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Effects of paclobutrazol on cultivars of Chinese bayberry (*Myrica rubra*) under salinity stress

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Abstract

Salt stress is one of the most critical factors hindering the growth and development of plants. Paclobutrazol (PBZ) is widely used to minimize this problem in agriculture because it can induce salt stress tolerance in plants. This study investigated the effects of PBZ on salt tolerance of seedlings from two Chinese bayberry cultivars (*i.e.*, Wangdao and Shenhong). Plants were treated with three salt concentrations (0, 0.2, and 0.4 % NaCl) and two PBZ concentrations (0 and 2.0 μmol L^{-1}). Application of PBZ increased a relative water content, proline content, chlorophyll (*a*+*b*) content, and antioxidant enzyme activities in both cultivars, resulting in a better acclimation to salt stress and an increase in dry matter production. We concluded that PBZ ameliorated the negative effects of salt stress in Chinese bayberry seedlings.

Additional key words: antioxidative enzyme; cellular damage; chlorophyll; gas exchange; photosynthesis.

Introduction

Salt-affected soil is a major form of land degradation (Dudal and Purnell 1986). One billion hectares of land are salt-affected worldwide (Wicke *et al*. 2011), out of which approximately 76 million ha are affected by human-induced salinization and sodification (Oldeman *et al.* 1991). The total area of saline soil in China is about 1 billion ha, out of which about 36.9 million ha of soil are undergoing secondary salinization, caused mainly by improper utilization of land resources, such as excessive irrigation and/or lack of adequate salt leaching (Li *et al.* 2005). Salinity stress has received increasing attention in recent years because it greatly reduces agricultural productivity (Parihar *et al.* 2015). The establishment of permanent vegetative cover is crucial in stopping further degradation of these saline land resources. Growing salt-tolerant trees and shrubs on salt-affected soils (for wood and nonwood products, and hydrological benefits) can be a productive use of moderately to highly salt-affected land (Marcar and Khanna 1997). Therefore, it is necessary to explore salt-tolerant plants to enable optimal utilization of salt-affected soils.

Soil salinity is a major environmental stress factor which limits plant growth due to reduction in photosynthesis (Meloni *et al.* 2003). It imposes both ionic toxicity and osmotic stress in plants, leading to nutritional disorders and oxidative stress (Serrano and Rodriguez 2002; de Azevedo Neto *et al.* 2006). Osmotic adjustment in terms of proline is considered an important physiological adaptation to saline environment (Woodward and Bennett 2005), facilitating the maintenance of cell turgor (Ramanjulu and Sudhakar 2001, Kumar *et al.* 2000, 2003). In addition to its role in osmotic adjustment, it may

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Abbreviations: CAT – catalase; Chl – chlorophyll; DM – dry mass; FM – fresh mass; MDA – malondialdehyde; PBZ – paclobutrazol; REC – relative electrolyte conductivity; ROS – reactive oxygen species; RWC – relative water content; SOD – superoxide dismutase.
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also protect membranes from damage and stabilize structures and activities of proteins and enzymes (Hessini *et al.* 2009). One of the biochemical changes in plants under salt stress is the excessive generation of reactive oxygen species (ROS) (Hasegawa *et al*. 2000). ROS can have detrimental effects on normal metabolism through oxidative damage to lipids, proteins, and nucleic acids (Olmos *et al.* 1994, Shalata and Tal 1998). In order to overcome oxidative stress, plants have developed oxygen radical detoxifying enzymes, such as superoxide dismutase (SOD), catalase (CAT), and antioxidant molecules, such as ascorbic acid and α-tocopherol (Prochazkova *et al.* 2001). Application of PBZ has ameliorated the negative effects of salt stress in peach and mango (EI-Khashab *et al.* 1997, Kishor *et al.* 2009). Exogenous application of PBZ has influenced a range of developmental and physiological process of plants under salt stress, such as growth of roots and stem (Manivannan *et al.* 2008), water relation (Sharma *et al.* 2011), antioxidant enzymes (Srivastav *et al.* 2003, Kishor *et al.* 2009), and photosynthetic pigments (Jaleel *et al.* 2007).

Chinese bayberry [*Myrica rubra* (*Lour.*) *Zucc.*] belongs to Myricaceae family, and is widely cultured as a good subtropical fruit crop of agro-forestry system in China (Zheng *et al.* 2010). Chinese bayberry is a fast-growing tree species that can bear fruits in about 2–3 years after grafting, with its economic life of about hundred years. Chinese bayberry is widely planted throughout the world for its great ecological, fruit and medicinal values (Chen *et al.* 2004, Sun *et al.* 2013). There are about nine varieties of red bayberry widely cultivated in saline land of Zhejiang Province (Shan and Wang 2012). The survey of Chinese bayberry resources in Zhejiang Province showed that Wangdao cultivar (WD), originated in Zhou Shan Dinghai district (ocean region), is a highly salt-tolerant cultivar (Wang *et al.* 2001). In contrast, Shenhong cultivar (SH) is a relatively salt-sensitive cultivar that has been cultivated in large areas of Zhejiang because of its relatively small seeds and large fruits (Wang *et al.* 2001). Although selection and breeding are the ultimate means to produce salt-tolerant species, exogenous application of osmoprotectants, growth promoters, and antioxidant compounds to plants has been considered short-term solution to ameliorate the negative effects of salt stress on plants (Hoque *et al.* 2007, Li *et al.* 2014). Biochemical and physiological parameters have been developed as effective indices for tolerance screening in plant breeding programs (Parida and Das 2005, Ashraf and Foolad 2007, Parihar *et al.* 2015). In this study, we intended to investigate whether PBZ can induce physiological responses that affect dry matter, photosynthesis, relative water content (RWC), proline, photosynthetic pigments, relative electrolyte conductivity (REC), malondialdehyde (MDA), and antioxidant enzyme activity, enabling Chinese bayberry seedlings to alleviate the negative effect of salt stress. Moreover, this experiment also examined sole and combined effects of salinity (NaCl) and PBZ on growth and physiology of two different cultivars of red bayberry seedlings. The investigation could improve the growth of Chinese bayberry and promote sustainable use of saline land resources.

