EFFECTS OF BOSCALID EXPOSURE ON PHOTOSYNTHETIC AND ANTIOXIDANT RESPONSES OF RICE SEEDLINGS

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Abstract. The widespread application of succinate dehydrogenase inhibitor fungicides (SDHIs) poses risks for ecosystems. This study investigated the impacts of boscalid (0–10 mg/L) exposure on photosynthesis and antioxidant systems in rice seedlings. The results showed that 1.5–2.5 mg/L boscalid increased chlorophyll (Chl) contents, photosynthetic rate (Pn) and Rubisco activities along with up-regulating the genes *psbA* and *psaB*, leading to the improvement of photosynthesis. However, 10 mg/L boscalid triggered the generation of $O_2^{\bullet-}$, enhancing the activities of NADPH oxidase, superoxide dismutase (SOD), ascorbate peroxidase (APX) isozymes as well as glutathione S-transferases (GSTs), suggesting reactive oxygen species (ROS) overproduction and a defensive response in the seedlings. Meanwhile, 10 mg/L boscalid decreased Chl contents, Pn and Rubisco activities accompanied by down-regulation of the genes *atpB*, *psbA* and *psaB*, as well as Lhcb1 protein, resulting in the impairment of photosynthesis via inhibition of ATP synthesis and photosynthetic electron transfer. Additionally, SOD and APX isozymes together with GSTs and reduced glutathione (GSH) alleviated boscalid-induced toxicity suggesting their role in the defense and detoxification in rice seedlings.
Keywords: *boscalid, toxic effects, oxidative stress, photosynthesis, rice*

Introduction

Fungicides belong to a class of pesticides used to control plant diseases caused by various pathogenic microorganisms. Among them, succinate dehydrogenase inhibitor fungicides (SDHIs) have been extensively applied for agricultural production to reduce fungal diseases that occur on corn, grains, vegetables and other plants (Huang et al., 2022). SDHIs can interfere with the respiration of pathogenic bacteria by inhibiting the activity of mitochondrial succinate dehydrogenase, thus achieving sterilization. With constant use of SDHIs, some of their residues have been widely detected in water and soil across China and abroad (Gulkowska et al., 2014; Ma et al., 2018). Therefore, persistent application of such fungicides poses a threat to the ecological environment. So far, there have been numerous studies mainly focused on the ecological risks of SDHIs on aquatic organisms, such as microalgae (Qian et al., 2018), *Daphnia magna* (Aksakal, 2020) and zebrafish (Huang et al., 2022), as well as soil biota (Han et al., 2022a; Yao et al., 2022). Whereas, studies investigating the toxic effects of SDHIs on higher plants are still limited.

Increasing studies revealed that exposure to SDHIs induced the emergence of reactive oxygen species (ROS) in living organisms (Ji et al., 2023; Qian et al., 2018; Wang et al., 2020a; Yao et al., 2018). NADPH oxidase constitutes one of the main sources of ROS in plants under hostile environmental conditions (Hafsi et al., 2022). To prevent oxidative damage induced by ROS, plants have developed a variety of enzymatic and non-enzymatic antioxidants, including catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), peroxidase (POD), glutathione S-transferases (GSTs) and reduced glutathione (GSH) (Mittler, 2002; Marchand et al.,

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2016; Zhang et al., 2021). These antioxidant enzymes exist in cells as multiple isozymes that collaborate in protecting organelles and relieving tissue injury (Mittler, 2002; Wang et al., 2018). Besides, some of such enzymes have been served as biomarkers to evaluate the levels of environmental pollutants (Wang et al., 2018). However, excessive ROS may break the balance between antioxidant and prooxidant systems following oxidative damage through oxidation of intracellular macromolecules (Gayathri et al., 2022; Li and Kim, 2022). Chloroplast is more vulnerable to oxidative damage than other organelles under adverse conditions, and consequently its structural and functional integrity tend to be destroyed herewith influencing the physiological metabolism (such as photosynthesis) (Challabathula et al., 2022; Gayathri et al., 2022; Li and Kim, 2022). However, studies involving oxidative damage induced by SDHIs on photosynthesis and defense systems are little. The relevant mechanisms are still poorly understood.

