ($P \leq 0.05$).

Similarly, before exposure to heat stress, the combined treatment of SA and CaCl₂ was superior, substantially decreasing the H₂O₂ levels in leaves at the beginning of heat stress by 30.2%, 27.2%, 19.1%, 21.7%, and 17.4% in treatments 8 to 12, respectively, compared with that in the control (Table 5). Furthermore, treatments 2, 5, and 6 also decreased significantly the levels of H₂O₂ compared with that in the control ($P \leq 0.05$). After a 6-d incubation period at high temperature, the content of H₂O₂ increased in most treatments, except in treatments 12 and 15, in which its content decreased. Overall, the application of both SA and CaCl₂ in H₂O₂ level showed a smaller decline, but not in treatment 14. The content of H₂O₂ in all treatments was also significantly lower than that in the control after 20 d of recovery ($P \leq 0.05$), except in treatment 4.

OSMOREGULATORY SYSTEM. The total soluble protein in leaves of *Rhododendron* 'Fen Zhen Zhu' increased from the beginning of heat stress in all treatments except in treatments 5, 6, and 7, which showed a minor decline in total soluble protein content after 20 d of recovery, and in treatments 1, 13, 14, and 16, in which the total soluble protein content increased after an initial slight reduction (Fig. 3). With the exception of treatments 4, 5, and 8, the soluble protein content in each group was significantly higher than that in the control ($P \leq 0.05$), regardless of the time period. The highest growth rate was observed in treatment 4, in which the growth rate increased by 56.5% under heat stress compared with that under optimal temperature conditions. Although the control showed the largest decline in total soluble protein content (19.3%) compared with that before the stress, after the 20 d recovery period, it increased by 25% compared with that before the stress, which was similar to treatments 14 and 16. Among the plants treated with individual exogenous application of SA and CaCl₂, plants in treatment 6 showed significantly higher growth compared with those in

Fig. 2. Chlorophyll *a* (A), chlorophyll *b* (B), total chlorophyll (C), and carotenoid (D) content of *Rhododendron* 'Fen Zhen Zhu' as a function of exogenous salicylic acid (SA) and/or CaCl₂ concentration during heat stress and recovery. Treatment numbers are described in Table 1; 0 d = before the heat stress (38 °C), 6 d = after the heat stress (38 °C), R20 d = after 20 d recovery (25 °C). Values within same column show significant differences at $P \leq 0.05$ significance level among treatments according to the Duncan's multiple range test. Data are means of three replicates $\pm$ SE.

other treatments under heat stress ($P \leq 0.05$); there was no significant difference between treatments 6 and 7 after 6 d of exposure to heat stress ($P > 0.05$). In addition, treatments 9, 12, and 15 were superior to others in total soluble protein at different times.

ANTIOXIDANT ENZYME SYSTEM. SOD and POD activities in leaves were examined to investigate whether a change in MDA and H₂O₂ content is associated with higher antioxidant activity. As shown in Tables 6 and 7, SOD and POD activities decreased initially and then increased with time in most treatments. Heat stress significantly decreased the SOD activity, and the decrease was greater in treatments with individual or combined exogenous application of SA and CaCl₂ (Table 6). The SOD activity in the control group was reduced by 8.7% under heat stress compared with that in plants exposed to 0 d of stress. After the 20-d recovery period, it reached only $\approx 94.7\%$ of that observed in the control. The SOD activity was increased in the plants pretreated with SA and CaCl₂, alone or in combination. In treatment 10, the SOD activity reached the highest level, increasing by 10.5% before the stress compared with that in untreated plants, and maintained

its high-activity level regardless of whether the plants were under stress conditions or in the recovery. Meanwhile, the SOD activity in treatment 8 was significantly higher than that in the control ($P \leq 0.05$), and treatments 9, 10, and 14 had a better effect on SOD activity under heat stress. Lower concentrations of SA or CaCl₂ were sustained among treatments. In addition, treatments 2 and 5 have a better effect in SA and CaCl₂ respectively on improving SOD activity. Similar to the SOD pattern, the POD activity was also affected in leaves exposed to heat (Table 7). The treatments induced the POD activity; the increase in POD activity was significant in plants treated with SA (treatment 2) and CaCl₂ (treatment 5) ($P \leq 0.05$). However, combined SA and CaCl₂ application is more beneficial to enhance POD activity, especially in treatment 11, which increased POD activity by 6.8% before the stress treatment compared with the control; it was the smallest decline in POD activity in all treatments after the stress incubation, and recovered well. Regardless of the time, POD and SOD activities increased more after the simultaneous application of SA and CaCl₂ compared with that in plants with individual exogenous SA and CaCl₂ application.