Materials and methods

Plant material and growth conditions: The experiment was conducted in controlled environment at Zhejiang A & F University, China (30°23′N, 119°72′E) in early March, 2011. One-year-old seedlings of Chinese bayberry (*M. rubra*) from two cultivars (*i.e*., WD and SH) were selected. The seedlings (one seedling per pot) were transplanted into plastic pots (30 cm was inner diameter, 28 cm height) containing 10 kg of silt soil and perlite mixture at a ratio of 3:1 (v/v). The soil was loam with pH 4.72, 12.42 g(organic matter) kg^{-1}, 141.08 g(total N) kg^{-1}, 2.13 g(total P) kg^{-1}, and 20.80 g(total K) kg^{-1}. All pots were irrigated on daily basis to keep them well watered and to maintain 75% field capacity of soil.

Paclobutrazol (PBZ) and salt-stress treatment: After 8 weeks, about 90 seedlings from each cultivar (WD or SH) were selected on the basis of uniformity, vigor, leaf size, and leaf shape. These seedlings were divided into six treatment groups (*see* the text table below). Each treatment was replicated three times and each replicate included five pots. The experiment was laid out in a completely randomized factorial design, with three salt concentrations (0, 0.2, or 0.4% NaCl) and two PBZ concentrations (0 and 2.0 μmol L^{-1}). In the present study, the PBZ treatment served as a main plot, NaCl treatment as subplot, and cultivar as sub-subplot factor.

Group	Treatment
P0N0 (control)	distilled water without PBZ
P1N0	distilled water with PBZ (2.0 μmol L^{-1})
P0N2	0.2 % NaCl [2.0 g(NaCl) kg^{-1}(soil)] without PBZ
P1N2	0.2 % NaCl [2.0 g(NaCl) kg^{-1}(soil)] with 2.0 μmol(PBZ) L^{-1}
P0N4	0.4 % NaCl [4.0 g(NaCl) kg^{-1}(soil)] without PBZ
P1N4	0.4 % NaCl [4.0 g(NaCl) kg^{-1}(soil)] with 2.0 μmol(PBZ) L^{-1}

Both NaCl and PBZ treatments were given as soil drench. To avoid osmotic shock, NaCl solution (20 or 40 g of NaCl dissolved in 3 L of distilled water) was gradually added to soil in eight steps up to final concentrations of 0.2 % and 0.4 % NaCl in 4 d. Sole salt treatments were prepared by adding 375 ml NaCl solution in pots at 06:30 or 17:30 h. In the combined treatment (salt stress + PBZ), 2.0 μmol(PBZ) L^{-1} (99 %, *EKEAR*, Shanghai, China) was added in NaCl solution. Solution leached out was collected and recycled in respective pots.

In preliminary experiments, 0.5, 1, 2, 4 μmol(PBZ) L^{-1} were used for treatments. Among all the treatments, 2 μmol(PBZ) L^{-1} increased a plant height significantly and the higher concentration reduced growth and pigment content drastically. At lower concentrations, there was no change in the growth and pigment content. Therefeore, 2 μmol(PBZ) L^{-1} concentration was used to study the effect of PBZ on *M. rubra*.

In the previous experiment, we found out that two months were enough to check differences in the growth of seedlings and between treatments. Sixty days after treatments, the second or third leaves from the top were used as material for physiological analyses. The leaf samples were collected from plants of each treatment and cleaned with moistened cloth to remove any surface contamination. Plants were immediately frozen in liquid nitrogen and stored at –70°C.

Biomass: Sixty days after treatments, seedlings from all treatments were uprooted for biomass analysis. The shoots and roots were oven-dried to constant mass at 85°C for 24 h and weighted using an electronic scale to determine the biomass.

Photosynthetic rate: The current-year leaves from six to eight randomly selected seedlings were chosen for measurement of net photosynthesis (P_N) on leaves of growing plants using portable *LI–6400* infrared gas analyzer (*LI-COR, Inc*., Lincoln, NE, USA) equipped with normal 2 × 3 cm chamber with *LI-COR 6400-02B* LED light source. Photosynthetic gas exchange measurements were taken from 8:00 to 11:00 h at PAR of 1,000 μmol(photon) m^{-2} s^{-1}. Leaf temperature and chamber CO_2 concentration were kept at about 29–32°C and 400 μmol(CO_2) mol^{-1}, respectively.

Leaf relative water content (RWC) and proline content: Leaf discs (10 mm diameter, 20 pieces) were collected from each treatment to determine RWC, which was calculated as RWC [%] = [(FM – DM)/(TM – DM)] ×100, where FM is fresh mass, TM is the turgid mass after rehydrating samples for 24 h in dark, and DM is the constant dry mass after oven-drying at 85°C for 48 h.

Free proline content was assessed as described by Bate *et al.* (1973). Powdered frozen leaves (0.2 g) were weighed into 10-ml centrifuge tubes and then 5 ml of 3 % aqueous sulfosalicylic acid was added for precipitation of protein. Samples were mixed before being heated in boiling water for 30 min and filtered through *Whatman* No. 2 filter paper. The filtrate (2 ml) was mixed with 2 ml of acid ninhydrin and 2 ml of glacial acetic acid in a test tube. It was maintained for 1 h at 100°C. The reaction was stopped using an ice bath. The mixture was extracted with toluene, and absorbance of fraction containing toluene aspirated from liquid phase was read at 520 nm with a spectrophotometer (*UV-2550, Shimadzu,* Japan). Proline concentration was determined using calibration curve in μg(proline) g^{-1}(FM).

Photosynthetic pigments: First, 5 g of leaf sample was grounded to powder in liquid nitrogen and repeatedly extracted with 15 ml of 80% acetone. The photosynthetic pigments were extracted at 4°C for 24 h in darkness. The absorbance of the supernatant was measured spectrophotometrically at 645 and 663 nm by a spectrophotometer (*U–3900*, *Hitachi*, Japan) after centrifugation at 10,000 × *g* for 10 min. The chlorophyll (Chl) concentration was determined using methods described by Lichtenthaler (1987).