Boscalid is one of the most widely used SDHI fungicides (Qian et al., 2018). As a persistent fungicide, it can be found in a variety of environments (Bhatt et al., 2023). The current study was hence performed to explore the effects of boscalid on photosynthetic and antioxidant response in rice (*Oryza sativa* L.) seedlings. The findings will afford new scientific basis for ecological risk assessment of boscalid pollution in agriculture.

Materials and methods

Plant material

Rice seeds were bought from Huainan Seeds Company. After seed germination, they were cultured in nutrient solution (Yoshida et al., 1976). The solutions were renewed every five days. Rice seedlings at three-leaf stage with unanimous growth were subjected to boscalid exposure. Referring to the literature (Qian et al., 2018), the tested concentrations of boscalid were set at 0 (control, Ck), 1.5, 2.5, 5 and 10 mg/L in present experiment. Triplicates were conducted for each concentration, and twenty-four rice seedlings were selected for each triplicate. After exposure of 30 d, leaves were collected for tests as below.

NBT visualization of superoxide anion radicals production

Nitroblue tetrazolium (NBT) visualization of the production of superoxide radical ($O_2^{\cdot -}$) was performed with reference to Romero-Puertas et al. (2004). Leaf sections were collected and immediately immersed into staining solution (0.1% NBT (w/v), 10 mmol/L sodium azide and 50 mmol/L Tris-HCl pH 6.5), and then treated by vacuum filtration for 2 min, repeating 4 times with an interval of 3 min. After that, they were placed in the incubator for illumination until dark blue spots appeared, and subsequently decolorized in boiling 90% (v/v) ethanol.

Protein extraction and protein gel blot analysis

Protein extraction and gel blot were performed according to Wang et al. (2020b) and Rong et al. (2018) with slight modification. To extract crude protein, 0.2 g leaf was grounded to be fine powder with liquid nitrogen, and then homogenized in 2 mL extraction buffer (0.1 mol/L Tris–HCl pH 8.0, 10% (v/v) glycerol, 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.2% (v/v) Triton X-100, 5% (w/v) PVPP, 1 mmol/L phenylmethylsulphonyl fluoride (PMSF), 1 mmol/L benzamidine, 1 mg/mL

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leupeptin and 2 mg/mL apratinin). The homogenates were centrifuged at 12,000 *g* for 15 min at 4°C, and the supernatant was used for protein gel blot and isozyme patterns analysis listed below. Total protein content was determined in accordance with Bradford (1976).

For protein gel blot, the total protein extract was mixed with 125 mmol/L Tris–HCl (pH 6.8), 25% (v/v) Glycerol, 5% (w/v) SDS, 20% (v/v) 2-Mercaptoethanol and 0.1% (w/v) bromophenol blue, and denatured by boiling for 5 min. After cooling, the mixture was centrifuged at 12,000 *g* for 5 min. The total protein was separated on SDS-PAGE (10% (m/v) separating gel, 5% (m/v) stacking gel), and then transferred onto PVDF membrane. The membranes were blocked with 8% (m/v) non-fat milk/TBST buffer for 3 h. Afterwards, they were washed with TBST buffer and followed by incubating in primary antibodies RbcL (1:5000, Agrisera), Lhcb1 (1:2000, Agrisera) and Actin (1:2000, Abmart) overnight at 4°C, respectively. After that, the membranes were transferred to corresponding secondary antibody goat anti-rabbit (1:40000, Boster) or goat anti-mouse (1:40000, Stressgen) at room temperature for 3 h.

Determination of SOD, CAT, POD, APX and NADPH oxidases isozymes

Isozymes were assayed by native polyacrylamide gel electrophoresis (PAGE) using Mini-PROTEIN 3 electrophoresis system (Bio-Rad, USA). Electrophoresis ran with constant voltage of 80 V to the separating gel, and whereafter resumed by 120 V to the end using the solution (25 mmol/L Tris, 192 mmol/L glycin, pH 8.3) as electrode buffer. Superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) isozymes were visualized on basis of García-Limones et al. (2002), and NADPH oxidases isozyme was determined by the methods of Sagi and Fluhr (2001).