Table 4. Effects of heat stress, salicylic acid (SA) and/or CaCl₂ application on malondialdehyde (MDA) content of *Rhododendron* ‘Fen Zhen Zhu’ after 0 and 6 d of high temperature [38 °C (0 d and 6 d)] and 20 d recovery of optimum temperature [25 °C (R20 d)].

Treatment no. ^z	MDA content [mean ± SE (μM·g ⁻¹ FW)]		
	0 d	6 d	R20 d
1	0.173 ± 0.005 a ^y	0.212 ± 0.004 a	0.185 ± 0.008 a
2	0.130 ± 0.001 gh	0.117 ± 0.002 i	0.145 ± 0.001 hij
3	0.144 ± 0.002 ef	0.139 ± 0.001 def	0.178 ± 0.001 ab
4	0.156 ± 0.001 c	0.144 ± 0.001 cdef	0.162 ± 0.001 de
5	0.154 ± 0.000 cd	0.136 ± 0.002 efg	0.168 ± 0.001 cd
6	0.156 ± 0.001 c	0.158 ± 0.001 b	0.157 ± 0.000 ef
7	0.156 ± 0.001 c	0.149 ± 0.001 bcd	0.177 ± 0.001 b
8	0.126 ± 0.002 h	0.092 ± 0.005 j	0.139 ± 0.001 j
9	0.135 ± 0.002 g	0.126 ± 0.005 ghi	0.149 ± 0.000 gh
10	0.148 ± 0.001 de	0.133 ± 0.006 fgh	0.168 ± 0.001 cd
11	0.142 ± 0.001 f	0.137 ± 0.003 defg	0.140 ± 0.001 ig
12	0.146 ± 0.001 ef	0.142 ± 0.005 def	0.147 ± 0.001 ghi
13	0.148 ± 0.002 de	0.154 ± 0.003 bc	0.152 ± 0.001 fgh
14	0.149 ± 0.002 de	0.124 ± 0.003 hi	0.146 ± 0.001 ghij
15	0.149 ± 0.003 de	0.145 ± 0.005 cde	0.153 ± 0.001 fg
16	0.166 ± 0.001 b	0.146 ± 0.004 cde	0.170 ± 0.000 c

^zTreatments described in Table 1.

^yMeans followed by different lower case letter indicate significant among treatments based on Duncan’s multiple range test at $P = 0.05$ ($n = 3$).

Table 5. Effects of heat stress, salicylic acid (SA) and/or CaCl₂ application on hydrogen peroxide (H₂O₂) content of *Rhododendron* ‘Fen Zhen Zhu’ after 0 and 6 d of high temperature [38 °C (0 d and 6 d)] and 20 d recovery of optimum temperature [25 °C (R20 d)].

Treatment no. ^z	H ₂ O ₂ content [mean ± SE (mm·g ⁻¹ protein FW)]		
	0 d	6 d	R20 d
1	342.924 ± 19.0449 a ^y	397.736 ± 11.429 a	372.453 ± 12.041 a
2	254.796 ± 9.495 cd	271.764 ± 13.002 fgh	261.281 ± 12.585 de
3	289.531 ± 14.512 abcd	300.090 ± 9.409 cdefg	275.911 ± 4.327 cde
4	330.165 ± 7.693 ab	340.804 ± 10.878 b	325.471 ± 18.099 abc
5	268.968 ± 12.742 cd	282.121 ± 12.651 efgh	257.930 ± 19.696 de
6	287.390 ± 28.231 bcd	307.105 ± 6.364 bcdef	274.753 ± 5.540 cde
7	292.080 ± 8.885 abcd	320.846 ± 8.779 bcde	290.197 ± 15.058 bcde
8	239.240 ± 3.905 d	252.363 ± 13.097 h	243.027 ± 15.438 e
9	249.648 ± 0.940 cd	262.030 ± 10.891 gh	241.089 ± 16.690 e
10	277.545 ± 16.052 bcd	287.939 ± 5.190 defgh	266.713 ± 18.181 de
11	268.313 ± 28.848 cd	280.143 ± 13.232 fgh	260.288 ± 6.649 de
12	283.070 ± 24.018 bcd	271.537 ± 10.589 fgh	256.502 ± 15.602 de
13	303.863 ± 17.006 abc	307.957 ± 5.220 bcdef	281.444 ± 19.758 bcde
14	293.978 ± 13.234 abcd	325.614 ± 20.285 bcd	270.216 ± 10.886 de
15	303.815 ± 13.744 abc	303.813 ± 7.222 bcdef	306.558 ± 14.264 bcd
16	328.796 ± 17.522 ab	331.922 ± 22.791 bc	328.884 ± 28.260 ab

^zTreatments described in Table 1.