Relative electrolyte leakage rate and lipid peroxidation: Membrane permeability was estimated by measuring relative electrolyte conductivity (REC) of leaf in accordance with protocol described by Nayyar (2003). Leaf tissue of 0.2 g was briefly rinsed with deionized water and immersed in a test tube with 30 ml of deionized water for 12 h. Electrical conductivity (initial EC) of solution was measured using conductivity meter (*Model DJS – 1C*; *Shanghai Analytical Instrument Co.,* Shanghai, China). The samples were heated to 100°C for 20 min and electrical conductivity (final EC) in bathing solution and results were read again.

Membrane permeability was calculated as:

$$\text{EC (\%)} = \left(\frac{\text{initial EC}}{\text{final EC}} \right) \times 100$$

TBARS were determined and expressed as malondialdehyde (MDA) equivalents, according to the method of Shahidi and Hong (1991). Fresh leaves (1.0 g) were grounded in 10% trichloroacetic acid and then centrifuged at 3,000 *g* for 10 min. Two milliliters of supernatant were mixed with 2 ml of 0.6 % thiobarbituric acid (TBA) and incubated for 30 min at 100°C to develop (TBA)2 – MDA adduct. The mixtures were immediately cooled down in an ice bath. After centrifugation at 5,000 × *g* for 20 min, the absorbance of the supernatant was recorded at 532, 600, and 450 nm with a spectrophotometer (*UV-2550, Shimadzu,* Japan). Lipid peroxidation was expressed in [μmol g^{-1}(FM)] using following formula:

$6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$.

Antioxidative enzyme activities: Superoxide dismutase (SOD) (EC 1.15.1.1) activity was determined using nitroblue tetrazolium (NBT) method (Fu and Huang 2001).

One unit of SOD activity was defined as amount of enzyme required to produce 50% inhibition of reduction of NBT at 560 nm. The activity of catalase (CAT) (EC 1.11.1.6) was determined using methods described by Fu and Huang (2001). One unit of CAT activity (per fresh mass, FM) was defined as μmol(H_2O_2) degraded per min.

Data analysis: Statistical analyses were performed with software statistical package for social science (*SPSS*) *version 11.5* (*IBM*, New York, USA). Three-way analyses of variance (*ANOVA*) were used to test the effects of PBZ, salt stress, and cultivars and their interaction on physiological and biochemical variables. Three replicates were performed for each physiological and biochemical variables.

Results

Biomass: Salt stress alone resulted in significant decrease in shoot DM, root DM, and total DM in both WD and SH (Table 1). The shoot DM decreased by 21.1 and 37.3% with application of 0.2 and 0.4% NaCl in WD, respectively, and it decreased by 34.3 and 50.4% under 0.2 and 0.4% NaCl treatment in SH, respectively. The roots biomass decreased by 17.9 and 17.2% in WD with application of 0.2 and 0.4% NaCl, respectively. The roots biomass decreased by 19.4 and 38.7% in SH with application of 0.2 and 0.4% NaCl, respectively. The combination of salt stress and PBZ significantly increased roots biomass both in WD and SH, compared to sole effects of salt stress. However, shoot biomass did not change significantly in both WD and SH under a combined application of NaCl and PBZ compared to sole NaCl. The combination of salt stress (NaCl) and PBZ significantly increased total biomass in WD compared to salt stress alone. The combined treatment significantly increased total DM in SH compared to 0.4% NaCl alone. The roots/shoot ratio in WD was hardly affected by 0.2% NaCl, while it significantly increased with application of 0.4% NaCl compared with control. Salt stress significantly increased the root/shoot ratio in SH compared with control. On other hand, the root/shoot ratio significantly increased with combined application of NaCl and PBZ compared to sole effect of NaCl and control.

Photosynthetic rate: Significant decreases in P_N were detected in both WD and SH under salt stress alone (Fig. 1). Decrease in P_N of WD and SH was 25.6 and 27.4%, respectively, after the treatment by 0.2% NaCl, whereas it was 53.6 and 69.1% in WD and SH,

Table 1. The shoot, roots, total plant dry mass and ratio of root dry mass to shoot dry mass in WD and SH exposed to different salt stress, paclobutrazol (PBZ) treatments, and their combination. Treatments: P0N0 – distilled water without PBZ; P1N0 – distilled water with PBZ; P0N2 – 0.2% NaCl without PBZ; P1N2 – 0.2% NaCl with PBZ; P0N4 – 0.4% NaCl without PBZ; and P1N4 – 0.4% NaCl with PBZ. *Different letters* within a column denote statistically significant differences between treatments at $P<0.05$ level according to *Tukey*'s test. Values are expressed as means ± SD, $n = 3$. *$0.01 \le P < 0.05$; **$0.001 \le P < 0.01$; ***$P < 0.001$; ns – not significant. F_c – cultivar effect; F_n – salt stress effect; F_p – PBZ effect. $F_{c\times n}$ – cultivars × salt stress interaction effect; $F_{c\times p}$ – cultivars × PBZ interaction effect; $F_{n\times p}$ – salt stress × PBZ interaction effect; $F_{c\times n\times p}$ – cultivars × salt stress × PBZ interaction effect.

