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Original Article

Regulation on Antioxidant Defense System in Rice Seedlings (*Oryza sativa* L. ssp. *indica* cv. 'Pathumthani 1') Under Salt Stress by Paclobutrazol Foliar Application

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Abstract

The present study investigated the effect of paclobutrazol (PBZ) foliar application on oxidative metabolism in salt-stressed rice (*Oryza sativa* L. cv. 'Pathumthani 1'; PTT1) seedlings. Fourteen-days-old rice seedlings, grown in the pots were pretreated with 15 mg L^{-1} paclobutrazol supplied as foliar spray. One week after pretreatment, the rice seedlings were exposed to salt stress (150 mM NaCl) for 12 days. It was observed that salinity enhanced the production of reactive oxygen species (ROS), including superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$). It also increased reactive oxygen species-associated oxidative damage, measured in terms of lipoxygenase activity, conjugated dienes, malondialdehyde content and relative electrolyte leakage. Increase in these parameters was associated with the decrease in the activity of enzymatic antioxidants [superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT)] and the levels of non-enzymatic antioxidants [ascorbic acid (AsA), total glutathione and α-tocopherol contents). Pretreatment of seedlings with paclobutrazol significantly lowered reactive oxygen species accumulation and membrane damage ($p < 0.05$), which can be correlated with the increased antioxidant activity (both enzymatic and non-enzymatic traits) under salt stress. The study concluded that paclobutrazol-treatment up-regulates the antioxidant defense system and recuperates the salt-induced oxidative damage in 'Pathumthani 1' rice seedlings under salt stress.

Keywords: antioxidants; membrane damage; *Oryza sativa*; reactive oxygen species; salinity stress; salt adaptation

Introduction

Salinity is the major problem in agriculture that limits the plant development and crop productivity. It induces the production of free radicals especially reactive oxygen species (ROS) (Puyang *et al*., 2015; Chakraborty *et al*., 2016). ROS such as superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) and singlet oxygen ($^1O_2$) are the most prevalent free radicals in plants under salt stress (Puyang *et al*., 2015; Chakraborty *et al*., 2016). High level of ROS triggers cellular disorders such as lipid peroxidation catalyzed by lipoxygenase (LOX), leading to the production of conjugated dienes (CD) and malondialdehyde (MDA) (Gill and Tuteja, 2010). Lipid peroxidation alters membrane properties, thereby causing cell defects such as ion leakage, cellular decompartmentalization and dysfunction (Sharma *et al*., 2012).To alleviate the oxidative damage induced by ROS, plants have evolved antioxidant defense system, including enzymatic antioxidants such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), peroxidase (POX), monodehydroascorbate reductase (MDHAR), glutathione reductase (GR) and dehydroascorbate reductase

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(DHAR), and non-enzymatic antioxidants such as ascorbic acid (AsA), glutathione, α-tocopherol, phenolic compounds and carotenoids (Gill and Tuteja, 2010).

Recent studies have reported that paclobutrazol (PBZ) protects plants from abiotic stresses such as drought (Somasundaram *et al*., 2009), chilling (Pinhero *et al*., 1997), flooding (Lin *et al*., 2006) and salinity (Srivastav *et al*., 2010). PBZ is reported to enhance the activities and/or contents of enzymatic and non-enzymatic antioxidants in *Catharanthus roseus*, *Arachis hypogaea*, *Vigna unguiculata* and *Citrus karna* (Jaleel *et al*., 2007; Sankar *et al*., 2007; Manivannan *et al*., 2008; Sharma *et al*., 2011).

'Pathumthani 1' (PTT1), a well-known rice cultivar in Thailand, is popular for its high yield, good quality (enriched aroma, long grain and soft texture) and three crop rotations per annum (as it is a short-day photoperiod insensitive) (Laohakunjit and Kerdchoechuen, 2007). However, the crop is sensitive to the salt stress at both seedling and reproductive stages (Cha-um *et al*., 2007). Although, PBZ has been shown to alleviate stress injury in *indica* rice (Khunpon *et al*., 2017), the effect of PBZ on free radicals, oxidative damage and antioxidant defense system in PTT1 rice under salinity stress has not been studied. In the present study, it was hypothesized that PBZ may enhance ROS scavenging activities *via* enzymatic and non-enzymatic antioxidants, leading to reduce free radical accumulation and oxidative damage of PTT1 rice seedlings grown under salt stress conditions.

Materials and Methods

Plant materials

Seeds of rice (*Oryza sativa* L. ssp. *indica* cv. 'PTT1'), obtained from Pathumthani Rice Research Center, Thailand, and were soaked in distilled water for 18 h and placed in a plastic tray (20 × 30 × 5 cm) covered with moist tissue papers. These were allowed to germinate at the room temperature (25±1 °C) under dark conditions for 72 h. The germinated seedlings were transferred to plastic pots (30 cm in diameter and 30 cm in height) containing 3 kg of clay soil (electrical conductivity = 1.787 dS m^{-1}; pH = 6.42; organic matter = 7.29%; total nitrogen = 0.26%; total phosphorus = 0.05%; total potassium = 1.04%). Seedlings were grown under 70±5% relative humidity, 26±5 °C ambient temperature, and 500-1,000 μmol m^{-2} s^{-1} photosynthetic photon flux densities, with 10 h d^{-1} photoperiod conditions for 14 days.

PBZ pretreatment, salt stress and experimental design

Fourteen-days-old rice seedlings in plastic pots were foliar sprayed (50 mL pot^{-1}) with distilled water or 15 mg L^{-1} PBZ using a handheld pressure sprayer (model SOLO 430-2G, Southern States, USA). Seven days after pretreatment, control and PBZ treated seedlings were transferred to 15-L plastic buckets (1 pot per bucket) containing 12 L tap water (as control) or 150 mM NaCl as salt stress treatment (Khunpon *et al*., 2017). The experiment was designed in a completely randomized design (CRD) with three replications (n = 3) per treatment. Rice seedlings were randomly uprooted at –7 (7 days before salt exposure), and at 0, 1, 3, 5, 7, 9 and 12 days after the salt treatment to determine ROS content, oxidative damage and the activity of antioxidant defense system. Data were analyzed by one-way analysis of variance (ANOVA) and significance was determined at $p \leq 0.05$, according to Tukey's HSD test, using SPSS version 17.0.