Measurement of GSTs activity and GSH content

Glutathione S-transferases (GSTs) activity and reduced glutathione (GSH) content were determined referring to the method of Zhang and Ge (2008) with minor modification. For GSTs activity assay, 0.2 g fresh leaf was homogenized in 1.5 mL extract solution (phosphate buffer (pH 6.5), 1 mmol/L EDTA). The homogenates were centrifuged at 4°C, 10,000 *g* for 10 min. The supernatant was subjected to GSTs activity determination using a kit (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China). For GSH content assay, 0.2 g leaf was homogenized with 2 mL 1 mmol/L EDTA, and then immediately acidified by adding 100 μL 50% (v/v) $HClO_4$. The homogenates were centrifuged at 12,000 *g* for 10 min. 250 μL of the supernatant was mixed with 2.4 mL 50 mmol/L phosphate buffer (pH 8.0), 150 μL 1% (v/v) *O*-phthaldialdehyde and 1.2 mL H_2O. The fluorescence intensity at 425 nm after excitation at 343 nm was detected.

RNA isolation and qPCR

0.1 g leaf was prepared for total RNA extraction using a RNAprep pure plant kit (Beijing Tiangen, Inc. China) followed by the manufacturer's instructions. First-strand cDNA was synthesized from 2 μg RNA using revertAid Reverse transcriptase (Thermo Fisher Scientific Biotech Co., Ltd. China) according to the instructions.

Quantitative real-time PCR (qPCR) was performed using a StepOnePlus™ Real-Time PCR System (ABI, USA) with TB Green™ Fast qPCR Mix (TaKaRa, Japan).

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PCR amplification procedures included predenaturation at 95°C for 30 s, followed by 40 cycles (95°C for 5 s and 60°C for 35 s) and a final dissociation cycle (95°C for 15 s, 60°C for 1 min and 95°C for 15 s). Each experiment was replicated three times. The relative expression of the target genes was calculated by comparative Ct method (Livak and Schmittgen, 2001). Among them, the gene ubiquitin (*Ubq*) was served as the reference.

The genes and the corresponding primer sequences were as below: *Ubq* (forward, 5′-TTCCATGCTGCTCTACCACAG-3′ and reverse, 5′-AGGGTTCACAAGTCTGCCTATT-3′); *atpB* (forward, 5′-TCATTGCTATTGTTCAAGCACG-3′ and reverse, 5′-ATTCATGTA-CGAGGAGCAGGGT-3′); *psbA* (forward, 5′-ATCTGTAGTTGATAGCCAAGGTCG-3′ and reverse, 5′-TAGGTCTAGAGGGAAGTTGTGAGC-3′); *psaB* (forward, 5′-GCGGCGGTACTTGTGATATT-3′ and reverse, 5′-CCCTGCCATAATGTGATGTG-3′).

Determination of Rubisco activity, Chl content and net photosynthetic rate

Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity was estimated by the methods of Wang et al. (2020b) with minor modification. 1 g leaf was ground into fine powder with liquid nitrogen, and then rapidly homogenized with extraction buffer (33 mmol/L Tris-HCl (pH 8.0), 33 mmol/L $MgCl_2$, 0.67 mmol/L Na_2EDTA and 10 mmol/L $NaHCO_3$). After centrifugation at 12,000 *g* for 5 min at 4°C, the supernatant was directly used for the evaluation of Rubisco activity. The initial Rubisco activity was measured by preparing reaction mixture (5 mmol/L HEPES (pH 8.0), 1 mmol/L $KHCO_3$, 2 mmol/L $MgCl_2$, 0.25 mmol/L DTT, 0.1 mmol/L Na_2EDTA, 1 U glyceraldehyde-3-phosphate dehydrogenase, 1 U creatine phosphokinase, 1 U 3-phosphoglyceric phosphokinase, 0.5 mmol/L ATP, 0.015 mmol/L NADH, 0.5 mmol/L phosphocreatine, 10 mL extract). The reaction was then initiated by adding 0.4 mmol/L RuBP. The changes in absorbance at 340 nm were monitored for 90 s.

Chlorophyll (Chl) extraction was based on Wang et al. (2020b). 0.2 g leaf were chilled with liquid nitrogen and grounded to be fine powder. The pigments were extracted with 100% (v/v) acetone. After centrifugation at 12,000 *g* for 10 min at 4°C, the supernatant was taken for absorbance detection at 665 nm and 649 nm, respectively.