PBZ [μmol L^{-1}]	NaCl [%]	Cultivar	Shoot dry mass [g per plant]	Root dry mass	Total dry mass	Root/shoot ratio
0	0	WD	18.46 ± 1.39^{a}	8.04 ± 0.66^{c}	26.50 ± 2.05^{ab}	0.44 ± 0.004^{e}
0	0	SH	16.61 ± 1.28^{b}	5.06 ± 0.11^{ef}	21.67 ± 1.37^{de}	0.31 ± 0.02^{g}
0	0.2	WD	14.60 ± 0.88^{c}	6.60 ± 0.79^{d}	21.20 ± 1.64^{de}	0.45 ± 0.03^{de}
0	0.2	SH	10.92 ± 0.77^{e}	4.08 ± 0.12^{g}	15.00 ± 0.66^{gh}	0.38 ± 0.04^{f}
0	0.4	WD	11.60 ± 1.07^{de}	6.66 ± 0.34^{d}	18.26 ± 1.36^{f}	0.58 ± 0.03^{bc}
0	0.4	SH	8.23 ± 0.72^{f}	3.10 ± 0.27^{h}	11.33 ± 0.99^{i}	0.38 ± 0.01^{f}
2	0	WD	18.73 ± 0.94^{a}	10.60 ± 0.58^{a}	29.33 ± 1.48^{a}	0.57 ± 0.01^{c}
2	0	SH	16.62 ± 0.90^{b}	7.12 ± 0.27^{d}	23.74 ± 1.08^{cd}	0.43 ± 0.02^{e}
2	0.2	WD	15.73 ± 0.56^{bc}	9.67 ± 0.17^{b}	25.40 ± 0.72^{bc}	0.61 ± 0.01^{ab}
2	0.2	SH	11.48 ± 0.87^{de}	5.59 ± 0.39^{e}	17.07 ± 1.24^{fg}	0.49 ± 0.01^{d}
2	0.4	WD	12.93 ± 0.36^{d}	8.01 ± 0.06^{c}	20.94 ± 0.42^{e}	0.62 ± 0.01^{a}
2	0.4	SH	9.13 ± 0.68^{f}	4.47 ± 0.71^{fg}	13.60 ± 1.28^{h}	0.49 ± 0.06^{d}
		F_c	***	***	***	***
		F_n	***	***	***	***
		F_p	*	***	***	***
		$F_{c\times n}$	*	ns	ns	*
		$F_{c\times p}$	ns	*	ns	ns
		$F_{n\times p}$	ns	*	ns	*
		$F_{c\times n\times p}$	ns	ns	ns	*

respectively, with application of 0.4% NaCl compared with control. In both WD and SH, increases in P_N were significant due to a combined effect of salt stress and PBZ compared to the effect of sole NaCl. The combined effect of 0.2% NaCl and PBZ caused 19.5 and 25.7% increase in P_N of WD and SH plants, respectively, compared to the plants with sole 0.2% NaCl. Application of 0.4% NaCl and PBZ treatment caused an increase of 57.0 and 52.8% in P_N of WD and SH, respectively.

RWC and proline content: RWC evaluates physiological water status of plants. Salt stress (NaCl) alone resulted in a significant decrease of RWC in both WD and SH (Fig. 2*A*). The RWC decreased by 3.6 and 11.1% in WD with application of 0.2 and 0.4 % NaCl, respectively, whereas it decreased by 6.2 and 21.9% in SH after treatments by 0.2 and 0.4% NaCl, respectively. The RWC was barely affected by PBZ in both WD and SH with the application of 0.2% NaCl. However, the RWC significantly increased in both WD and SH after the combined application of 0.4% NaCl and PBZ compared to effects of sole salt stress (NaCl).

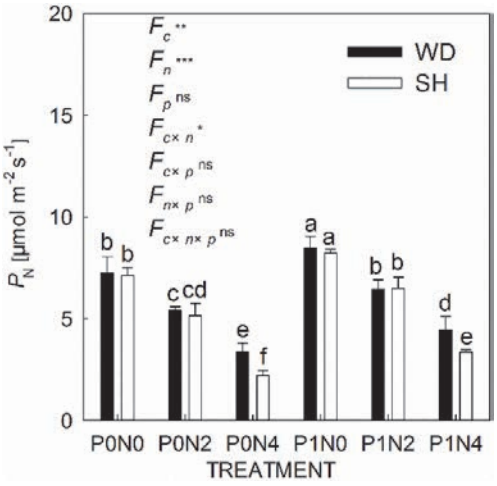

Fig. 1. Photosynthetic rate (P_N) in WD and SH exposed to different salt stress concentrations, PBZ treatments and their combination. WD – Wangdao cultivar, SH – Shenhong cultivar. Treatments: P0N0 – distilled water without PBZ; P1N0 – distilled water with PBZ; P0N2 – 0.2% NaCl without PBZ; P1N2 – 0.2% NaCl with PBZ; P0N4 – 0.4% NaCl without PBZ; and P1N4 – 0.4% NaCl with PBZ. *Different letters* above bars denote statistically significant differences between treatments at $P<0.05$ level according to *Tukey*'s test. Values are expressed as means ± SD, $n = 3$. *$0.01\leq P<0.05$; **$0.001\leq P<0.01$; ***$P<0.001$; ns – not significant. F_c – cultivar effect; F_n – salt stress effect; F_p – PBZ effect. $F_{c\times n}$ – cultivar× salt stress interaction effect; $F_{c\times p}$ – cultivar × PBZ interaction effect; $F_{n\times p}$ – salt stress × PBZ interaction effect; $F_{c\times n\times p}$ – cultivars × salt stress × PBZ interaction effect.

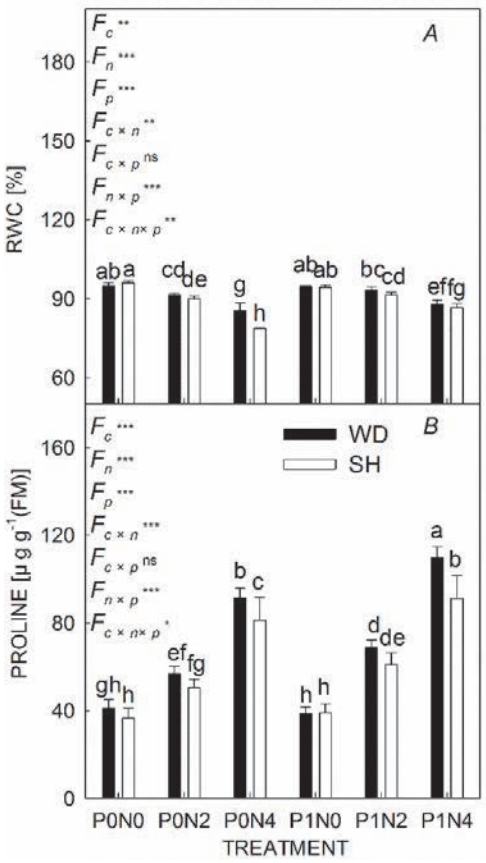

Fig. 2. Relative water content (RWC, *A*) and proline content (*B*) in WD and SH exposed to different salt stress, PBZ treatments and their combination. WD – Wangdao cultivar, SH – Shenhong cultivar. Treatments: P0N0 – distilled water without PBZ; P1N0 – distilled water with PBZ; P0N2 – 0.2% NaCl without PBZ; P1N2 – 0.2% NaCl with PBZ; P0N4 – 0.4% NaCl without PBZ; and P1N4 – 0.4% NaCl with PBZ. *Different letters* above bars denote statistically significant differences between treatments at $P<0.05$ level according to *Tukey*'s test. Values are expressed as means ± SD, $n = 3$. *$0.01\leq P<0.05$; **$0.001\leq P<0.01$; ***$P<0.001$; ns – not significant. F_c – cultivar effect; F_n – salt stress effect; F_p – PBZ effect. $F_{c\times n}$ – cultivar × salt stress interaction effect; $F_{c\times p}$ – cultivar × PBZ interaction effect; $F_{n\times p}$ – salt stress × PBZ interaction effect; $F_{c\times n\times p}$ – cultivars × salt stress × PBZ interaction effect.