^yMeans followed by different lower case letter indicate significant among treatments based on Duncan’s multiple range test at $P = 0.05$ ($n = 3$).

The SOD and POD activities in all treated plants were significantly higher than those in untreated plants ($P \leq 0.05$).

Discussion

The genus *Rhododendron* includes heat-sensitive plant species, with the optimum growth temperature between 10 and 25 °C. However, during summer months in China, *Rhododendrons* must endure high temperatures of over 35 °C, which increases the cost of management practices employed in commercial cultivation of these plants in greenhouses. The plants may alter their morphology and physiology under heat stress, decreasing their ornamental values. In the present study,

Rhododendron ‘Fen Zhen Zhu’ plants exposed to heat stress developed the following signs of stress: yellowing of the leaf tips, browning and drying of the leaves, and different levels of injury depending on the treatment (Fig. 1; Table 3). Such morphological changes might have occurred due to a substantial decline in light-harvesting pigment content (Fig. 2), which increased the damaging effects of ROS (Tables 4 and 5). This mechanism could be used to extend heat tolerance. Chloroplasts, mitochondria, and peroxisomes are the major sources of ROS production in plant cells. Production of ROS by these sources is enhanced by unfavorable environmental conditions that limit CO₂ fixation, leading to an increase in photoinhibition and overproduction of superoxide radicals and H₂O₂ (Radwan,

2012). Similar findings were reported in three cultivars of *Raphanus sativus* exposed to 40 °C (Chen et al., 2014). In addition, the total soluble protein content decreased during heat stress (Fig. 3) as did the SOD and POD activities (Tables 6 and 7). Furthermore, the 2-month-long observations revealed that the plants that were subjected to heat stress, in particular those treated with SA and CaCl₂, were growing better than those that were not subjected to heat stress.

It has long been hypothesized that SA and Ca²⁺ protect against various adverse environmental conditions by regulating the antioxidant levels through activation or inhibition of the

antioxidant enzymatic system, which is responsible for scavenging generated ROS (Apel and Hirt, 2004; Farooq et al., 2008; Kumar et al., 2012; Tan et al., 2011). Decreased MDA, H₂O₂, and chlorophyll content and increased activities of SOD and POD could be the reason for diminished growth performance of the experimental plants under heat stress. Exogenous application of SA and CaCl₂ alleviated the heat-induced reduction in growth of experimental plants (Figs. 2–3; Tables 3–7). Similar improvement in growth by exogenous SA and CaCl₂ treatment, individual and combined, was reported in *S. lycopersicum* grown under heat stress conditions (Manaa et al., 2014). The decrease in injury levels may be due to the inhibited decline in chlorophyll content and increased accumulation of MDA and H₂O₂. The experimental results showed that pigment content decreased during heat treatment; however, *Rhododendron* leaves sprayed with SA and CaCl₂, alone or in combination, 3 d before exposure to heat stress showed fewer or no injuries, which helped reduce pigment loss induced by heat stress. Specifically, treatment 15 (2.0 mM SA + 7.0 mM CaCl₂) prevented most the loss of pigments before and after stress treatment. In addition, before the heat stress treatment, the greatest decrease in pigment content was observed in treatment 6 (7.0 mM CaCl₂). Slight decrease in chlorophyll *a* and carotenoid contents was observed in treatment 15. Similarly, slight decrease in chlorophyll *b* and total chlorophyll contents was observed in treatment 10 (0.5 mM SA + 10.5 mM CaCl₂) and treatment 13 (1.0 mM SA + 10.5 mM CaCl₂), respectively. These treatments contained a high concentration of Ca²⁺, which may be because SA-treated cells can maintain Ca²⁺ homeostasis and antioxidant systems under heat stress, and increased cytosolic Ca²⁺ protected the chloroplast structure (Wang and Li, 2006). Lipid peroxidation levels were assessed by MDA, the final product of lipid peroxidation. H₂O₂ is produced during lipid catabolism as a side product of fatty acid oxidation. MDA and H₂O₂ play an important role in plant tissue metabolism, but any excess production of MDA and H₂O₂ is harmful, especially to the membranes. Accumulation of elevated H₂O₂ and MDA

Fig. 3. Total soluble protein content of *Rhododendron* ‘Fen Zhen Zhu’ as a function of exogenous salicylic acid (SA) and/or CaCl₂ concentration during heat stress and recovery. Treatment numbers are described in Table 1; 0 d = before the heat stress (38 °C), 6 d = after the heat stress (38 °C); R20 d = after 20 d recovery (25 °C). Values within same column show significant differences at *P* ≤ 0.05 significance level among treatments according to the Duncan’s multiple range test. Data are means of three replicates ±SE.