ROS determination

Superoxide radical ($O_2^{\bullet-}$) content

$O_2^{\bullet-}$ content was determined using the protocol of Jiang *et al*. (2010) with some modifications. Leaves and roots (500 mg) were homogenized in a mixture of 6 mL of 65 mM potassium-phosphate (K-P) buffer (pH 7.8), 2 mL of 0.1 M ethylenediaminetetra-acetic acid (EDTA) and 2 mL of 10 mM hydroxylammonium chloride at 4 °C for 1 min. The homogenate was centrifuged at 12,000 ×*g* at 4 °C for 15 min. Then, 2 mL of the supernatant was mixed with 2 mL of 17 mM 4-aminobenzenesulfonic acid and 2 mL of 7 mM α-naphthylamine and the mixture was placed in a water bath at 40 °C for 15 min. Then, 2 mL of ether was added to the mixture and the contents were centrifuged at 3,000 ×*g* for 15 min. Finally, the absorbance of the mixture was measured at 530 nm using a visible spectrophotometer (Genesys 20, Thermo Scientific, USA). The $O_2^{\bullet-}$ content was determined from the linear equation of the standard curve of sodium nitrite and expressed as nmol g^{-1} FW (fresh weight).

Hydrogen peroxide (H_2O_2) content

H_2O_2 content was determined according to the method of Velikova *et al*. (2000) with some modifications. Leaves and roots (500 mg) of were homogenized in 5 mL of 1% (w/v) trichloro-acetic acid (TCA) at 4 °C for 1 min. The homogenate was centrifuged at 20,000 ×*g* at 4 °C for 20 min. Then 0.5 mL of the supernatant was added to a solution containing 0.1 mL of 1 M KI and 2.4 mL of 10 mM K-P buffer (pH 7.0). The absorbance was measured at 390 nm using a visible spectrophotometer). The H_2O_2 content was calculated from the linear equation of the standard curve prepared with H_2O_2 and expressed as nmol g^{-1} FW.

Hydroxyl radical ($OH^{\bullet}$) content

$OH^{\bullet}$ content was determined using the protocols of Schopfer *et al*. (2001), Yang *et al*. (2008) and Ruenroengklin *et al*. (2009) with some modifications. Five hundred milligrams of leaves and roots were homogenized in 15 mL of 20 mM K-P buffer (pH 6.0) at 4 °C for 1 min. The homogenate was centrifuged at 3,000 ×*g* at 4 °C for 20 min. One milliliter of the supernatant was mixed with 1.5 mL of 20 mM K-P buffer (pH 6.0) containing 20 mM 2-deoxy-D-ribose at 25 °C for 30 min. Then, 1 mL of 0.5% (w/v) 2-thiobarbituric acid (TBA) in 1.4% (w/v) TCA was added to the reaction mixture. The mixture was heated in boiling water at 100 °C for 10 min and then cooled to room temperature. The absorbance of the mixture was measured at the excitation wavelength of 532 nm and the emission wavelength of 553 nm using a visible spectrophotometer. The $OH^{\bullet}$ content was calculated using the molar extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol g^{-1} FW.

Determination of oxidative damage

Lipoxygenase (LOX) activity

Enzyme extraction was determined using the method of Sovrano *et al*. (2006) and Wongsheree *et al*. (2009) with some modifications. Five hundred milligrams of leaves and roots were homogenized in 10 mL of 100 mM K-P buffer (pH 6.8) containing 1.5% (w/v) polyvinyl pyrrolidone (PVPP) at 4 °C for 1 min. The homogenate was centrifuged at 12,000 ×*g* at 4 °C for 20 min and the supernatant was refereed as enzyme extract and used for LOX assay. Protein content was determined according to the methods of Lowry *et al*. (1951) using bovine serum albumin as standard.

LOX (EC 1.13.11) activity was determined according to the method of Sovrano *et al*. (2006) and Wongsheree *et al*. (2009) with some modifications. It was assayed in a 2 mL reaction mixture containing 100 µL of 10 mM linoleic acid, 1.85 mL of 0.1 M K-P buffer (pH 6.8) and 50 µL of enzyme extract at 4 °C for 5 min. The increase in absorbance due to the formation of conjugated diene fatty acid hydroperoxide was observed with UV/VIS spectrophotometer (Lambda 25, Perkin Elmer Instruments, UK) at 234 nm. The LOX activity was calculated using the molar extinction coefficient of 25 mM^{-1} cm^{-1} and expressed as µmol mg^{-1} protein min^{-1}.

Conjugated dienes (CD) content

CD content was measured according to the method of Juntachote *et al*. (2007) with some modifications. Leaves and roots (500 mg) were homogenized in 10 mL of distilled water at 4 °C for 1 min. The 500 µL of the homogenate was mixed with 5 mL of extract solution (3:1: hexane : isopropanol, v/v) for 1 min and centrifuged at 2,000 × *g* at 4 °C for 5 min. The absorbance of the reaction mixture was measured at 233 nm. The CD content was calculated from the molar extinction coefficient of 25,200 M^{-1} cm^{-1} and expressed as µmol g^{-1} FW.