Net photosynthetic rate (Pn) was determined at 9:00 a.m.–11:00 a.m., with a handheld photosynthesis system (TPS-2, PP-SYSTEMS, USA). During determination, external CO_2 concentration was about 380 μL/L, illumination intensity were set at 1200 μmol/m^2/s. Six leaves at the same position of rice seedlings were selected for measurement in each treatment.

Statistical analysis

Statistically significant differences were analyzed by one-way ANOVA followed by Duncan's test using the SPSS 22.0 software. Differences were considered to be significant at $p < 0.05$.

Results

Changes in $O_2^{\cdot-}$ production and NADPH oxidase isozyme activities in the leaves

The reaction of intracellular $O_2^{\cdot-}$ with NBT produced dark-blue hydrazone spots, and the density of spots displayed the generation of $O_2^{\cdot-}$. As shown in *Figure 1A*, with

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increasing concentrations of boscalid in the solution, $O_2^{\cdot-}$ production exhibited a J-shaped dose-response curve and increased significantly compared with the control at 10 mg/L boscalid.

The electrophoresis gel bands' integrated densities (denoting enzyme activities) indicated the total activities of isozymes (the same as follows). It was shown that the activities of NADPH oxidase isozymes also displayed a J-shaped dose-response curve and were distinctly enhanced in response to 10 mg/L boscalid (*Fig. 1B*).

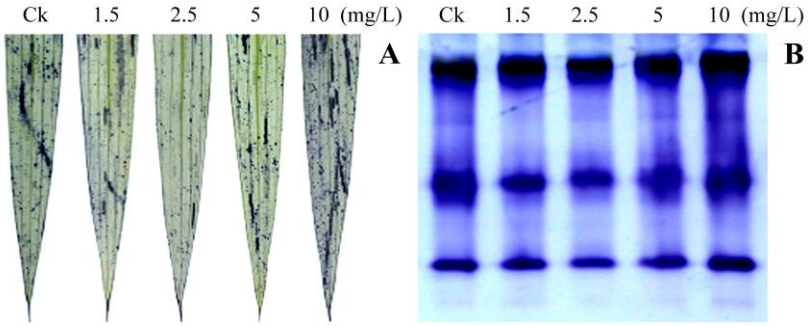

Figure 1. *Changes in $O_2^{\cdot-}$ production (A) and activities of NADPH oxidase isozyme (B) in rice leaves exposed to boscalid for 30 d. 0 (control, Ck), 1.5, 2.5, 5 and 10 mg/L denote the treatment concentrations of boscalid added in solutions. The same below*

Changes in SOD, POD, CAT and APX isozymes in the leaves

As illustrated in *Figure 2A, D*, the activities of both SOD and APX isozymes exhibited a J-shaped dose-response curve with the increase of boscalid and were apparently enhanced at 5–10 mg/L boscalid compared with the control. Unlikely, the activities of POD and CAT isozymes showed a U-shaped dose-response curve (*Fig. 2B, C*).

