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## Subcellular Localization and Responses of Antioxidant Systems of Three *Rhododendron* Cultivars under Salt Stress

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Effects of NaCl stress on reactive oxygen species (ROS) and the antioxidant systems in the mitochondria, cytosol and chloroplasts of the three *Rhododendron* cultivars were studied for elucidating the response mechanism of the antioxidant systems in *Rhododendron* to salinity stress at the subcellular level. According to the morphological damage index, the order of the resistance to salt stress was as follows: ‘Yanzhi Mi’ > ‘Hong Shan Hu’ > ‘Hong Yue’. ROS including hydrogen peroxide ( $H_2O_2$ ) and superoxide radicals ( $O_2^{\cdot-}$ ) was mainly located in the cytosol, and the level in the mitochondria and chloroplasts was lower, and the differences of the two subcellular compartments were not significantly. Salinity stress induced the increase of production rate of  $O_2^{\cdot-}$  and  $H_2O_2$  level in three subcellular compartments and resulted in the accumulation of malondialdehyde (MDA). MDA in the three subcellular compartments of ‘Yanzhi Mi’ leaves did not change significantly under salt stress, which further indicated that ‘Yanzhi Mi’ had better salt tolerance. The activities of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD) and glutathione reductase (GR), and the level of non-enzymatic antioxidants including ascorbate (AsA) and glutathione (GSH) were higher in the cytosol of the three cultivars than the other two subcellular compartments. Ascorbate peroxidase (APX) in the chloroplasts and catalase (CAT) in the mitochondria were highest. With the exception of CAT, other antioxidants were enhanced to varying degrees under salinity stress. The results indicated that the salt-sensitive cultivars were more severely damaged by peroxidation under salt stress, which caused the stress response of ROS-scavenging system, especially the significant increase of SOD and POD in salt-sensitive rhododendrons. But this did not effectively alleviate the peroxidation damage under salt stress, which may be related to the decrease of CAT activity, and also indicated the importance of CAT activity in salt tolerance of rhododendron.

**Key words:** antioxidant capacities, chloroplasts, cytosol, mitochondria, plant salt tolerance, reactive oxygen species

### INTRODUCTION

Under salt stress conditions, plants can produce large amounts of reactive oxygen species (ROS), which are highly reactive and toxic and adversely affect plant's subcellular compartments and metabolism (Ashraf, 2009).  $O_2^{\cdot-}$  and  $H_2O_2$ , the two main forms of ROS, are located in different subcellular compartments such as the cytosol, chloroplasts, mitochondria, peroxisomes and apoplasts due to the high metabolic activity that normally occurs in these subcellular compartments. ROS can cause oxidative damage to the subcellular compartments, the corresponding ROS-scavenging mechanisms are needed to maintain the balance of ROS (Mittler, 2002; Shanker and Shanker, 2016). ROS-scavenging mechanisms are primarily divided into enzymatic antioxidants comprising of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR), and non-enzymatic antioxidants such as ascorbate (AsA) and glutathione (GSH) (Das and Roychoudhury, 2014). In plants, AsA–GSH cycle makes an important impact in controlling redox homeostasis. APX, using AsA as the electron donor to reduce  $H_2O_2$  to water, prevents the

$H_2O_2$  accumulation under the stress conditions. GR uses NADPH as an electron donor to convert oxidized glutathione (GSSG) to GSH. AsA and GSH are two kinds of non-enzymatic antioxidants that can react directly with ROS and remove ROS as the substrate of the enzymes (Das and Roychoudhury, 2014; Anjum *et al.*, 2010). These antioxidants locate in almost all subcellular compartments, such as chloroplasts, cytosol, mitochondria, apoplasts and peroxisomes, and play a key role in controlling the ROS level in these subcellular compartments (Ashraf, 2009; Das and Roychoudhury, 2014).

The distribution of ROS and the antioxidants in subcellular compartments was found to be different under various abiotic stresses. For instance, the level of  $H_2O_2$  and the activities of SOD, APX and CAT in the chloroplasts of winter wheat leaves under low temperature stress conditions were higher than those in the mitochondria (Li *et al.*, 2014). Under salt stress, the chloroplasts of wheat showed higher SOD, APX and GR activities than those in the mitochondria and cytosol. The distributions of SOD, APX and GR in the mitochondria and cytosol were also different. SOD and GR activities in the mitochondria were higher than that in the cytosol, while APX activity in the cytosol was higher than that in the mitochondria (Sairam *et al.*, 2002). While SOD, GR, APX, AsA and GSH of maize during water stress were mainly located in the cytosol, and CAT had the highest activity in the peroxisomes (Tan *et al.*, 2010). White

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clover under water stress showed the highest SOD, APX and GR activities in the chloroplasts, CAT activity was the highest in the peroxisomes. The level of ROS in subcellular compartments was not consistent with the degree of peroxidation damage. According to the  $\text{H}_2\text{O}_2$  level, the subcellular distribution of  $\text{H}_2\text{O}_2$  in white clover under water stress was chloroplasts > cytosol > peroxisomes > mitochondria, however, the level of malondialdehyde (MDA) was highest in the chloroplasts, followed by the peroxisomes, mitochondria and cytosol in decreasing order (Wang *et al.*, 2008).

The effects of salt stress on crop plants have been studied extensively (Chawla *et al.*, 2013; Hernández *et al.*, 2000; Hichem *et al.*, 2009). However, few authors have focused their attentions on the effects of soil salinization on ornamental plants, whose ornamental value and application may be affected by the excess salinity in soil (Acosta-Motos *et al.*, 2015). Rhododendron is a famous ornamental plant in the world, whose wild germplasm resources are mainly distributed in China (Chamberlain *et al.*, 1996). Rhododendron plants favor acidic soil and are salt-tolerant to salinity stress. The salinization of soil has become a serious problem worldwide, drastically limits the introduction and cultivation of wild rhododendrons, and the further popularization and landscape application of some horticultural cultivars. At present, the studies on the antioxidant systems of rhododendron under abiotic stresses have been published. The study of Wang *et al.* (2009) showed that the balance between ROS generation and ROS scavenging was beneficial to the adaptability of rhododendron plants to low temperature in winter. Zhou *et al.* (2017) also found that the antioxidant system played a significant role in growth adaptability of *R. chrysanthum* in high mountains. However, the effects of salt stress on ROS-scavenging system of rhododendrons at the subcellular level are still unclear. Therefore, the seedlings of three *Rhododendron* cultivars including 'Yanzhi Mi', 'Hong Shan Hu' and 'Hong Yue', were used as the experimental materials to analyze the changes of ROS and antioxidants in the chloroplasts, mitochondria and cytosol under NaCl stress in the present study.