Malondialdehyde (MDA) content

MDA content was determined according to the method of Hodges *et al*. (1999) with some modifications. Five hundred milligrams of leaves and roots were homogenized in 5 mL of 0.1% (w/v) TCA at 4 °C for 1 min. The homogenate was centrifuged at 10,000 ×*g* at 4 °C for 20 min. Then, 0.5 mL of the supernatant was mixed with 0.5 mL of 20% (*w/v*) TCA containing 0.5% (*w/v*) TBA. The mixture was heated in boiling water for 10 min, cooled to room temperature (25±1 °C) and centrifuged at 10,000 ×*g* for 5 min. The absorbance of the mixture was measured at 532 nm and corrected for non-specific absorbance at 600 nm. The MDA content was calculated using the molar extinction coefficient of 155 $mM^{-1}cm^{-1}$ and expressed as nmol g^{-1} FW.

Electrolyte leakage (EL)

EL was determined according to the method of Chan *et al*. (1985) with some modifications. Ten discs of rice leaves and roots tissues were placed in an Erlenmeyer flask containing 25 mL of deionized water at 25 °C for 30 min. The initial conductivity (E1) of the mixture was measured with a conductivity meter (FiveEasy FE 30, Mettler Toledo, Switzerland). Then, the contents in the Erlenmeyer flask were heated at 98 °C for 15 min, and the conductivity (E2) the mixture was re-measured after cooling. EL was determined using the formula: Membrane permeability units were calculated as: EL (%) = (E1/E2) × 100.

Extraction and assay of enzymatic antioxidant activities

Antioxidant enzyme extraction was determined using to the method of Sunohara and Matsumoto (2004) with slight modifications. Two hundred milligrams of rice leaves or roots were sliced and homogenized in 10 mL of 25 mM K-P buffer (pH 7.8) containing 1 mM AsA, 0.4 mM EDTA and 2% PVPP at 4 °C for 1 min. The homogenate was centrifuged at 15,000 × *g* at 4 °C for 20 min and the supernatant was filtered through filter paper (Whatman No.1, England). The filtrate was collected to determine the activities of APX (EC 1.11.1.11) and CAT (EC 1.11.1.6). For the SOD (EC 1.15.1.1) assay, the supernatant was dialyzed against 10 mM K-P buffer (pH 7.8) using regenerated cellulose tubular membrane (Cellu•Sep, USA) at 4 °C for overnight. The dialyzed extract was centrifuged at 15,000 × *g* at 4 °C for 20 min. The filtrate was collected to determine the SOD activity. The protein content in the enzyme extract was determined according to the met of Lowry *et al*. (1951), using bovine serum albumin as standard.

SOD activity was determined according to the method of Sunohara and Matsumoto (2004) with some modifications. It was assayed in a 2 mL reaction mixture comprising of 0.04 mL of xanthine oxidase, 0.2 mL of 0.1 mM cytochrome C from horse heart, 0.2 mL of 500 mM K-P buffer (pH 7.8), 0.2 mL of 1 mM xanthine (dissolved in 10 mM NaOH), 1.32 mL of distilled water and 0.04 mL of enzyme extract for 1 min. The rate of reduction of cytochrome C was measured by the increase in absorbance at 550 nm using a UV/VIS spectrophotometer. The SOD activity was expressed as U mg^{-1}protein. One unit is determined as the amount of enzyme that inhibits the rate of cytochrome c reduction by 50% under specific conditions.

APX activity was determined according to the method of Sunohara and Matsumoto (2004) with some modifications. It was assayed in a 2 mL reaction mixture comprising 0.02 mL of 10 mM H_2O_2, 0.5 mL of 1 mM ascorbic acid, 0.5 mL of 100 mM K-P buffer (pH 7.0), 0.5 mL of 0.4 mM EDTA, 0.38 mL of distilled water and 0.1 mL of enzyme extract for 1 min. It was measured in terms of disappearance/decomposition of AsA at 290 nm, and calculated using ε= 2.8 $mM^{-1}cm^{-1}$). The APX activity was expressed as µmol AsA mg^{-1} protein min^{-1}.

CAT activity was determined according to the method of Sunohara and Matsumoto (2004) with some modifications. It was assayed in a 2 mL reaction mixture comprising of 1.9 mL of 50 mM K-P buffer (pH 7.0) containing 25 mM H_2O_2 and 0.1 mL of enzyme extract for 1 min. It was measured in terms of decomposition of H_2O_2 at 240 nm and calculated using ε = 0.0394 $mM^{-1}cm^{-1}$). The CAT activity was expressed as µmol H_2O_2 mg^{-1} protein min^{-1}.

Determination of non-enzymatic antioxidant contents

Ascorbic acid (AsA) content

AsA content was determined according to the method of Deepa *et al*. (2006) with slight modifications. Five hundred milligrams of rice leaves and roots were homogenized in 10 mL of 3% (*w/v*) metaphosphoric acid at 4 °C for 1 min. The homogenate was centrifuged at 3,000 × *g* at 4 °C for 20 min. Two mL of the supernatant was added to 5 mL of 3% (w/v) metaphosphoric acid and then titrated with 0.1 mM 2,6-dichloroindophenol to the end point. The AsA content was expressed as mg AsA g^{-1} FW against AsA (Sigma Aldrich, USA) standard curve.

α-Tocopherol content

It was assayed according to the method of Contreras-Guzmán and Strong (1982) with slight modifications. Five hundred milligrams of rice leaves and roots were immersed in 20 mL of ethanol in a water bath at 85 °C for 30 min. The solution was allowed to cool and then filtered into a separating funnel and 20 mL of heptane was added to it. The solution was shaken for 5 min and thereafter 20 mL of 1.25% (w/v) sodium sulphate was added. The solution was again shaken for 2 min and allowed to separate into layers. The upper layer was separated to eliminate the interfering substances by the slight saponification method. The mixture containing 7 mL of upper layer solution, 5 mL of 50% (w/v) potassium hydroxide and 5 mL of 5% (w/v) AsA was shaken for 2.5 min and allowed to separate into layers. Five milliliters of the upper layer solution were mixed with 10 mL of 80% (v/v) ethanol, shaken for 1 min and allowed to separate into layers. The upper layer was selected to assay α-tocopherol by a reaction with cupric ions to be complexed with 2,2'-biquinoline (cuproine). A volume of 0.5 mL of α-tocopherol in ethanol was processed in the same way as the sample and used as standard. The absorbance of the mixture was measured at 545 nm with a VIS spectrophotometer. The α-tocopherol content was expressed as μg α-tocopherol g^{-1}FW.