Table 6. Effects of heat stress, salicylic acid (SA) and/or CaCl₂ application on superoxide dismutase (SOD) activity of *Rhododendron* ‘Fen Zhen Zhu’ after 0 and 6 d of high temperature [38 °C (0 d and 6 d)] and 20 d recovery of optimum temperature [25 °C (R20 d)].

Treatment no. ^z	SOD activity [mean ± SE (U·g ⁻¹ ·min ⁻¹ FW)]		
	0 d	6 d	R20 d
1	331.866 ± 4.582 e ^y	305.092 ± 8.818 g	314.218 ± 1.685 d
2	340.647 ± 3.773 de	330.434 ± 3.716 ef	343.582 ± 3.975 bc
3	347.259 ± 0.471 bcde	328.145 ± 3.645 f	335.298 ± 6.118 c
4	342.145 ± 2.947 cde	324.318 ± 0.623 f	334.332 ± 5.559 c
5	349.431 ± 8.518 abcde	338.403 ± 7.263 cdef	352.286 ± 5.987 ab
6	341.040 ± 3.905 cde	327.533 ± 3.893 f	338.545 ± 3.676 bc
7	344.510 ± 10.298 cde	329.206 ± 1.838 ef	337.019 ± 3.801 c
8	359.095 ± 2.758 abc	358.551 ± 4.994 a	351.708 ± 2.868 ab
9	363.020 ± 11.188 ab	351.344 ± 1.526 abc	345.685 ± 6.643 abc
10	366.669 ± 4.396 a	354.917 ± 3.040 ab	359.301 ± 1.209 a
11	350.772 ± 1.505 abcd	337.875 ± 3.461 cdef	345.576 ± 3.400 abc
12	345.048 ± 2.589 cde	333.829 ± 2.797 def	348.781 ± 3.251 abc
13	365.502 ± 4.466 a	348.071 ± 3.784 abcd	346.618 ± 5.313 abc
14	356.077 ± 3.456 abcd	345.352 ± 3.860 abcd	352.007 ± 5.188 ab
15	355.114 ± 3.832 abcd	342.966 ± 5.808 bcde	348.731 ± 1.715 abc
16	342.828 ± 4.537 cde	337.465 ± 3.205 cdef	336.758 ± 2.814 c

^zTreatments described in Table 1.

^yMeans followed by different lower case letter indicate significant among treatments based on Duncan’s multiple range test at *P* = 0.05 (*n* = 3).

Table 7. Effects of heat stress, salicylic acid (SA) and/or CaCl₂ application on peroxidase (POD) activity of *Rhododendron* 'Fen Zhen Zhu' after 0 and 6 d of high temperature [38 °C (0 d and 6 d)] and 20 d recovery of optimum temperature [25 °C (R20 d)].

Treatment no. ^z	POD activity [mean ± SE (U·g ⁻¹ ·min ⁻¹ FW)]		
	0 d	6 d	R20 d
1	99.544 ± 5.460 f ^y	75.497 ± 3.274 h	87.879 ± 2.227 d
2	138.867 ± 8.997 abcde	119.079 ± 6.786 cdefg	122.964 ± 9.565 abc
3	121.516 ± 8.154 def	100.861 ± 2.486 g	110.281 ± 2.752 bcd
4	126.972 ± 10.441 cde	107.838 ± 5.875 fg	111.839 ± 6.891 bcd
5	133.299 ± 9.383 bcde	118.928 ± 6.523 cdeg	126.641 ± 13.669 abc
6	120.561 ± 9.741 ef	103.270 ± 2.884 g	105.069 ± 7.661 cd
7	123.305 ± 9.926 def	112.887 ± 9.317 efg	120.241 ± 11.492 abc
8	153.680 ± 11.405 abc	138.889 ± 5.526 abc	135.412 ± 9.657 ab
9	157.221 ± 7.423 ab	132.040 ± 6.606 abcde	140.262 ± 3.104 a
10	150.788 ± 6.668 abc	140.619 ± 15.530 abc	142.450 ± 8.903 a
11	161.490 ± 6.932 a	150.440 ± 3.760 a	147.572 ± 6.923 a
12	151.424 ± 11.476 abc	135.079 ± 3.354 abcd	126.241 ± 7.814 abc
13	148.331 ± 3.972 abcd	128.066 ± 5.268 bcdef	125.315 ± 4.117 abc
14	155.808 ± 6.993 ab	143.363 ± 6.100 ab	137.548 ± 6.739 ab
15	148.087 ± 4.050 abcd	135.241 ± 7.010 abcd	130.443 ± 5.828 abc
16	139.560 ± 1.945 abcde	114.167 ± 2.487 defg	125.376 ± 11.414 abc