The effects of sole salt stress (NaCl) induced a significant increase in the proline content in both WD and SH (Fig. 2*B*). It revealed that sole effects of PBZ was insignificant, however, the combined effect of NaCl and PBZ resulted in the significant increase in proline. The combined application of PBZ and 0.2% NaCl induced the significant increase of 21% in the proline content in both WD and SH, whereas 20 and 12.2% increase in proline was observed after the treatment of 0.4% NaCl and PBZ, respectively, compared to the sole effect of NaCl. Moreover, there were significant differences in the proline content between WD and SH when exposed to 0.4% NaCl, with WD showing higher values than SH.

Table 2. The chlorophyll (Chl) *a*, Chl *b*, and total Chl (*a*+*b*) and the ratio of Chl *a*/*b* in WD and SH exposed to different salt stress, paclobutrazol (PBZ) treatments, and their combination. Treatments: P0N0 – distilled water without PBZ; P1N0 – distilled water with PBZ; P0N2 – 0.2% NaCl without PBZ; P1N2 – 0.2% NaCl with PBZ; P0N4 – 0.4% NaCl without PBZ; and P1N4 – 0.4% NaCl with PBZ. *Different letters* within a column denote statistically significant differences between treatments at $P<0.05$ level according to *Tukey*'s test. Values are expressed as means ± SD, $n = 3$. *$0.01 \le P < 0.05$; **$0.001 \le P < 0.01$; ***$P < 0.001$; ns – not significant. F_c – cultivar effect; F_n – salt stress effect; F_p – PBZ effect; $F_{c\times n}$ – cultivars × salt stress interaction effect; $F_{c\times p}$ – cultivars × PBZ interaction effect; $F_{n\times p}$ – salt stress × PBZ interaction effect; $F_{c\times n\times p}$ – cultivars × salt stress × PBZ interaction effect.

PBZ	NaCl [%]	Cultivar	Chl *a* [mg g^{-1}FM]	Chl *b*	Chl (*a*+*b*)	Chl *a*/*b*
0	0	WD	2.05 ± 0.08^{b}	0.67 ± 0.01^{b}	2.72 ± 0.09^{b}	3.05 ± 0.10^{cde}
0	0	SH	2.00 ± 0.10^{b}	0.69 ± 0.04^{ab}	2.69 ± 0.14^{b}	2.89 ± 0.06^{f}
0	0.2	WD	1.66 ± 0.04^{ef}	0.51 ± 0.01^{cd}	2.18 ± 0.03^{de}	3.24 ± 0.09^{cde}
0	0.2	SH	1.52 ± 0.01^{g}	0.47 ± 0.02^{de}	2.00 ± 0.03^{f}	3.23 ± 0.06^{cdef}
0	0.4	WD	1.58 ± 0.06^{fg}	0.41 ± 0.02^{e}	1.99 ± 0.04^{f}	3.88 ± 0.11^{a}
0	0.4	SH	1.35 ± 0.11^{h}	0.42 ± 0.06^{e}	1.77 ± 0.17^{g}	3.27 ± 0.04^{cde}
2	0	WD	2.21 ± 0.08^{a}	0.72 ± 0.09^{ab}	2.93 ± 0.15^{a}	3.10 ± 0.07^{cdef}
2	0	SH	2.24 ± 0.03^{a}	0.76 ± 0.04^{a}	3.00 ± 0.03^{a}	2.97 ± 0.01^{ef}
2	0.2	WD	1.86 ± 0.02^{c}	0.55 ± 0.01^{c}	2.41 ± 0.02^{c}	3.41 ± 0.05^{bc}
2	0.2	SH	1.79 ± 0.02^{cd}	0.53 ± 0.01^{cd}	2.32 ± 0.02^{cd}	3.41 ± 0.03^{bc}
2	0.4	WD	1.72 ± 0.10^{de}	0.47 ± 0.05^{de}	2.18 ± 0.14^{de}	3.70 ± 0.17^{ab}
2	0.4	SH	1.59 ± 0.06^{fg}	0.47 ± 0.01^{de}	2.06 ± 0.06^{ef}	3.39 ± 0.06^{bcd}
		F_c	***	***	**	**
		F_n	***	***	***	***
		F_p	***	ns	***	ns
		$F_{c\times n}$	*	ns	ns	*
		$F_{c\times p}$	ns	ns	ns	ns
		$F_{n\times p}$	ns	ns	ns	ns
		$F_{c\times n\times p}$	ns	ns	ns	ns

Photosynthetic pigments: Significant decreases in Chl *a*, Chl *b*, and Chl (*a*+*b*) were detected in both WD and SH under salt stress (Table 2). The Chl *a*, Chl *b*, and Chl (*a*+*b*) decreased by 19, 23.9, and 23.5%, respectively, in WD with the application of 0.2% NaCl and decreased by 24, 31.9, and 25.7%, respectively, in SH with the application of 0.2% NaCl compared with control. The Chl *a*/*b* ratio of both WD and SH was not changed significantly with the application of 0.2% NaCl compared with control. However, the Chl *a*/*b* ratio significantly increased with the application of 0.4% NaCl compared with control. In both cultivars, increases in Chl *a*, and Chl (*a*+*b*) were significant due to the combined application of PBZ and salt stress (NaCl) compared to the sole application of NaCl.