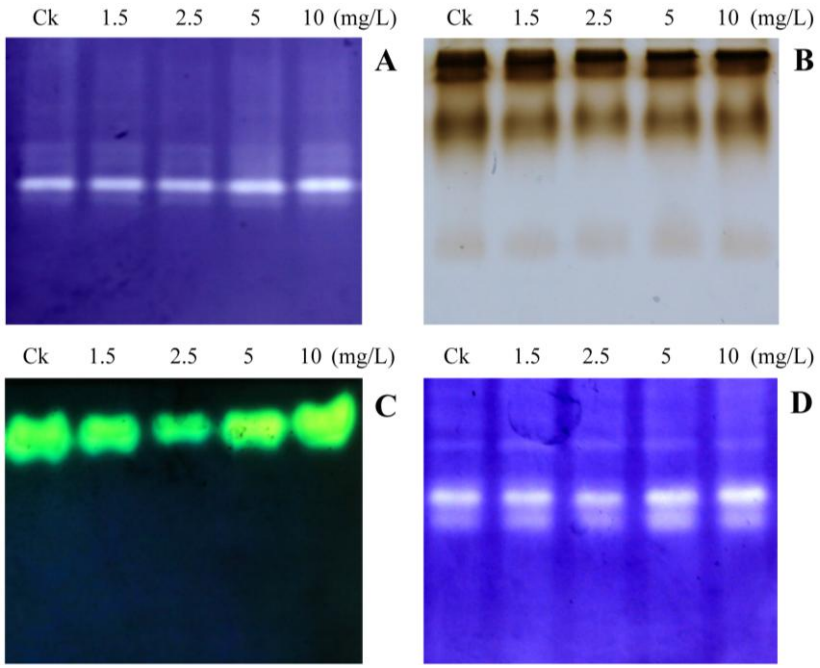

Figure 2. *Patterns of SOD (A), POD (B), CAT (C) and APX (D) isozymes in rice leaves exposed to boscalid for 30 d*

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Alteration in GSTs activities and GSH contents in the leaves

With increasing boscalid, GSTs activities and GSH contents appeared J- and inverted J-shaped dose-response curves, respectively (*Fig. 3*). By contrast, GSTs was induced at 10 mg/L boscalid, while GSH content was obviously decreased.

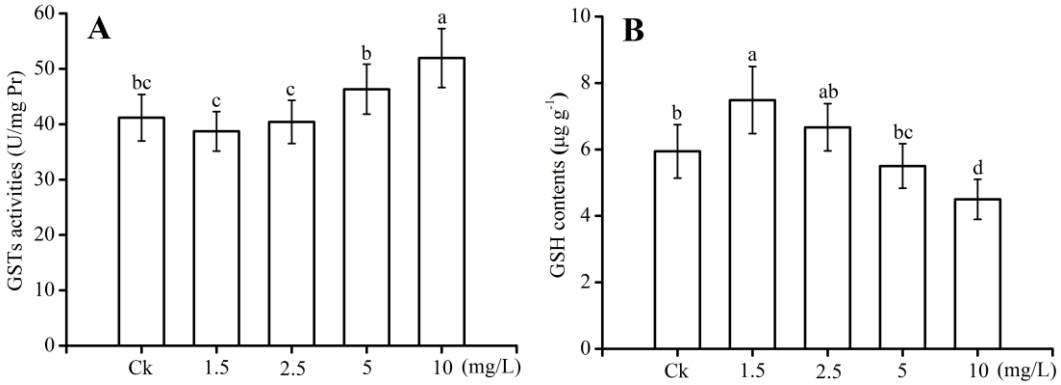

Figure 3. *Activities of GSTs (A) and contents of GSH (B) in rice leaves exposed to boscalid for 30 d. The same letters represent no significant difference at $p < 0.05$*

Variation in Chl contents, Pn and Rubisco activities in the leaves

As seen in *Figure 4*, Chl contents, Pn and Rubisco activities differentially presented inverted J-shaped dose-response curves. Generally, Pn and Rubisco activities showed the same trend, and they were both noticeably increased at 2.5 mg/L boscalid and declined at 10 mg/L in comparison to the control ($p < 0.05$). Unlikely, Chl contents were increased at 1.5 mg/L boscalid ($p < 0.05$).

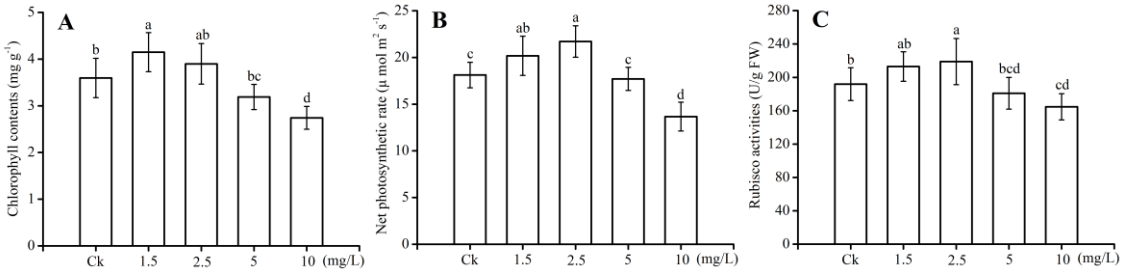

Figure 4. *Chlorophyll contents (A), Net photosynthetic rate (B) and activities of Rubisco (C) in rice leaves exposed to boscalid for 30 d. The same letters represent no significant difference at $p < 0.05$*