Total glutathione content

Total glutathione content was determined according to the method of Gronwald *et al*. (1987) with slight modifications. Five hundred milligrams of rice leaves and roots were homogenized in 12 mL of 5% (w/v) TCA at 4 °C for 1 min. The homogenate was centrifuged at 12,000 × *g* at 4 °C for 10 min. The supernatant was diluted twice, with 0.5 M K-P buffer (pH 8.0) in a ratio of 1:1 and then with 0.1 M K-P buffer (pH 8.0) in a ratio of 1:9. The reaction mixture of 2.2 mL comprised of 0.1 M K-P buffer (pH 7.5) containing 5 mM EDTA; 0.2 mL of 1 mM NADPH (prepared in 0.1 M K-P buffer with pH 7.5) containing 5 mM EDTA; and 0.2 mL (1 unit) of GR (prepared in 0.1 M K-P buffer with pH 7.5). These components were equilibrated in test tube at 25 °C for 2 min. The reaction was initiated by adding 0.2 mL of 6 mM 5-5'-dithiobis (2-nitrobenzoicacid) (prepared in 0.1 M K-P buffer with pH 7.5) and 0.2 mL of diluted supernatant in the reaction mixture. The change in absorbance at 412 nm was measured every 1 min for 5 min by using a VIS spectrophotometer. Total glutathione content was determined from the linear equation of the standard curve prepared with reduced glutathione and was expressed as μg reduced glutathione g^{-1} FW.

Results

Reactive oxygen species content

The ROS levels in leaves and roots of salt-stressed rice seedlings continuously increased during the experimental period as compared to the control plants (Fig. 1). $O_2^{\bullet-}$ in leaves and roots of rice seedlings significantly increased 1 day after salt treatment, whereas H_2O_2 and $OH^{\bullet}$ significantly increased 3 days after salt treatment. After 7 days of salt treatment, $O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$ content in the leaves of NaCl-stressed seedlings increased by 47.9%, 19.9% and 22.1%, respectively, as compared to the control and these parameters increased by 40.4%, 23.1% and 27.3%, respectively, on day 12 of salinization. Seven days after salt treatment, the content of $O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$ in the roots was increased by 52.2%, 34.4% and 28.7%, respectively, as compared to the control and increased by 48.0%, 45.7% and 33.4%, respectively, on day 12 of salinization (Fig. 1).

Application of 15 mg L^{-1} PBZ increased the contents of $O_2^{\bullet-}$ and H_2O_2 on Day 0 to Day 1 after the pretreatment in both leaves and roots under normal condition (prior to the salt treatment) by 4.9-13.8% (Fig. 1). PBZ pretreatment alleviated the adverse effects of ROS accumulation under salinity and caused a significant reduction in the accumulation of $O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$ in the rice seedlings as compared to the control (Fig. 1). The contents of $O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$ in the leaves of PBZ pretreated seedlings were decreased by 15.5%, 11.8%, and 10.3%, respectively, on Day 7 and11.0%, 7.6% and 6.0%, respectively, on Day 12 after the salt treatment as compared to the control. ROS content ($O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$) in the roots of seedlings treated with PBZ were 16.0%, 12.3% and 14.6% lower on Day 7, and 13.5%, 9.8% and 14.1% lower on Day 12 after salt treatment, respectively, than that of the control.

Oxidative damage

Oxidative damage related parameters were significantly enhanced in both leaves and roots of NaCl-stressed seedlings 3 days after exposure to the 150 mM NaCl as compared to that of the control (Fig. 2). Salt stress increased LOX activity, CD and MDA contents, and EL in the leaves of PBZ untreated seedlings by 27.6%, 5.0%, 24.0% and 26.6%, respectively, on Day 7, and by 27.3%, 8.6%, 58.6% and 31.3% in comparison to the control on Day 12. In the roots, these parameters were increased by 30.2%, 8.6%, 26.4% and 20.6% to that of the control on Day 7 and 40.0%, 14.5%, 36.7% and 36.9% to that of the control on Day 12 after salinization, respectively (Fig. 2).

In addition, PBZ pretreatment significantly decreased the oxidative damage in comparison with PBZ untreated seedlings under saline conditions (Fig. 2). LOX activity, CD and MDA contents, and EL in the leaves of PBZ treated seedlings were lower than those in the PBZ untreated seedlings by 12.8%, 3.2%, 9.3% and 7.1% on Day 7, and by 11.9%, 3.7%, 6.4% and 2.4% on Day 12 after salt stress, respectively. In the roots, these were lowered by 13.0%, 4.2%, 10.5% and 8.2% on Day 7, and by 12.7%, 4.9%, 8.3% and 2.4% on Day 12 after salt stress, respectively, than PBZ untreated seedlings.