## MATERIALS AND METHODS

### Plant materials and Salt-stress treatments

Four-month-old cutting seedlings of the three *Rhododendron* cultivars, 'Yanzhi Mi', 'Hong Shan Hu' and 'Hong Yue' were obtained from a nursery production company (Yuhua Dujuan in Changzhou, Jiangsu province). The seedlings were cultivated in the soil (3:1, v/v, mixture of peat and perlite). According to the previous preparatory experiment, on the first and seventh day of the experiment, the seedlings were watered evenly with 0.5% of NaCl solution until a small amount of solution flowed out of the bottom of the basin (Salt content of the cultivation substrate was determined on the 1st, 3rd, 7th, 10th and 14th day of treatment, the actual salt concentration in the soil were between 0.47% and 0.63%). Ten seedlings in a batch were used in the experiment.

After 14 days of treatment, when the three cultivars presented different degrees of salt stress symptoms, the middle leaves of the seedlings were sampled and immediately stored at  $-80^\circ\text{C}$  for further analysis. As the control, the other seedlings were watering with overnight tap water.

The experiment was conducted in the greenhouse of Nanjing Forestry University at the temperature of  $25 \pm 2^\circ\text{C}$  and relative humidity of 70–80%, with light intensity of  $165 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  and photoperiod of 12-h light/12-h dark. The same experiment was conducted twice again to confirm the results.

### Statistics of morphological damage index

The morphological damage index was divided into five grades. Grade 0: no symptom; Grade 1: the leaves appeared to have white spots and the edges were yellow; Grade 2: 1/3 of the leaf area was yellow; Grade 3: the edges of the new leaves were curled and 1/2 of the leaf area was yellow; Grade 4: the edges of the new leaves were severely curled and withered, and more than 1/2 of the leaf area was yellow. The morphological damage index was as follows: morphological damage index (%) =  $\Sigma$  (corresponding number of leaves  $\times$  grades) / (the highest grade  $\times$  total number of leaves)  $\times$  100.

### Isolation of subcellular compartments

Isolation of the subcellular compartments was conducted by Sairam *et al.* (2002) with some modifications, and all the procedures were carried out on ice. Ten g of leaves were grinded with liquid nitrogen and then added to 30 ml of cool protoplast extraction buffer (50  $\text{mmol}\cdot\text{L}^{-1}$  Tris-HCl, 0.35  $\text{mmol}\cdot\text{L}^{-1}$  sorbitol, 2  $\text{mmol}\cdot\text{L}^{-1}$  EDTA, 2.5  $\text{mmol}\cdot\text{L}^{-1}$  DTT, pH7.5). After filtering through 4 layers of gauze, the filtrate was centrifuged at  $500\times g$  for 5 min in a refrigerated centrifuge. The precipitate was discarded, and the supernatant was centrifuged at  $2000\times g$  for 10 min to separate the chloroplasts, which was precipitated and the mixture of cytosol and mitochondria in the upper layer. Then the supernatant fraction was further centrifuged at  $12000\times g$  for 20 min, and the precipitate was the mitochondria, and the supernatant was the cytosol. The chloroplasts and mitochondria were washed once with extraction buffer and then centrifuged at  $2000\times g$  and  $12000\times g$  respectively. The pellet was suspended in 5 ml of extraction buffer.

### Measurement of physiological indexes

#### MDA content

MDA content was analyzed according to Velikova *et al.* (2000) using thiobarbituric acid (TBA) test. 0.5 ml of extraction buffer of subcellular fraction was homogenized in 5 ml of 0.1% pre-cooled trichloroacetic acid (TCA) solution. The homogenate was centrifuged at  $10000\times g$  for 20 min and 0.5 ml of the supernatant was mixed with 1 ml of 0.5% TBA in 20% TCA, then incubated in boiling water for 50 min, and the reaction was stopped by placing the reaction tubes in an ice bath. Then the samples were centrifuged at  $10000\times g$  for 5 min, and the absorbancy of supernatant was read at

532 nm. The value for non-specific absorption at 600 nm was subtracted.

#### *H<sub>2</sub>O<sub>2</sub> content*

H<sub>2</sub>O<sub>2</sub> content was measured by the potassium iodide method according to Velikova *et al.* (2000). A half ml of the subcellular suspension was added to 5 ml of 0.1% TCA, and then centrifuged at 12000×g for 15 min. One ml of the supernatant was added to 1 ml of phosphate buffer (10 mmol·L<sup>-1</sup>, pH 7.0) and 2 ml of 1 mol·L<sup>-1</sup> potassium iodide solution, shaken well, and kept standing for 10 min. The absorbance was recorded at 390 nm.

#### *O<sub>2</sub><sup>·-</sup> production rate*

The change of O<sub>2</sub><sup>·-</sup> production rate was measured following the method from Wang *et al.* (2013) with minor modifications. A half ml of the subcellular suspension was added to 5 ml of phosphate buffer (65 mmol·L<sup>-1</sup>, pH 7.8), and then centrifuged at 10000×g for 10 min, and the supernatant was the assay solution. One ml of the supernatant was mixed with 0.2 ml of 10 mmol·L<sup>-1</sup> hydroxylamine hydrochloride and 1 ml of phosphate buffer (65 mmol·L<sup>-1</sup>, pH 7.8). The mixture was reacted at 25°C for 20 min. After 1 ml of 17 mol·L<sup>-1</sup> p-aminobenzenesulfonic acid and 1 ml of 7 mmol·L<sup>-1</sup> α-naphthylamine were added, the mixture was incubated at 25°C for 20 min. The absorbance was measured at 530 nm.

#### *Enzyme assays*

SOD, POD, CAT, APX and GR activities were assessed by Turkan *et al.* (2005) with some modifications. A half ml of the subcellular suspension was added to 5 ml of ice-cold phosphate buffer (50 mmol·L<sup>-1</sup>, pH 7.8). The homogenate was centrifuged at 10000×g and 4°C for 15 minutes. The supernatant was used for measuring the activities of SOD, POD and CAT. A half ml of the subcellular suspension was added to 5 ml of ice-cold phosphate buffer (25 mmol·L<sup>-1</sup>, pH 7.0). The homogenate was centrifuged at 10000×g (4°C, 15 minutes), and the supernatant used for measuring APX activity. A half ml of the subcellular suspension was added to 5 ml of Tris-HCl buffer (50 mmol·L<sup>-1</sup>, pH 7.0) containing 1 mmol·L<sup>-1</sup> EDTA, 1% PVP and 5 mmol·L<sup>-1</sup> MgCl<sub>2</sub>, and then centrifuged at 10000×g for 20 min and the supernatant was the GR assay solution. The procedure was conducted at 4°C.

SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium. The 3-ml reaction system contained 1.5 ml of phosphate buffer (50 mmol·L<sup>-1</sup>, pH 7.8), 0.3 ml of 130 mmol·L<sup>-1</sup> methionine, 0.3 ml of 750 μmol·L<sup>-1</sup> NBT, 0.3 ml of 100 μmol·L<sup>-1</sup> EDTA-Na<sub>2</sub>, 0.05 ml of initial enzyme extract, 0.3 ml of 20 μmol·L<sup>-1</sup> riboflavin and 0.25 ml of distilled water. The mixture was reacted under 8000 lx for 10 minutes. One unit (U) of SOD activity was defined as the amount of enzyme that was required to cause 50% inhibition of the reduction of nitroblue tetrazolium as monitored at 560 nm.

POD activity was assayed by recording by following the consumption of H<sub>2</sub>O<sub>2</sub> at 470 nm in 3 minutes. The

reaction mixture (3 ml) included 2.6 ml of 0.3% guaiacol solution, 0.3 ml of 0.6% H<sub>2</sub>O<sub>2</sub> and 0.1 ml of enzyme extract. The absorbance value per minute was changed by 0.01 as one unit of enzyme activity.

CAT activity was assayed by recording by following the consumption of H<sub>2</sub>O<sub>2</sub> at 240 nm in 4 minutes. The assay mixture contained 1.5 ml of phosphate buffer (50 mmol·L<sup>-1</sup>, pH 7.8), 0.3 ml of 100 mmol·L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub>, 0.2 ml of enzyme and 1 ml of distilled water. The reaction was started with the addition of H<sub>2</sub>O<sub>2</sub>. The absorbance value per minute was changed by 0.01 as one unit (U) of enzyme activity.

APX activity was assayed by recording the decrease in optical density due to AsA at 290 nm in 1 minute. The 4-ml reaction mixture contained 3.4 ml of potassium phosphate buffer (25 mmol·L<sup>-1</sup>, pH 7.0), 0.2 ml of 0.5 mmol·L<sup>-1</sup> AsA, 0.2 ml of 1 mmol·L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> and 0.2 ml of enzyme. The reaction was started with the addition of H<sub>2</sub>O<sub>2</sub>. 0.01 change of absorbance at 290 nm was defined as one unit of APX activity.

GR activity was measured by following the decrease in absorbance at 340 nm in 4 minutes due to NADPH oxidation. The 4-ml reaction system contained 3120 μl of Tris-HCl buffer (50 mmol·L<sup>-1</sup>, pH 7.5) containing 0.1 mmol·L<sup>-1</sup> EDTA and 5 mmol·L<sup>-1</sup> MgCl<sub>2</sub>, 80 μl of 10 mmol·L<sup>-1</sup> NADPH, 200 μl of 10 mmol·L<sup>-1</sup> GSSG and 0.6 ml of enzyme. The absorbance value per minute was changed by 0.01 as one unit of enzyme activity.

#### *Nonenzymatic antioxidants assays*

AsA content was determined using the method from Kampfenkel *et al.* (1995). A half ml of the subcellular suspension was added to 5 ml of 6% TCA, and centrifuged at 15600×g for 10 min. The supernatant was used for the determination of AsA. The AsA reaction system was consisted of 0.2 ml of supernatant, 0.6 ml of phosphate buffer (0.2 mol·L<sup>-1</sup>, pH 7.4), 0.2 ml of double distilled water, 1.0 ml of 10% TCA, 0.8 ml of 42% phosphoric acid (H<sub>3</sub>PO<sub>4</sub>), 0.8 ml of 2% 2, 2'-bipyridine, and 0.4 ml of 3% ferric chloride (FeCl<sub>3</sub>). The absorbance was measured at 525 nm. The content of AsA in the sample was determined from the standard curve and the content of AsA (μg·g<sup>-1</sup>) was calculated.

GSH content was measured according to Law *et al.* (1983). A half ml of the subcellular suspension was added to 5 ml of pre-cooled 5% TCA containing 5 mmol·L<sup>-1</sup> EDTA-Na<sub>2</sub>, and centrifuged at 12000×g for 20 min. The supernatant was collected for determination of GSH content. The GSH reaction mixture included 1 ml of supernatant, 1 ml of phosphate buffer (0.1 mmol·L<sup>-1</sup>, pH 7.7) and 0.5 ml of 4 mmol·L<sup>-1</sup> 5-(dithiobis-2-nitrobenzoic acid). The absorbance was recorded at 412 nm. GSH content in the sample was determined from the standard curve and the content of GSH (μmol·g<sup>-1</sup>) was calculated.

#### **Statistical analyses of the data**

The data were analyzed using Excel 2007 and a one-way ANOVA using the SPSS 19.0 software. All results were represented as mean ± SE from at least three inde-

pendent series of experiments. The data were compared by least significant difference (LSD) with three times all the above indexes used for analyzing significance ( $P < 0.05$ ).

## RESULTS

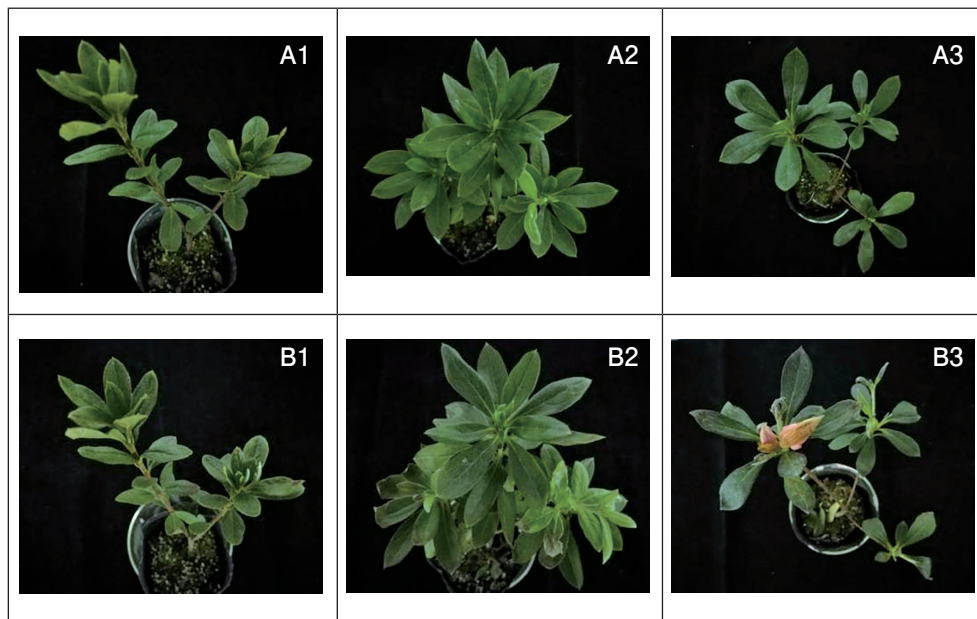
### Analysis of morphological index of salt stress