Expression levels of key photosynthesis-related genes and proteins in the leaves

From *Figure 5A*, the mRNA levels of *atpB* gene was found to be remarkably decreased with increasing of boscalid ($p < 0.05$). Whereas, the levels of both *psbA* and *psaB* were both followed inverted J-shaped dose-response curves, and they were evidently up-regulated at 1.5–2.5 mg/L boscalid and down-regulated at 10 mg/L ($p < 0.05$) (*Fig. 5B, C*). Lhcb1 and RbcL proteins were probed by gel blot, and their abundances differentially exhibited U-shaped dose-response curves (*Fig. 5D*).

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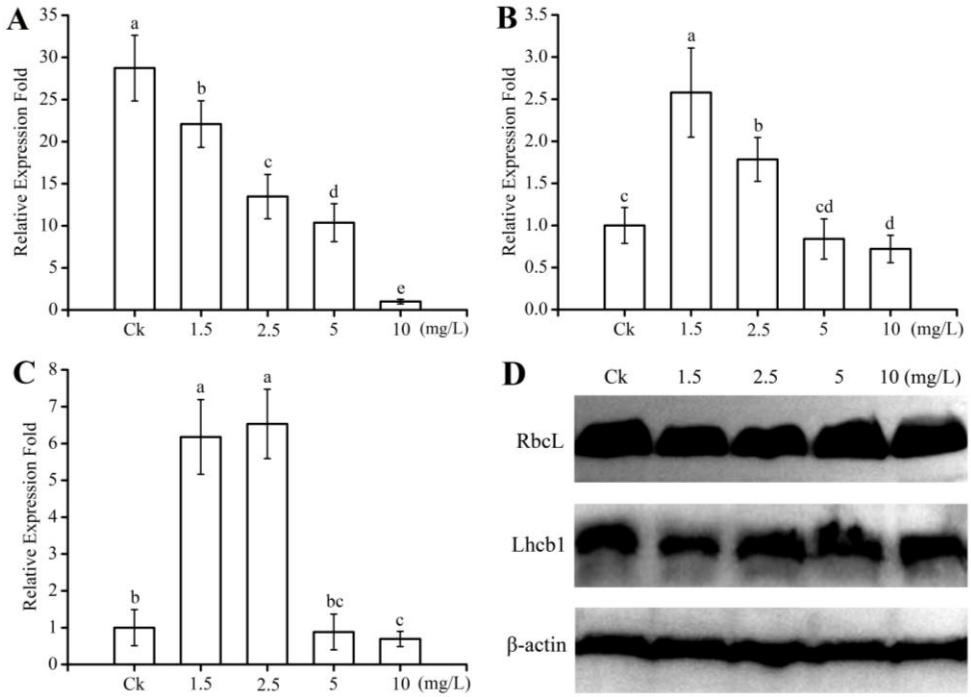

Figure 5. *Expression levels of photosynthesis-related genes atpB (A), psbA (B), psaB (C) and proteins (Lhcb1 and RbcL) (D) in rice leaves exposed to boscalid for 30 d. The same letters represent no significant difference at $p < 0.05$*

Discussion

So far, studies relating to toxic effects of SDHIs on higher plants are still limited. Photosynthesis is one of the most sensitive physiological processes to oxidative stress (Wang et al., 2022). Hereby, this study aimed to investigate the phytotoxicity of boscalid (a typical representative of SDHIs) to photosynthetic and antioxidant systems in rice seedlings.

$O_2^{\cdot -}$ is one important species of ROS, which can serve as an indicator of cell damage or toxicity (Lin et al., 2009). In this study, 10 mg/L boscalid promoted $O_2^{\cdot -}$ accumulation in rice leaves. Meanwhile, NADPH oxidase, SOD and APX isozymes at 10 mg/L boscalid were evidently enhanced above those of the respective controls. This signified ROS overproduction, and SOD and APX isozymes were contributed to a defense system that enhanced ROS-scavenging capability, thereby protecting rice seedlings from oxidative damage. As well, GSTs/GSH system is also involved in cellular defense against oxidative stress in organisms (Zhang et al., 2021). Therein, GSTs are multi-functional superfamily of enzymes that detoxify of xenobiotics and endogenous compounds, including harmful products resulted from oxidatively modified lipids and proteins, by means of conjugating electrophilic substrates to GSH (Han et al., 2022b; Wang et al., 2018). As shown in *Figure 3*, GSTs was induced at 10 mg/L boscalid, while GSH content was obviously decreased. The activated GSTs facilitated the conjugation of GSH to toxic substance, and hereby led to the consumption of GSH suggesting their involvement with the detoxification mechanism in rice seedlings.