Cellular damages: Application of 0.4% NaCl induced a significant increase of REC in both WD and SH, whereas the REC of both WD and SH were insignificantly changed by the treatment of 0.2% NaCl compared with control (Fig. 3*A*). The combined application of 0.4% NaCl and PBZ significantly increased REC in SH, whereas it was insignificant in WD compared with control. The REC in WD was not changed significantly after the combined treatment of 0.4% NaCl salt stress and PBZ compared to 0.4% NaCl salt stress alone, however, REC significantly decreased in SH. Salt stress significantly increased MDA in both WD and SH (Fig. 3*B*). The combination of salt stress and PBZ significantly decreased MDA in both WD and SH compared to salt stress alone.

Antioxidant enzymes activity: A significant increase in SOD and CAT activity were detected in both WD and SH after 0.2% NaCl application, with enhancing values in WD contrary to SH (Fig.4*A*,*B*). The treatment of 0.4% NaCl induced a significant increase of SOD and CAT activities in WD compared to SH. The combined effect of NaCl and PBZ significantly increased SOD and CAT activity in both WD and SH compared to the sole application of NaCl and control.

Relationships between various characteristics: The proline content was linearly and positively correlated with SOD and CAT activity in WD compared to nonlinear and negative correlation of proline with SOD and CAT in SH (Fig. 5*A*,*B*). The MDA content was negatively correlated with Chl (*a*+*b*) content in both cultivars (Fig. 5*C*,*D*). The Chl (*a*+*b*) content was positively correlated with P_N in both WD and SH.

Discussion

Soil salinity is one of the major factors limiting crop production. Salt stress causes wide variety of physiological and biochemical changes in plants (Chartzoulakis *et al.* 2002). Plants grown under salt stress conditions showed reduced biomass accumulation, CO_2 assimilation, RWC, and Chl content and increased MDA, REC, and anti-oxidant enzyme activities. There were large differences in salt tolerance between genotypes of various crop species (Mc Kersie and Leshem 1994, He *et al.* 2016). Numerous studies have suggested that PBZ could protect plants from salinity in *Citrus* (Sharma *et al.* 2011, 2013).

One of the first responses of plants to salt stress was a reduced production of biomass (Marcar *et al.* 2002, Jaleel *et al.* 2007, Manivannan *et al.* 2008, Cha-um and Kirdmanee 2010), which is considered as an index of plants tolerance (Mekawy *et al.* 2015). In this study, we found out that the decrease of biomass production due to salinity was greater in SH than that of WD cultivar which indicated that WD possessed stronger salt tolerance compared to SH. The shoot DM was affected more by salt stress than root DM and led to higher root/shoot ratios (Table 1), which might be attributed to significant reduction of photosynthetic activity in leaves due to salinity (Fig. 1) being in accordance with another study on tomatoes (Al-Karaki 2000). However, the positive effects of PBZ on root DM under salt stress were observed in both cultivars of WD and SH (Table 1). Stimulation of root growth by PBZ under salt stress can be possibly explained by the fact that PBZ significantly increased root length under salt stress (Jaleet *et al.* 2007).

Inhibition of photosynthesis is well-known symptom of salt stress (Chartzoulakis *et al.* 2002, Marcar *et al.* 2002). In present study, WD exhibited lesser reduction in P_N compared to SH under salt stress (Fig. 1), which indicated a lesser decrease in biomass of WD than that of SH under salt stress. WD plants might benefit from their better water status, as indicated by less pronounced reduction in RWC (Fig. 2*A*). In present study, the use of PBZ improved RWC and P_N in both WD and SH (Figs. 1,2*A*). This occurred probably due to the fact that triazole compounds, such as PBZ, reduce gibberellin (GA) biosynthesis and increase abscisic acid (ABA) and cytokinin content (Fletcher *et al.* 2000), which ultimately helps plants maintain better water balance under stress (Fletcher and Nath 1984). Higher ABA content induced by PBZ might result in partial stomatal closure and thereby it lowered transpiration rate in leaves. It has been reported that PBZ decreases water loss and increase tolerance of plants to salt stress (Kishor *et al.* 2009, Sharma *et al.* 2011). We suggested that PBZ may enhance salinity tolerance of Chinese bayberry seedlings through enhanced RWC.

Proline accumulation is the primary defense response when plants adapt to salt stress (Ramanjulu and Sudhakar 2001, Kumar *et al.* 2000, 2003, Woodward and Bennett 2005, Cha-um and Kirdmanee 2010) facilitating the

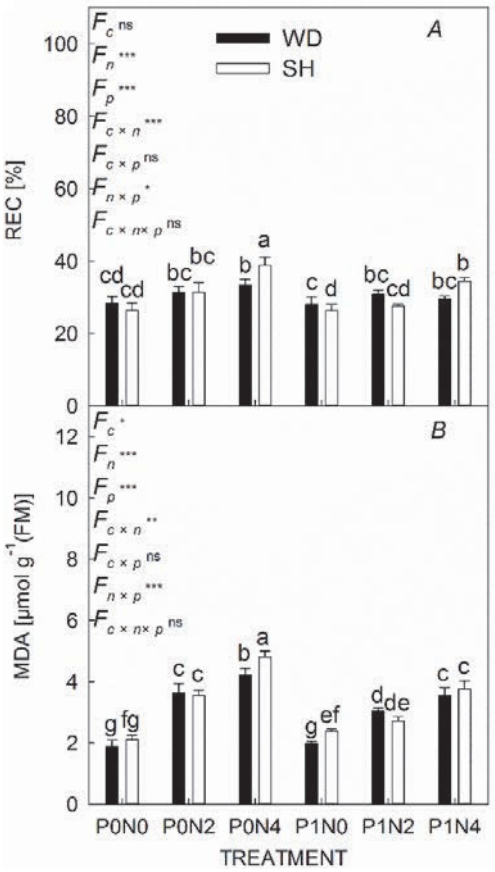

Fig. 3. The relative electrolyte conductivity (REC, *A*) and the malondialdehyde (MDA, *B*) in WD and SH exposed to different salt stress, PBZ treatments and their combination. WD – Wangdao cultivar, SH – Shenhong cultivar. Treatments: P0N0 – distilled water without PBZ; P1N0 – distilled water with PBZ; P0N2 – 0.2% NaCl without PBZ; P1N2 – 0.2% NaCl with PBZ; P0N4 – 0.4% NaCl without PBZ; and P1N4 – 0.4% NaCl with PBZ. *Different letters* above bars denote statistically significant differences between treatments at $P<0.05$ level according to *Tukey*'s test. Values are expressed as means ± SD, $n = 3$. *$0.01\leq P<0.05$; **$0.001\leq P<0.01$; ***$P<0.001$; ns – not significant. F_c – cultivar effect; F_n – salt stress effect; F_p – PBZ effect. $F_{c\times n}$ – cultivar × salt stress interaction effect; $F_{c\times p}$ – cultivar × PBZ interaction effect; $F_{n\times p}$ – salt stress × PBZ interaction effect; $F_{c\times n\times p}$ – cultivars × salt stress × PBZ interaction effect.