According to the external morphology and the salt damage index of leaves of three *Rhododendron* cultivars (Figs. 1 and 2), the injury degree of rhododendron seedlings was gradually aggravated with the prolongation of stress time. After salinity stress, 'Yanzhi Mi' exhibited the least amount of salt damage, with an index of 8.54%. The most serious salt damage was observed in

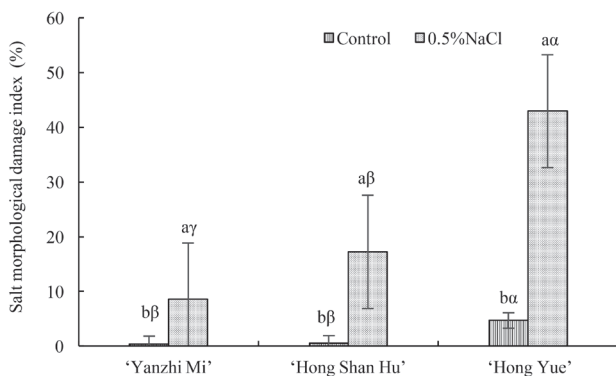
'Hong Yue' with an index of 42.95%. The stress degree of 'Hong Shan Hu' was between 'Yanzhi Mi' and 'Hong Yue', its salinity index was 17.21%. The morphological damage index of 'Hong Yue' control group was higher than that of the other two cultivars, which was related to slight damage during transportation.

### ROS production

$O_2^{\cdot-}$  production rate was significantly accelerated in the three subcellular compartments of the three cultivars under salt stress, except for that in the cytosol of 'Yanzhi Mi' (Fig. 3). Among the three subcellular compartments, the fastest production rate of  $O_2^{\cdot-}$  was in the cytosol, followed by the chloroplasts and mitochondria in decreasing order both in the salt-treated and control



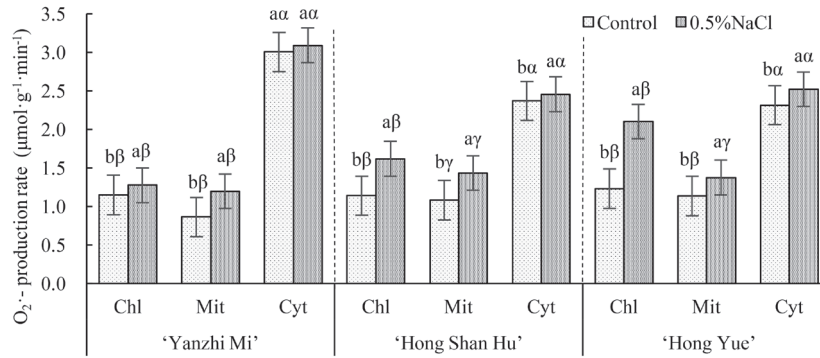
**Fig. 1.** Growth status of three *Rhododendron* cultivars before and after 0.5% salt treatment. Note: A1: 'Yanzhi Mi' control; A2: 'Hong Shan Hu' control; A3: 'Hong Yue' control; B1: 'Yanzhi Mi' 0.5% salt stress; B2: 'Hong Shan Hu' 0.5% salt stress; B3: 'Hong Yue' 0.5% salt stress.



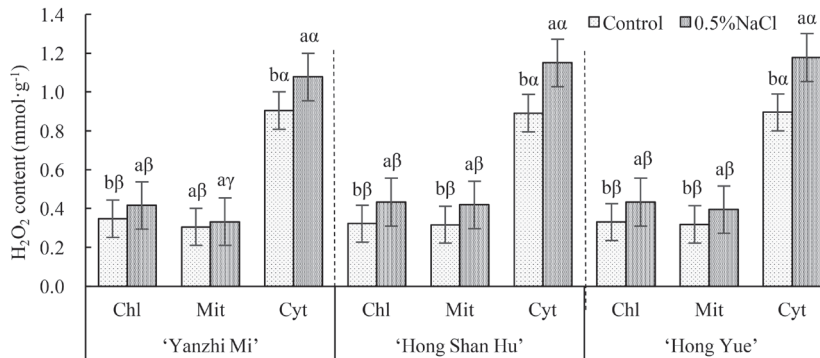
**Fig. 2.** Morphological damage index of the three cultivars under salt stress. Note: Lowercase letters (a, b) indicate the difference between the two treatments of the same *Rhododendron* cultivar, and Greek letters ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) represent the difference between the three *Rhododendron* cultivars of the same treatment, and different letters indicate significant difference ( $p < 0.05$ ) according to LSD's multiple-range tests.

seedlings, and the production rate of  $O_2^{\cdot-}$  in the cytosol was significantly higher than that in the chloroplasts and mitochondria. In the control group seedlings,  $O_2^{\cdot-}$  production rate in the chloroplasts and mitochondria of 'Yanzhi Mi' and 'Hong Yue' did not reach the significant level, while  $O_2^{\cdot-}$  production rate in the chloroplasts of 'Hong Shan Hu' was significantly higher than that in the mitochondria. Under salt stress,  $O_2^{\cdot-}$  production rate in the chloroplasts and mitochondria of 'Yanzhi Mi' had no evident difference, while the  $O_2^{\cdot-}$  production rate in the chloroplasts of 'Hong Shan Hu' and 'Hong Yue' was obviously higher than that in the mitochondria.

Under salt stress,  $H_2O_2$  contents were enhanced obviously in the three subcellular compartments of the three cultivars, except for that in the mitochondria of 'Yanzhi Mi' (Fig. 4). Among the three subcellular compartments, the highest  $H_2O_2$  accumulation was observed in the cytosol, followed the chloroplasts and mitochondria in the salt-treated and control seedlings.  $H_2O_2$  con-



**Fig. 3.** O<sub>2</sub><sup>-</sup> production rate in different subcellular compartments of three *Rhododendron* cultivars exposed to salt stress. Note: The values are given as mean  $\pm$  SE. Chl: chloroplasts; Mit: mitochondria; Cyt: cytosol. Lowercase letters (a, b) indicate the difference between the two treatments of the same *Rhododendron* cultivar, and Greek letters ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) represent the difference between the three subcellular compartments (the chloroplasts, mitochondria and cytosol) under the same treatment of the same cultivar, and different letters indicate significant difference ( $p < 0.05$ ) according to LSD's multiple-range tests.