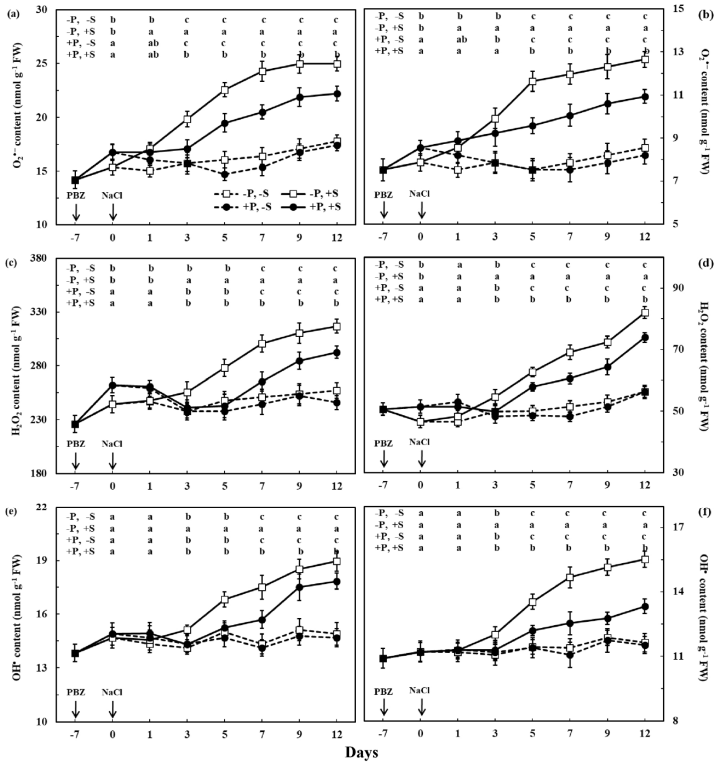

Fig. 1. $O_2^{\bullet-}$ content in leaves (a) and roots (b), H_2O_2 content in leaves (c) and roots (d), and $OH^{\bullet}$ content in leaves (e) and roots (f) of PTT1 rice seedlings with or without pre-treatment of 15 mg L^{-1} PBZ (P), and subsequently exposed to 0 or 150 mM NaCl (S) for -7, 0, 1, 3, 5, 7, 9 and 12 days. Values represent mean ± SE (n = 3). Different lowercase letters in each day show significant difference in each treatment at $p \leq 0.05$, according to Tukey's HSD

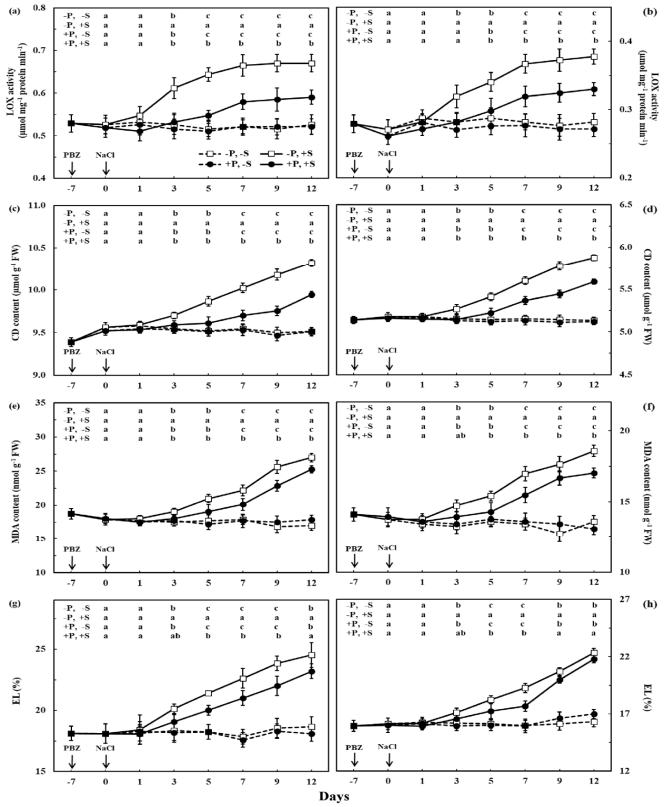

Fig. 2. LOX activity in leaves (a) and roots (b), CD content in leaves (c) and roots (d), MDA content in leaves (e) and roots (f), and EL in leaves (g) and roots (h) of PTT1 rice seedlings with or without pre-treatment with 15 mg L^{-1}PBZ (P) and subsequently exposed to 0 or 150 mM NaCl (S) for -7, 0, 1, 3, 5, 7, 9 and 12 days. Values represent mean ± SE (n = 3). Different lowercase letters in each day show significant difference in each treatment at $p \leq 0.05$, according to Tukey's HSD

Enzymatic antioxidants

The activities of SOD and APX in leaves and roots of PBZ untreated seedlings were slightly increased during the first 3 days of salt treatment, and thereafter declined significantly during the period of salt exposure. However, CAT activity significantly decreased throughout the experimental period (Fig. 3). Seven days after salt treatment, activities of SOD, APX and CAT in the leaves was significantly decreased by 7.4%, 5.3% and 4.4%, respectively, and declined by 46.3%, 12.0% and 10.7%, respectively, on Day 12. In roots, the activities of SOD, APX and CAT were decreased by 5.5%, 4.4% and 4.6%, respectively, on Day 7, and by 23.0%, 9.3% and 14.9%, respectively, on Day 12 after salt treatment.

PBZ pretreatment significantly enhanced activities of SOD, APX and CAT by 3.2-12.5% in both leaves and roots of rice seedlings grown under normal conditions (Fig. 3). The activities of SOD, APX and CAT increased during the first 5 days after NaCl exposure and slightly decreased thereafter. SOD, APX and CAT activities in the leaves of PBZ treated seedlings were higher than those in the untreated seedlings by 53.9%, 29.4% and 8.6%, on Day 7, and by 127.9%, 24.8% and 13.1% on Day 12 after salt treatment, respectively. In the roots, SOD, APX and CAT activities of PBZ treated seedlings were higher than the untreated seedlings by 32.7%, 18.2% and 10.6% on Day 7, and by 37.5%, 17.3% and 13.8%, respectively, on Day 12 after salt stress.