maintenance of water content (Yoshiba *et al.* 1997). In the present study, irrespective of PBZ treatment, the proline content in the salt-treated plants was higher than that of non-salt-treated plants. The higher proline content was observed in the salt-treated plants with PBZ compared to that of the salt-treated plants without PBZ (Fig. 2*B*). These results suggested that the salt-treated plants with PBZ had higher capacity for osmotic adjustment in terms of accumulating proline. Similar results were reported by Sharma *et al.* (2011). They revealed that PBZ increased

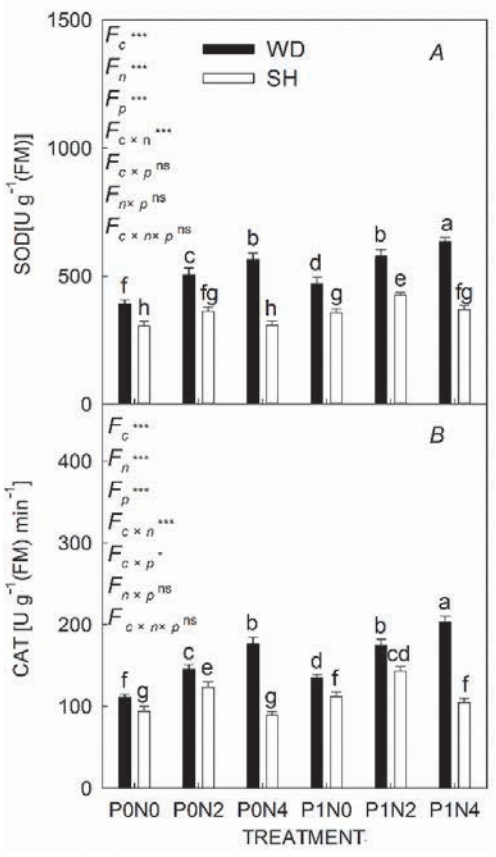

Fig. 4. Superoxide dismutase (SOD, *A*) and catalase (CAT, *B*) activity in WD and SH exposed to different salt stress, PBZ treatments and their combination. WD – Wangdao cultivar, SH – Shenhong cultivar. Treatments: P0N0 – distilled water without PBZ; P1N0 – distilled water with PBZ; P0N2 – 0.2% NaCl without PBZ; P1N2 – 0.2% NaCl with PBZ; P0N4 – 0.4% NaCl without PBZ; and P1N4 – 0.4% NaCl with PBZ. *Different letters* above bars denote statistically significant differences between treatments at $P<0.05$ level according to *Tukey*'s test. Values are expressed as means ± SD, $n = 3$. *$0.01 \le P < 0.05$; **$0.001 \le P < 0.01$; ***$P < 0.001$; ns – not significant. F_c – cultivar effect; F_n – salt stress effect; F_p – PBZ effect. $F_{c\times n}$ – cultivar × salt stress interaction effect; $F_{c\times p}$ – cultivar × PBZ interaction effect; $F_{n\times p}$ – salt stress × PBZ interaction effect; $F_{c\times n\times p}$ – cultivars × salt stress × PBZ interaction effect.

contents of proline in citrus (Karna khatta) under saline conditions, ameliorating RWC during stress conditions.

The REC is an indicator of cell membrane stability and integrity. It is commonly considered one of the best physiological indicators of salt tolerance in plants (Srivastav *et al.* 2003, Kishor *et al.* 2009). In this study, the WD cultivar showed a less pronounced increase in REC than that of SH under salt stress, indicating that WD exhibited higher tolerance to salt stress than that of SH (Fig. 3*A*). PBZ significantly reduced REC and MDA content in both WD and SH under salt stress (Fig. 3*A,B*). It has been reported that inhibition of electrolyte leakage by triazoles is correlated with ability to maintain membrane integrity in plants (Fletcher *et al.* 2000). Triazoles were found to alter sterol biosynthesis and to change composition of sterols in plasma membrane (Burden *et al.* 1987). PBZ, as a triazole compound, might possess a protective ability similar to that of triazoles. It was suggested that PBZ might have facilitated the increase in membrane stability observed in cucumber leaves through alterations in membrane lipid composition under chilling temperature (Whitaker and Wang 1987). Further studies are suggested to explore whether sterol composition can be changed by PBZ supplementation.

Even under optimal conditions, many metabolic processes produce ROS. The balance between production and removal of ROS is controlled by cellular osmoprotectants and antioxidant enzyme systems (Bohnert and Jensen 1996, Apel and Hirt 2004). However, excessive ROS are generated in plants suffering abiotic stress and damage the cellular components (Dat *et al.* 2000). The current results showed that 0.4% NaCl increased activities of both SOD and CAT in WD, but they changed it only slightly in SH (Fig. 4*A, B*). Under salt stress, the activities of SOD, CAT, peroxidase (POD), ascorbate peroxidase (APX), and glutathione reductase (GR) increased considerably, resulting in lower lipid peroxidation in salt-tolerant beet genotypes than in salt-sensitive genotypes (Bor *et al.* 2003). Salt-resistant maize also showed pronounced SOD, CAT, POD, and APX activity in comparison to salt-susceptible maize under salt stress (de Azevedo Neto *et al*. 2006). The slight changes in activities of SOD and CAT in SH under 0.4% NaCl indicated that scavenging function of antioxidant enzymes in SH was impaired or inhibited by severe salt stress. PBZ significantly increased SOD and CAT activity in two Chinese bayberry cultivars both under control and salt stress with significantly higher values in WD than that of SH (Fig. 4*A,B*). PBZ has increased antioxidant enzyme activity as reported in previous studies (Manivannan *et al.* 2008, Srivastav *et al.* 2010, Sharma *et al.* 2011). The strong positive relationship between concentrations of proline and antioxidant enzyme activity (SOD and CAT) was observed in WD (Fig. 5*A,B*). It has been reported that proline accumulation can activate antioxidant defense mechanisms (Türkan *et al.* 2005, Ben Ahmed *et al.* 2009). Proline can stabilize structures and activities of enzymes (Hessini *et al.* 2009). The higher accumulation of proline in WD under salt stress might increase or facilitate antioxidant enzyme activity.