**Fig. 4.** H<sub>2</sub>O<sub>2</sub> content in different subcellular compartments of three *Rhododendron* cultivars exposed to salt stress. Note: The values are given as mean  $\pm$  SE. Chl: chloroplasts; Mit: mitochondria; Cyt: cytosol. Lowercase letters (a, b) indicate the difference between the two treatments of the same *Rhododendron* cultivar, and Greek letters ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) represent the difference between the three subcellular compartments (the chloroplasts, mitochondria and cytosol) under the same treatment of the same cultivar, and different letters indicate significant difference ( $p < 0.05$ ) according to LSD's multiple-range tests.

tent in the cytosol was evidently higher than that in the chloroplasts and mitochondria, and there was no significant difference in H<sub>2</sub>O<sub>2</sub> contents between the mitochondria and chloroplasts.

### Lipid peroxidation

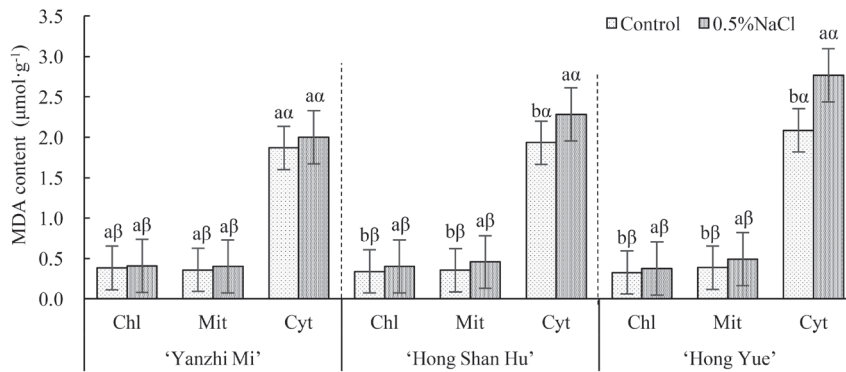
MDA contents were increased markedly in the three subcellular compartments of 'Hong Shan Hu' and 'Hong Yue' due to salt stress (Fig. 5), but MDA in 'Yanzhi Mi' did not change almost. Among the three subcellular compartments, the cytosol contained the highest MDA content, followed by the chloroplasts and mitochondria, however, MDA contents had no obvious difference between the chloroplasts and mitochondria.

### Enzymatic antioxidants

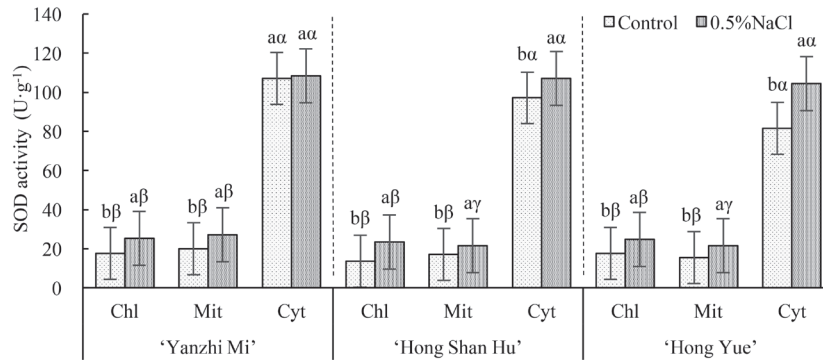
Compared with the control seedlings, SOD activities markedly increased in all subcellular compartments of the three cultivars under salt stress, except that SOD

activity in cytosol of 'Yanzhi Mi' was significantly unchanged (Fig. 6). Among the three subcellular compartments, the highest SOD activity was found in the cytosol. In the control seedlings, SOD activity of the three cultivars had no significant difference between the chloroplasts and mitochondria. Under salt stress, SOD activity in the chloroplasts of 'Hong Yue' and 'Hong Shan Hu' was significantly higher than that in the mitochondria, while SOD activities in the chloroplasts and mitochondria of 'Yanzhi Mi' did not reach the significant level.

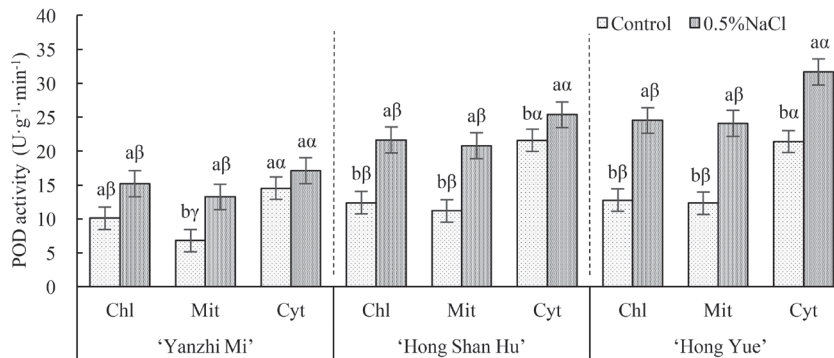
POD activities were not improved significantly in chloroplasts and cytosol of 'Yanzhi Mi' (Fig. 7), but improved markedly in the mitochondria. POD activities were improved significantly in the three subcellular compartments of 'Hong Shan Hu' and 'Hong Yue'. Among the three subcellular compartments, the highest POD activity was in the cytosol, followed by the chloroplasts and mitochondria in the salt-treated and control seedlings. POD activity in the cytosol was significantly higher than



**Fig. 5.** MDA contents in different subcellular compartments of three *Rhododendron* cultivars exposed to salt stress. Note: The values are given as mean  $\pm$  SE. Chl: chloroplasts; Mit: mitochondria; Cyt: cytosol. Lowercase letters (a, b) indicate the difference between the two treatments of the same *Rhododendron* cultivar, and Greek letters ( $\alpha$ ,  $\beta$ ) represent the difference between the three subcellular compartments (the chloroplasts, mitochondria and cytosol) under the same treatment of the same cultivar, and different letters indicate significant difference ( $p < 0.05$ ) according to LSD's multiple-range tests.



**Fig. 6.** SOD activity in different subcellular compartments of three *Rhododendron* cultivars exposed to salt stress. Note: The values are given as mean  $\pm$  SE. Chl: chloroplasts; Mit: mitochondria; Cyt: cytosol. Lowercase letters (a, b) indicate the difference between the two treatments of the same *Rhododendron* cultivar, and Greek letters ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) represent the difference between the three subcellular compartments (the chloroplasts, mitochondria and cytosol) under the same treatment of the same cultivar, and different letters indicate significant difference ( $p < 0.05$ ) according to LSD's multiple-range tests.