Non-enzymatic antioxidants

Non-enzymatic antioxidants in the leaves and roots of rice seedlings were significantly decreased after 3 days of salt treatment and their content was the lowest at the end of experiment (day 12) (Fig. 4). Seven days after salt treatment, AsA, α-tocopherol and total glutathione content in the leaves was significantly decreased by 20.6%, 16.2% and 17.7%, respectively, and declined by 36.8%, 34.3% and 34.3%, respectively, on Day 12. In roots, these were decreased by 15.0%, 10.3% and 15.0%, respectively, on Day 7, and by 31.2%, 25.0% and 37.7%, respectively, on Day 12 after salt stress.

The seedlings pretreated with PBZ but unexposed to salinity showed a significant increase in AsA, α-tocopherol and total glutathione content in both leaves and roots by 10.5-21.5% (Fig. 4). In addition, seedlings pretreated with PBZ and exposed to salt stress also showed a significant increase in AsA, α-tocopherol and total glutathione content throughout the study period as compared to the untreated seedlings (Fig. 4). AsA, α-tocopherol and total glutathione content in the leaves of PBZ treated seedlings was higher by 22.2%, 15.0% and 23.2% after 7 days, and by 39.5%, 36.0% and 43.5% after 12 days of salt stress than those in the untreated seedlings, respectively, measured in terms of, respectively and it was, respectively, higher on Day 12. AsA, α-tocopherol and total glutathione content in the roots of seedlings pretreated with PBZ were 13.7%, 14.6% and 25.5% higher, respectively, than that of the control roots on Day 7, and were 21.4%, 21.2% and 50.0% higher, respectively, on Day 12 after salt treatment.

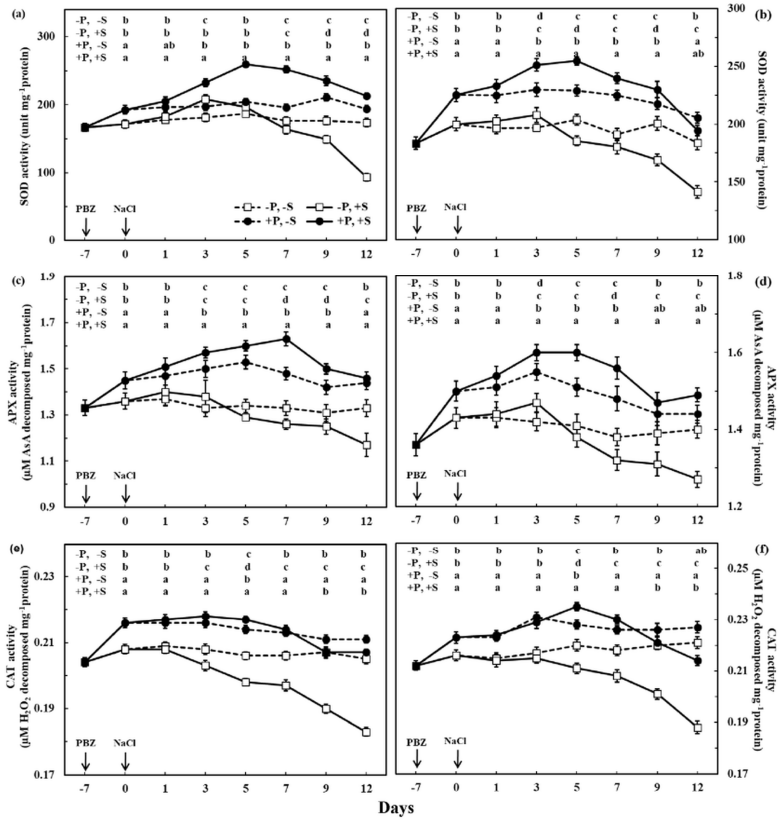

Fig. 3. SOD activity in leaves (a) and roots (b), APX in leaves (c) and roots (d) and CAT in leaves (e) and roots (f) of PTT1 rice seedlings with or without pre-treatment with 15 mg L^{-1}PBZ (P) and subsequently exposed to 0 or 150 mM NaCl (S) for -7, 0, 1, 3, 5, 7, 9 and 12 days. Values represent mean ± SE (n = 3). Different lowercase letters in each day show significant difference in each treatment at $p \leq 0.05$, according to Tukey's HSD