^zTreatments described in Table 1.

^yMeans followed by different lower case letter indicate significant among treatments based on Duncan's multiple range test at $P = 0.05$ ($n = 3$).

levels during heat stress was recorded (Tables 4 and 5), suggesting the presence of oxidative species in 'Fen Zhen Zhu' leaves. However, the levels of MDA and H₂O₂ after SA and CaCl₂ pretreatment, alone or in combination, were lower compared with the control, and application of SA and CaCl₂ in combination had a greater effect on the inhibition of MDA and H₂O₂, especially in treatment 8 (0.5 mM SA + 3.5 mM CaCl₂), which lowered the levels of ROS in plants, suggesting that SA and CaCl₂ might have interacted in managing plant stress.

However, improving growth by applying SA and CaCl₂ alone or in combination onto the plants during heat stress could be correlated with a substantial increase in total soluble protein content (Fig. 3), especially in plants exposed to treatments 12 and 15, both of which contained 7.0 mM CaCl₂. The considerable increase in total soluble protein content in our findings could be due to a sufficient calcium concentration, which is required at all stages of plant growth and development and which plays a vital role in regulating the polar growth of cells and tissues as well as the plant adaptation to various stress factors.

Various enzymatic scavenging activities, particularly for ROS, are found in different cellular compartments. The enzymatic ROS-scavenging pathway, an antioxidant defense mechanism, helps to counteract ROS and protect cells from oxidative damage (Radwan, 2012). SOD acts as a first line of defense against ROS by converting superoxides into H₂O₂, and thus decreasing their toxic effects to the cell (Wang et al., 2004). H₂O₂ has to be further detoxified by POD and catalase/ascorbate POD to water and oxygen (Shah et al., 2001). H₂O₂ detoxification prevents the oxidation of biological molecules and destruction of cells (Liochev and Fridovich, 1994). In the present study, exogenous application of SA and CaCl₂, alone or combined, could induce changes in SOD and POD activities similar to those in the control. A large amount of Ca²⁺ influx from apoplasts to protoplasts after 10 h of SA induction has been reported (Guo et al., 2015). In addition, the combined application of SA and CaCl₂ had a greater effect on SOD and POD activities. The effectiveness of the

combined treatments is optimal if the mixture contains at least 0.5 mM SA or 3.5 mM CaCl₂. This might be because a lower concentration of SA induced an increase in Ca²⁺ content, which led to the inhibition of SOD and POD activities. The study also revealed that treatment 5 was significantly higher than the application of CaCl₂ alone in improving SOD and POD activities ($P \leq 0.05$).

In conclusion, the present study showed that the heat stress causes yellowing of leaf tips and browning and drying of leaf parts in *Rhododendron* 'Fen Zhen Zhu'. In addition, the contents of chlorophyll and total soluble protein decreased, the MDA and H₂O₂ contents increased, and the activities of SOD and POD increased under heat stress. The plant morphology and physiology showed a slight improvement after 20 d of recovery. The exogenous application of SA and CaCl₂, alone or combined, improved plant growth by regulating the same physiological and biochemical mechanisms that respond to heat stress. Application of SA and CaCl₂ is effective in alleviating the decrease in chlorophyll content at higher concentrations of CaCl₂. At lower concentrations of SA and CaCl₂, the activity of SOD and POD increased as well as the accumulation of total soluble protein. This resulted in further scavenging of ROS and increased MDA and H₂O₂.

The present results provide a new perspective to the use of SA and CaCl₂ in the protection of *Rhododendron* 'Fen Zhen Zhu' from injuries induced by heat stress. This method could save energy costs in greenhouse management. Thus, the measurement of related physiological indexes and exploration of signal transduction pathways is a powerful tool to improve plant tolerance to abiotic stress.

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