**Fig. 7.** POD activity in different subcellular compartments of three *Rhododendron* cultivars exposed to salt stress. Note: The values are given as mean  $\pm$  SE. Chl: chloroplasts; Mit: mitochondria; Cyt: cytosol. Lowercase letters (a, b) indicate the difference between the two treatments of the same *Rhododendron* cultivar, and Greek letters ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) represent the difference between the three subcellular compartments (the chloroplasts, mitochondria and cytosol) under the same treatment of the same cultivar, and different letters indicate significant difference ( $p < 0.05$ ) according to LSD's multiple-range tests.

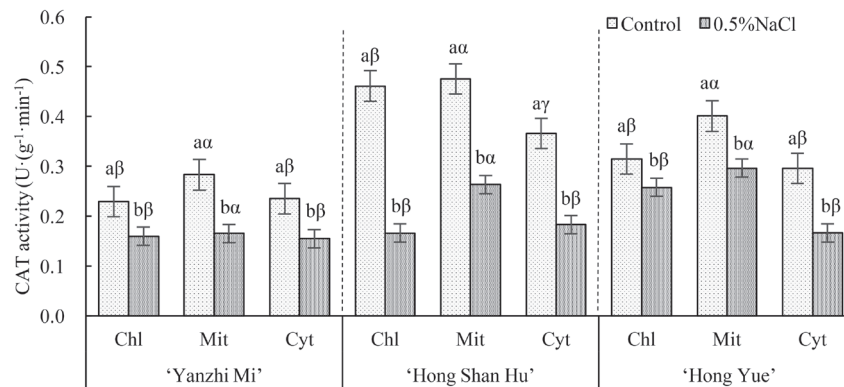
that in the chloroplasts and mitochondria, but POD activities in the chloroplasts and mitochondria were almost the same except that POD activity in the chloroplasts of ‘Yanzhi Mi’ in control group was dramatically higher than that in the mitochondria.

In contrast to SOD and POD, CAT activities were decreased dramatically in the three subcellular compartments of the three cultivars under salt stress (Fig. 8). In both the salt-treated and control seedlings, the highest CAT activity was found to be in the mitochondria, followed by the chloroplasts and cytosol, and CAT activities in the chloroplasts and cytosol were almost the same except for ‘Hong Shan Hu’ in control group.

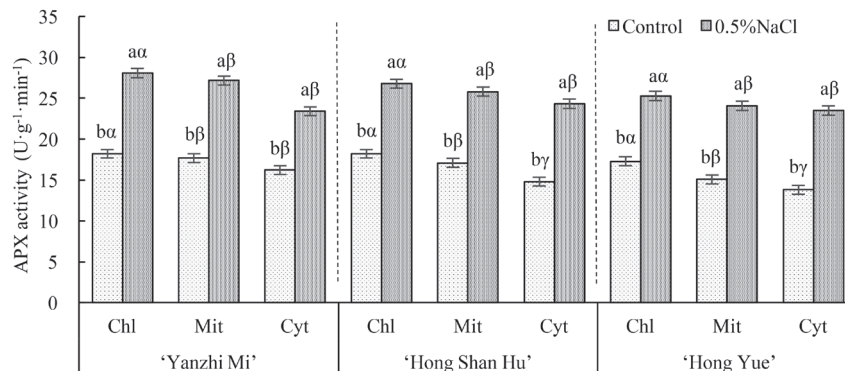
APX activities were promoted markedly in the three subcellular compartments of the three cultivars exposed to salt stress (Fig. 9). The highest activity of APX was found to be in the chloroplasts, followed by the mito-

chondria and cytosol in both the salt-treated and control plants. APX activity was distinctly higher in the chloroplast than that in the mitochondria and cytosol. In the control seedlings, the APX activity in the mitochondria of ‘Hong Shan Hu’ and ‘Hong Yue’ was evidently higher than that in the cytosol, but APX activity did not reach the significant difference between the mitochondria and cytosol of ‘Yanzhi Mi’. In the salt-treated seedlings, there was no significant difference between the mitochondria and cytosol of the three cultivars.

GR activities in the three subcellular compartments of the three cultivars were increased obviously under salt stress (Fig. 10). Among the three subcellular compartments, the cytosol contained the highest GR activity, followed by the chloroplasts and mitochondria, and GR activity between the chloroplasts and mitochondria did not reach the significant level.



**Fig. 8.** CAT activity in different subcellular compartments of three *Rhododendron* cultivars exposed to salt stress. Note: The values are given as mean  $\pm$  SE. Chl: chloroplasts; Mit: mitochondria; Cyt: cytosol. Lowercase letters (a, b) indicate the difference between the two treatments of the same *Rhododendron* cultivar, and Greek letters ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) represent the difference between the three subcellular compartments (the chloroplasts, mitochondria and cytosol) under the same treatment of the same cultivar, and different letters indicate significant difference ( $p < 0.05$ ) according to LSD's multiple-range tests.



**Fig. 9.** APX activity in different subcellular compartments of three *Rhododendron* cultivars exposed to salt stress. Note: The values are given as mean  $\pm$  SE. Chl: chloroplasts; Mit: mitochondria; Cyt: cytosol. Lowercase letters (a, b) indicate the difference between the two treatments of the same *Rhododendron* cultivar, and Greek letters ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) represent the difference between the three subcellular compartments (the chloroplasts, mitochondria and cytosol) under the same treatment of the same cultivar, and different letters indicate significant difference ( $p < 0.05$ ) according to LSD's multiple-range tests.

### Non-enzymatic Antioxidants

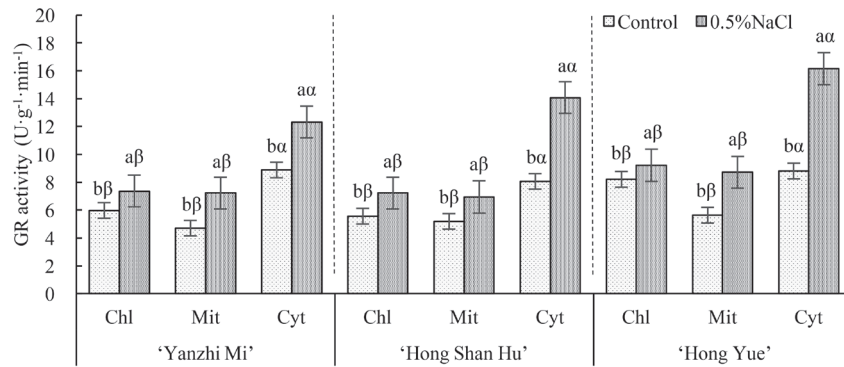
AsA contents in the three subcellular compartments of the three cultivars under salt stress were improved evidently in comparison to the control plants (Fig. 11). Among the three subcellular compartments, AsA content in the cytosol was obviously higher than that in the chloroplasts and mitochondria, and there was no evident difference in AsA contents between the chloroplasts and mitochondria.