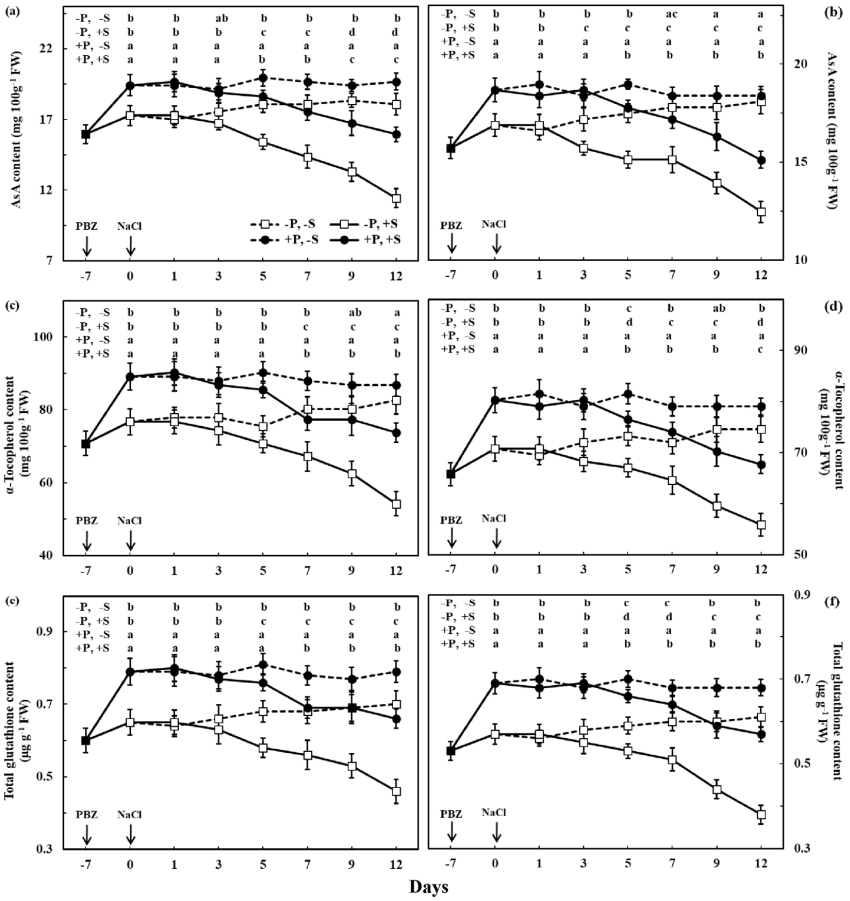

Fig. 4. AsA in leaves (a) and roots (b), α-tocopherol in leaves (c) and roots (d) and total glutathione in leaves (e) and roots (f) of PTT1 rice seedlings with or without pre-treatment with 15 mg L^{-1}PBZ (P) and subsequently exposed to 0 or 150 mM NaCl (S) for -7, 0, 1, 3, 5, 7, 9 and 12 days. Values represent mean ± SE (n = 3). Different lowercase letters in each day show significant difference in each treatment at $p \leq 0.05$, according to Tukey's HSD

Discussion

Salinity is one of the major environmental factors which greatly inhibit plant growth and development especially in the crop seedlings. Normally, oxygen in the plant cells is converted into ROS as byproduct of numerous metabolic processes, and the molecules are scavenged by antioxidant defense system (Sharma *et al*., 2012). The most commonly occurring ROS in plant cells are $O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$ and $^1O_2$. However, salt stress disturbs the equilibrium between accumulation and scavenging of ROS leading to the ROS overproduction. In the present study, NaCl stress significantly increased ROS accumulation such as $O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$ with increasing the time of salt exposure (Fig. 1). Among all the ROS, $O_2^{\bullet-}$ increased faster than H_2O_2 and $OH^{\bullet}$ during the initial period of salinity (Fig. 1). This is because $O_2^{\bullet-}$ is the first free radical produced in the plant cells by leakage of electron to oxygen molecules from electron transport chain, and it can be further converted into H_2O_2 and $OH^{\bullet}$ (Gill and Tuteja, 2010; Sharma *et al*., 2012). H_2O_2 can be produced from $O_2^{\bullet-}$ radical in the presence of SOD and is a stable ROS molecule with long half-life (Gill and Tuteja, 2010). In our study, $OH^{\bullet}$ levels increased slowly under salt stress conditions as compared to $O_2^{\bullet-}$ and H_2O_2 (Fig. 1). It is suggested that $OH^{\bullet}$ is generated from $O_2^{\bullet-}$ and H_2O_2 by reacting with the reduced forms of Fe^{2+} and Cu^{2+} in the Fenton and Haber-Weiss reactions (Dat *et al*., 2000; Arora *et al*., 2002; Sharma *et al*., 2012). ROS levels in the leaves were apparently higher than in the roots (Fig. 1). Since leaf tissues constitute the major sites of ROS generation (chloroplasts and mitochondria), thus, the leaves produced more ROS than roots under stress conditions through the leakage of electrons from electron transport chain (Møller *et al*., 2007; Gill and Tuteja, 2010; Sharma *et al*., 2012).

Foliar application of PBZ has been evaluated to alleviate the negative effects of salinity in many plants such as barley, wheat and mango (Özmen *et al*., 2003; Hajihashemi *et al*., 2007; Srivastav *et al*., 2010). In the present study, PBZ pretreatment significantly increased $O_2^{\bullet-}$and H_2O_2 levels 7 days after the foliar spray under normal conditions (Fig. 1). It is possible that PBZ induced ROS generation, especially $O_2^{\bullet-}$and H_2O_2, as they are signal molecules for the up-regulation of plant defense mechanism (Neill *et al*., 2002; Buetler *et al*., 2004). The accumulation of $OH^{\bullet}$ was not observed in PBZ pretreated seedlings under normal conditions as the molecule is generated after the $O_2^{\bullet-}$ and H_2O_2 formation, and these radicals might be scavenged by

antioxidant defense system soon after their formation. However, 10 days after pretreatment (Day 3), ROS levels were decreased. In this study, PBZ pretreatment significantly reduced ROS levels in the leaves and roots of PTT1 rice seedlings exposed to the salt stress throughout the experiment period. On Day 12 after salt treatment, PBZ treatment significantly decreased ROS levels with an average reduction of 10.3% (Fig. 1).

Previously studies have demonstrated that the increase in ROS results in lipid peroxidation, thereby causing oxidative damage and leakage of electrolytes (Lee *et al*., 2013; Puyang *et al*., 2015; Chakraborty *et al*., 2016). Lipid peroxidation is the main cause of membrane deterioration and ion leakage and is indicated by the enhanced MDA and CD levels, as these are the secondary end products of polyunsaturated fatty acid oxidation and the activity of LOX (Dermiral and Türkan, 2005; Gill and Tuteja, 2010; Sharma *et al*., 2012). Membrane lipid peroxidation cause cell defects such as ion leakage and cellular decompartmentalization (Blokhina *et al*., 2003; Gill and Tuteja, 2010). In the present study, oxidative damage in PTT1 rice seedlings exposed to salt stress increased with increasing exposure time, when assessed by LOX activity, CD and MDA content, and EL (Fig. 2), which coincided with the increase in ROS levels (Fig. 1). It is possible that ROS overproduction in PTT1 rice seedlings exposed to salt stress induced lipid peroxidation and membrane destruction. In the present study, oxidative damage in the leaves was apparently greater than that in the roots and it correlated with the higher levels of ROS in the leaves than in roots (Figs. 1, 2).