Oxidative damage has been reported to exert negative effects on photosynthetic efficiency (Liu et al., 2022). Net photosynthetic rate (Pn) is determined as a direct indicator to evaluate photosynthetic efficiency (Li and Yi, 2020). Furthermore,

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chlorophyll (Chl) is an important photosynthetic pigment participating in light absorption and energy transfer during photosynthesis (Yu et al., 2023). As well, Rubisco is the key enzyme of CO_2 fixation in photosynthesis that converts atmospheric CO_2 to carbohydrates (Flecken et al., 2020; Gonçalves et al., 2020). From *Figure 4*, Chl contents, Pn and Rubisco activities were all found to be increased at 1.5–2.5 mg/L boscalid and declined at 10 mg/L. So, it could be concluded that 1.5–2.5 mg/L boscalid improved photosynthesis, while 10 mg/L boscalid had detrimental impacts on photosynthesis due to inhibiting Rubisco activities and Chl anabolism.

Photosynthesis is a complex multistep reaction process controlled by many genes (Fernie and Bauwe, 2020). In present study, the expression patterns of some key photosynthesis-related genes were detected employing qPCR. The selected genes *atpB*, *psbA* and *psaB* encode for chloroplast proteins, which are located in ATP synthase, PSII (Photosystem II) and PSI complexes, respectively (Scheller et al., 2001; Yang et al., 2021; Zhang et al., 2018). Such those are extremely vital protein complexes on the thylakoid membrane of chloroplasts taking part in ATP synthesis and photosynthetic electron transfer during photosynthesis (Choquet and Vallon, 2000; Xu et al., 2023). Moreover, the abundances of RbcL (catalyzing CO_2 fixation) and Lhcb1 (participating in adaptation of photosynthesis to different light regimes) proteins were also assayed by gel blot. The results evidenced that 1.5–2.5 mg/L boscalid differentially increased the transcript levels of *psbA* and *psaB* genes. However, the level of *atpB* gene was decreased, as well as the abundances of Lhcb1 and RbcL proteins. Aside from down-regulation of *atpB* gene, 10 mg/L boscalid identically depressed the genes *psbA* and *psaB*, accompanied by the degradation of Lhcb1. These findings unveiled that 1.5–2.5 mg/L boscalid might improve photosynthesis by facilitating photosynthetic electron transfer. Whereas, 10 mg/L boscalid possibly impaired photosynthesis via inhibition of ATP synthesis and photosynthetic electron transfer.

Conclusion

The toxicity of boscalid to rice seedlings was dose-dependent. 1.5–2.5 mg/L boscalid enhanced Chl contents, Pn and Rubisco activities along with up-regulating the genes *psbA* and *psaB*, leading to the improvement of photosynthesis. However, 10 mg/L boscalid accelerated the production of $O_2^{\bullet -}$, underlying the activated activities of NADPH oxidase, superoxide dismutase (SOD), ascorbate peroxidase (APX) isozymes as well as glutathione S-transferases (GSTs), suggesting reactive oxygen species (ROS) overproduction and defense response in the seedlings. Meanwhile, 10 mg/L boscalid decreased Chl contents, Pn and Rubisco activities accompanied by down-regulation of the genes *atpB*, *psbA* and *psaB*, as well as Lhcb1 protein, resulting in the impairment of photosynthesis possibly via inhibition of ATP synthesis and photosynthetic electron transfer. In addition, SOD and APX isozymes together with GSTs and reduced glutathione (GSH) devoted to alleviate boscalid-induced toxicity suggesting their participation in the defense and detoxification in rice seedlings. Consequently, widespread application of boscalid posed potential toxic effects on growth of rice seedlings.

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