Under salt stress, GSH contents in the three subcellular compartments of the three cultivars were enhanced to some extent, and the increase of GSH contents were significant in the chloroplasts and cytosol (Fig. 12). Among the three subcellular compartments, GSH content in the cytosol was higher than that in the chloroplasts and mitochondria, and GSH contents in the chloroplasts and mitochondria had no marked difference.

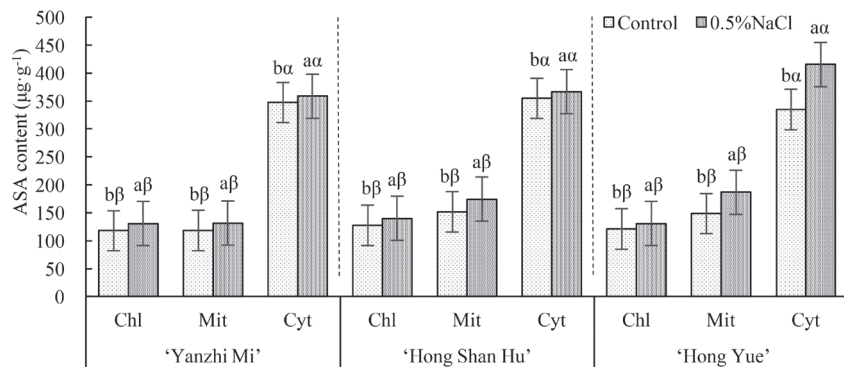
### DISCUSSION

#### Subcellular localization of ROS production and peroxidation damage under salt stress

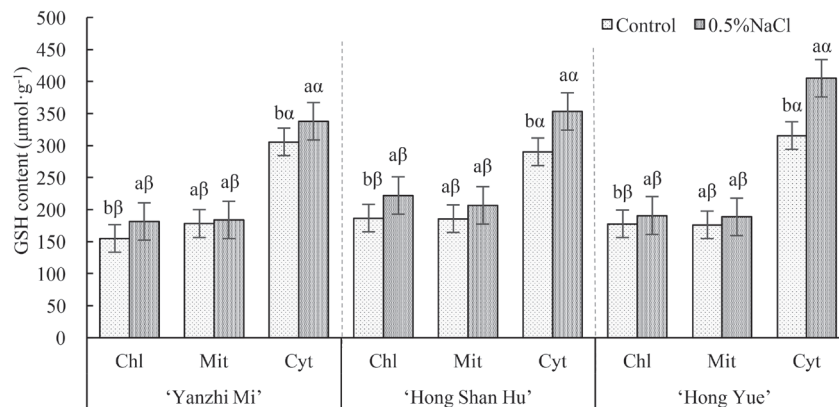
According to the external morphology and salt damage index of the three *Rhododendron* cultivars, the resistance of 'Yanzhi Mi' to salt stress was stronger than 'Hong Shan Hu' and 'Hong Yue'. Under salt stress, the levels of  $H_2O_2$  and  $O_2^{\cdot-}$  in the leaves of the three cultivars were enhanced, however, the increase amplification of  $H_2O_2$  and  $O_2^{\cdot-}$  in the mitochondria, cytosol and chloroplasts of the two salt-sensitive cultivars was more remarkable than salt-tolerant cultivar 'Yanzhi Mi'. The excessive accumulation of ROS in the two salt-sensitive rhododendrons resulted in serious peroxidation damage. In other plants such as tomato (Mittova *et al.*, 2003), rice (Chawla *et al.*, 2013) and wheat (Sairam *et al.*, 2002), the increasing rate of ROS and MDA in salt-sensi-



**Fig. 10.** GR activity in different subcellular compartments of three *Rhododendron* cultivars exposed to salt stress. Note: The values are given as mean  $\pm$  SE. Chl: chloroplasts; Mit: mitochondria; Cyt: cytosol. Lowercase letters (a, b) indicate the difference between the two treatments of the same *Rhododendron* cultivar, and Greek letters ( $\alpha$ ,  $\beta$ ) represent the difference between the three subcellular compartments (the chloroplasts, mitochondria and cytosol) under the same treatment of the same cultivar, and different letters indicate significant difference ( $p < 0.05$ ) according to LSD's multiple-range tests.



**Fig. 11.** AsA content in different subcellular compartments of three *Rhododendron* cultivars exposed to salt stress. Note: The values are given as mean  $\pm$  SE. Chl: chloroplasts; Mit: mitochondria; Cyt: cytosol. Lowercase letters (a, b) indicate the difference between the two treatments of the same *Rhododendron* cultivar, and Greek letters ( $\alpha$ ,  $\beta$ ) represent the difference between the three subcellular compartments (the chloroplasts, mitochondria and cytosol) under the same treatment of the same cultivar, and different letters indicate significant difference ( $p < 0.05$ ) according to LSD's multiple-range tests.



**Fig. 12.** GSH content in different subcellular compartments of three *Rhododendron* cultivars exposed to salt stress. Note: The values are given as mean  $\pm$  SE. Chl: chloroplasts; Mit: mitochondria; Cyt: cytosol. Lowercase letters (a, b) indicate the difference between the two treatments of the same *Rhododendron* cultivar, and Greek letters ( $\alpha$ ,  $\beta$ ) represent the difference between the three subcellular compartments (the chloroplasts, mitochondria and cytosol) under the same treatment of the same cultivar, and different letters indicate significant difference ( $p < 0.05$ ) according to LSD's multiple-range tests.

tive species were much higher than salt-tolerant species when they were exposed to salt stress. The results showed that the plants with stronger resistance had smaller changes in ROS and membrane quality under stress conditions. Salt-tolerant rice cultivar maintained the ultrastructures largely intact under salt stress (Lee *et al.*, 2013). *Rhododendron* seedlings with higher heat-resistant species maintained more stable cell membrane structure to improve their adaptability to heat stress (Gu *et al.*, 2016).