In our study, despite the initial rise in ROS in PTT1 rice seedlings pretreated with PBZ, we did not observe any oxidative damage as assessed in terms of LOX activity, CD and MDA contents and EL under normal conditions (Fig. 2). It is suggested that the ROS accumulation in PBZ treated plants was insufficient to destroy the cellular metabolism. PBZ significantly decreased the oxidative damage in leaves and roots of PTT1 seedlings exposed to salt stress with an average reduction of 6.6% as compared to the control on Day 12 (Fig. 2).

Antioxidant enzymes especially SOD and APX were upregulated during first three days after salt treatment, leading to increase in ROS accumulation and elevate the up-regulation of antioxidant enzymes at early salt exposure time (Figs. 1 and 3). However, a prolonged period of salt stress caused a decrease in SOD, APX and CAT activities, indicating that the stress might destroy the ability of enzyme by inhibiting enzyme activity or denaturing the enzyme structure leading to the generation and accumulation of ROS. In the present study, SOD activity increased faster than APX and CAT in salt stressed plants during the initial period of salt exposure (Fig. 3). We concluded that SOD might be demonstrated as the first line of ROS scavenging reaction (Gill and Tuteja, 2010; Sharma *et al*., 2012).

An average increase of 39.1% has been observed in the antioxidant enzyme activities in PBZ pretreated seedlings exposed to NaCl as compared to those without PBZ pretreatment after 12 days of exposure (Fig. 3). The increase in enzymatic antioxidants by PBZ possibly reduced ROS accumulation and oxidative damage in PTT1 rice seedling (Figs. 1 and 2). The results of this study are consistent with those of Özmen *et al.* (2003), Jaleel *et al*. (2007) and Manivannan *et al.* (2008) who reported that PBZ treatment could enhance antioxidant activity such as SOD, CAT, APX and POX under salt stress in barley, *Catharanthus roseus* and *Vigna unguiculata*, respectively.

Plants also have non-enzymatic antioxidants such as AsA, α-tocopherol and total glutathione which scavenge the overproduction of ROS by donating electron or hydrogen atom to ROS (Blokhina *et al*., 2003; Gill and Tuteja, 2010; Sharma *et al*., 2012). AsA is a potential scavenger of $^1O_2$, $O_2^{\bullet-}$and $OH^{\bullet}$ that scavenges H_2O_2 through ascorbate-glutathione cycle (AsA-GSH cycle). Moreover, it can regenerate tocopheroxyl radical to α-tocopherol and plays the major role in stomatal movements (Gill and Tuteja, 2010; Sharma *et al*., 2012). Tocopherol is the major lipid soluble antioxidant that can scavenge $^1O_2$, $OH^{\bullet}$ and lipid peroxide radicals (Blokhina *et al*., 2003; Gill and Tuteja, 2010; Sharma *et al*., 2012). Glutathione, a major low-molecular-weight thiol-containing antioxidant, can directly detoxify $^1O_2$, $O_2^{\bullet-}$, $OH^{\bullet}$ and H_2O_2, and can regenerate AsA through AsA-GSH cycle including the control with the redox state in plant cells (Munné-Bosch, 2005; Gill and Tuteja, 2010; Sharma *et al*., 2012). In our study, the contents of AsA, α-tocopherol and total glutathione in leaves and roots of PTT1 rice seedlings under salt stress gradually decreased throughout the experimental period (Fig. 4), indicating that salinity caused the reduction of antioxidant contents leading to the ROS overproduction. It is believed that salt exposure might cause the reduction of antioxidant content by counteracting with ROS accumulation or destroying non-enzymatic antioxidants. The levels of AsA and total glutathione rapidly decreased during the first 5 days of exposure as compared to the α-tocopherol (Fig. 4). Thus, AsA and glutathione could scavenge the ROS in the initial period of salt stress and afterwards only α-tocopherol could scavenge the ROS and lipid radical.

In this study, PBZ application significantly increased AsA, α-tocopherol and total glutathione under normal conditions. PBZ pretreatment also increased and restored non-enzymatic antioxidant in leaves and roots of PTT1 rice seedlings with an average increase of 35.3% as compared to the seedlings without PBZ pretreatment, 12 days after salt treatment (Fig. 4). It is suggested that the increase in antioxidant contents by PBZ possibly reduced ROS-induced damage, leading to the salt adaptation. These results are in accordance with the previous reports on groundnut and *Catharanthus roseus* that PBZ treatment could enhance non-enzymatic antioxidants such as AsA, α-tocopherol and glutathione under salt and water stress, respectively (Jaleel *et al*., 2007; Sankar *et al*., 2007). It is possible that the increase in AsA and total glutathione levels in PBZ treated plants could exhibit the stimulatory effect on the enzymes of the AsA-GSH cycle especially APX activity which important to detoxify H_2O_2 overproduction. These contents play an important role in controlling the cellular redox state of antioxidant defense system. In this experiment, PBZ pretreatment significantly enhanced ROS scavenging activities via enzymatic and non-enzymatic antioxidants in leaves and roots of PTT1 seedlings exposed to salt stress (Figs. 3 and 4). It is possible that PBZ might

induce ROS accumulation which is a signal molecule in the early period to induce the up-regulation of antioxidant defense system leading to reduce free radical accumulation and oxidative damage of PTT1 rice seedlings grown under salt stress conditions.

Conclusions

In conclusion, the present study demonstrated that salinity stress caused ROS accumulation, oxidative damage and antioxidant deficiency. PBZ pretreatment effectively protected rice seedlings from salt stress by enhancing antioxidant defense system, reducing ROS accumulation and oxidative damage. This represents the protective effects of PBZ in repairing the salt-induced damage in rice seedlings. Therefore, exogenous PBZ application might be a useful method to induce salt adaptation in PTT1 rice seedling.

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