Degradation of photosynthetic pigments in leaves is a parameter responsiveness of many plants to salt-stressed environment (Woodward and Bennett 2005, Kishor *et al.* 2009, Sharma *et al.* 2011). In this study, Chl (*a*+*b*) of both WD and SH was significantly lower under salt stress with values of decrease significantly greater in WD than that of SH (Table 2). Lesser reduction of Chl was observed in salt-tolerant clones compared to salt-sensitive clones *Eucalyptus camaldulensis* exposed to salt stress (Woodward and Bennett 2005). It has been reported that Chl degradation is a typical symptom of oxidative stress (Smirnoff 1993). MDA content is usually used to

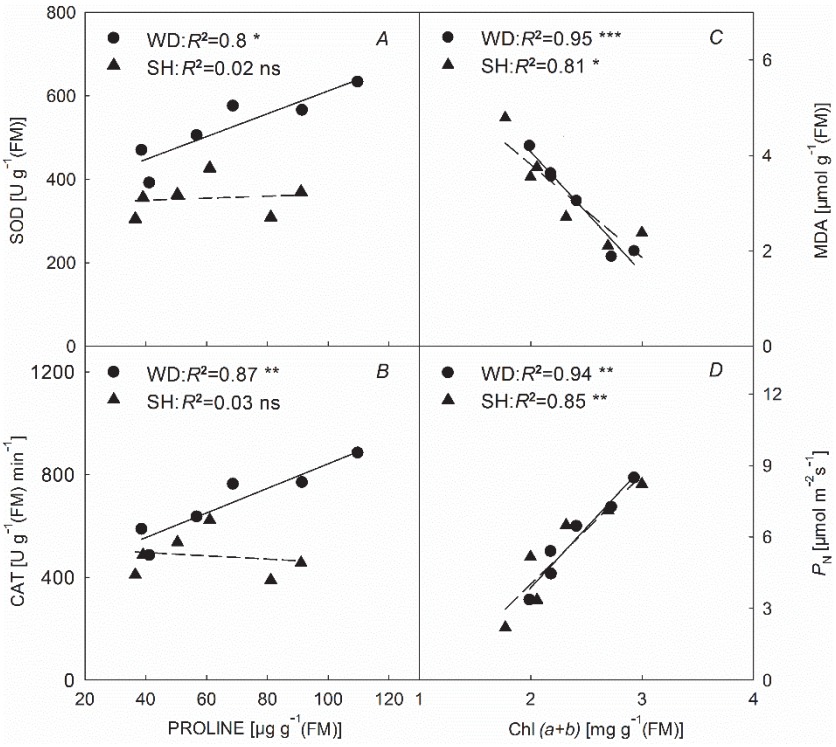

Fig. 5. Correlations between proline content and superoxide dismutase (SOD, *A*) activity and catalase (CAT, *B*) activity, between total chlorophyll [Chl *(a+b)*] and malondialdehyde (MDA) and and photosynthetic rate (P_N, *D*). WD – Wangdao cultivar (●), *solid line* – the best-fit regression for DW; SH – Shenhong cultivar (▲), *dotted line*, the best-fit regression for SH. Values are means of three replicates per cultivar and treatment. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; ns – not significant.

measure the extent of lipid peroxidation resulting from oxidative stress (Feng *et al.* 2003). In our current study, significant negative correlation was observed between total Chl and MDA content and P_N of each cultivar (Fig. 5*C,D*). It was revealed that salt stress may cause significant increases in MDA, which might harm Chl biosynthesis and reduce P_N. WD showed a less pronounced decrease in Chl (*a+b*) and P_N and less pronounced increase in the MDA content than that of SH, indicating that WD was more tolerant to salt stress than SH. Plants reduce absorbance of light under drought conditions by decreasing pigment contents, which is a kind of photoprotection (Elsheery and Cao 2008). Increased Chl *a/b* ratios may decrease emphasis on light collection relative to rates of PSII photochemistry. Both cultivars showed higher ratios of Chl *a/b* after the application of 0.4% NaCl. This could be explained as a decrease in a number of peripheral light-harvesting complexes. Under salt stress, PBZ treatment resulted in a significant increase in Chl *a*, Chl *b*, and total Chl content in both WD and SH (Table 2). This could be attributed to the ability of trizaoles to increase cytokinin content, which was associated with initiation of Chl biosynthesis (Fletcher *et al.* 2000).

Conclusion: Exposure to salt stress decreased CO_2 assimilation, Chl (*a+b*) and plant growth, but increased proline content, REC, MDA, and antioxidant enzymes (SOD and CAT) activities. WD showed less pronounced decrease in shoot dry matter, root dry matter, P_N, RWC, and Chl (*a+b*) and less pronounced increase in REC and MDA content than that of SH at 0.4% NaCl. We concluded that WD appeared to be more tolerant to salinity (NaCl) than SH cultivar. In our study, PBZ application protected Chinese bayberry seedlings from NaCl stress, by increasing root dry matter, P_N, Chl (*a+b*) content, RWC, proline content, and antioxidant enzyme activity (SOD and CAT) at 0.4% NaCl. Significantly positive relationships were observed between proline content and activities of antioxidant enzymes (SOD and CAT) in WD compared to insignificant relations in case of SH indicate that proline accumulation could activate the antioxidant defense mechanisms in WD under salt stress.

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