The cytosol, chloroplasts, mitochondria, peroxisomes and apoplasts in plants are the sources for ROS production (Mittler, 2002). In the present study, we analyzed the ROS levels in the three subcellular organelles like the cytosol, chloroplasts and mitochondria, and found that  $H_2O_2$  level and  $O_2^{\cdot-}$  production rate in the cytosol were significantly higher than that in the chloroplasts and mitochondria. Stress type or genotype may affect the subcellular distribution of ROS. In white clover exposed to PEG-induced water stress (Wang *et al.*, 2008) and wheat (Sairam *et al.*, 2002) exposed to salt stress, the chloroplasts showed higher  $H_2O_2$  accumulation compared with other subcellular compartments such as the cytosol and mitochondria. MDA, as a suitable marker for membrane lipid peroxidation (Moller *et al.*, 2007), in the present study, the excessive accumulation of ROS in the cytosol resulted in more serious oxidative damage indicated by the higher MDA level in the cytosol. In addition, MDA had the highest content in the chloroplasts and was consistent with the distribution of ROS in white clover (Wang *et al.*, 2008) and wheat (Sairam *et al.*, 2002). But the subcellular distribution of ROS is not always consistent with MDA, Mittova *et al.* (2003) investigated the response of the antioxidative systems of leaf cell mitochondria and peroxisomes of a cultivated tomato and a wild species to salt stress, the results showed that  $H_2O_2$  level in the peroxisomes was

higher than that in the mitochondria, however, MDA in the mitochondria was higher than that in the peroxisomes.

#### Subcellular localization of antioxidant systems damage under salt stress

There are three types of SODs classified by their metal cofactors, including Fe-SOD, Mn-SOD and Cu/Zn-SOD (Das and Roychoudhury, 2014), and Cu/Zn-SOD was the main form of the SOD in the cytosol (Tan *et al.*, 2010; Huseynova *et al.*, 2014). SOD had the highest activities in the cytosol and was 4.27–7.16 times that of the mitochondria and chloroplasts, which suggested Cu/Zn-SOD should be the main form and play an important role in the elimination of  $O_2^{\cdot-}$  in rhododendron plants. The highest  $O_2^{\cdot-}$  production rate and SOD activity in the cytosol of rhododendron leaves indicated that the production and elimination of  $O_2^{\cdot-}$  in rhododendrons were mainly carried out in the cytosol. POD, GR, AsA and GSH were primarily distributed in the cytosol of rhododendron leaves, however, the distribution of CAT and APX differed from the antioxidants above mentioned. CAT was the most active in the mitochondria and APX was mainly located in the chloroplasts, indicating that Chloroplast and mitochondria were the main sites for APX and CAT enzymes to scavenge  $H_2O_2$ , respectively. Tan *et al.* (2010) found that the antioxidants in maize leaves during water stress exhibited significantly higher activity in the cytosol than other subcellular compartments such as the chloroplasts and mitochondria. The research of Locato *et al.* (2009) showed the cytosol of tobacco under heat shock contained the highest activity of APX and GR. In addition, SOD, APX, GR and AsA of wheat (Sairam *et al.*, 2002) exposed to salt stress and APX, GR, AsA and GSH of white clover (Wang *et al.*, 2008) under water stress were the highest in the chloroplasts. Yu *et al.* (2014) also investigated CAT activities

in the three subcellular compartments of two *Sabina* species under low temperature stress and found the mitochondria had the highest activity. In the leaves of salt-stressed tomato (Sairam *et al.*, 2002) and water-stressed white clover (Tan *et al.*, 2010), CAT exhibited the higher activity in the peroxidases than that in the mitochondria and chloroplasts. In conclusion, stress types or genotypes may affect the subcellular distribution of antioxidants.

Maintaining the dynamic balance of ROS under stresses is crucial for plant adaptation to stress conditions (Das and Roychoudhury, 2014). Plants with stronger resistance usually showed stronger antioxidant mechanisms (Sairam *et al.*, 2002; Chawla *et al.*, 2013), but some studies showed that plants with weaker tolerance had increased activities of the antioxidant enzymes under stress conditions. In two *Jatropha* species, SOD, CAT and POD in salt-sensitive *J. curcas* appeared to be higher activities under 200 mmol·L<sup>-1</sup> salt concentration than salt-tolerant *J. cinerea* (Hishida *et al.*, 2014). A salt-sensitive rice cultivar IR-29 had higher H<sub>2</sub>O<sub>2</sub>-scavenging enzyme activities in salt-treated seedlings than a salt-tolerant cultivar Pokkali (Lee *et al.*, 2013). In the present study, SOD and POD mainly distributed in the cytosol, however, their activities in 'Yanzhi Mi' with salt-tolerance did not increase under salt stress, on the contrary, the increase of SOD and POD activities in salt-sensitive *Rhododendron* cultivars was remarkable. This should be related to the adaptability of plants to stress conditions. Mild stress can result in the oxidative stress and induce the response of plants with weak resistance to the stress, indicated by the increase of ROS-scavenging ability (Lee *et al.*, 2013; Hishida *et al.*, 2014), but the plants with strong resistance do not suffer from the oxidative stress under the mild stress, their antioxidant activity also do not change significantly. When the stress level increases, only those with strong resistance can maintain their higher antioxidant activity.

In the present study, SOD, POD and AsA-GSH cycle in the three *Rhododendron* cultivars under salt stress, their ROS-scavenging abilities were all enhanced, however, it did not effectively reduce peroxidation damage, which may be related to the decrease of CAT activity. Among the seven antioxidants tested in this study, only CAT decreased significantly under salt stress, suggesting that maintaining the activity of CAT enzymes is essential to improve the tolerance of rhododendrons to salt stress. Similar results have been reported in other plants such as mulberry (Harinasut *et al.*, 2003) and apple (Wang *et al.*, 2013) under salt stress, CAT activity was decreased or unchanged while other measured antioxidants were enhanced along with the enhancement of ROS and MDA. But CAT is not equally effective for salt tolerance in all plants. In rice (Chawla *et al.*, 2013) exposed to salt stress, the increase activity of CAT and other antioxidants did not reduce the ROS levels, and still resulted in the oxidative stress.

In summary, compared with the cultivars with weak salt resistance, the salt-resistant *Rhododendron* cultivar had smaller changes in ROS and membrane quality

under stress conditions. The enhancement of SOD, POD and AsA-GSH cycle activities did not prevent the accumulation of ROS and membrane peroxidation. CAT may be the key enzyme for scavenging ROS. Under salt stress, ROS and its scavenging systems were found in three subcellular compartments of the rhododendron. MDA, ROS and its scavenging system including SOD, POD, AsA, GSH and GR, were primarily distributed in the cytosol, indicating that the cytosol was the main site of ROS accumulation and elimination in rhododendron. However, there are differences in the distribution of APX and CAT. CAT and APX scavenging H<sub>2</sub>O<sub>2</sub> were principally conducted in the chloroplasts and mitochondria respectively.

## AUTHOR CONTRIBUTIONS

Pan Liu performed the experiments, collected and analyzed the physiological data and drafted the paper. Xingmin Geng took care of the study designing and preparation of experimental materials, and edited and reviewed the manuscript. Yukio Ozaki supervised the experiments on salt stress and contributed to the manuscript writing. Hui Zhao and Fang Zheng were responsible for data analysis, literature collection and manuscript proofreading.

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