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Salicylic acid mediates antioxidant defense system and ABA pathway related gene expression in *Oryza sativa* against quinclorac toxicity

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ABSTRACT

The auxin herbicide quinclorac is widely used for controlling weeds in transplanted and direct-seeded rice fields. However, its phytotoxic responses on rice are still unknown. Therefore, in the present investigation we studied the effects of different concentrations (0, 0.1 and 0.5 g/L) of quinclorac herbicide on the physiological and biochemical changes of two rice cultivars (XS 134 and ZJ 88) and further analyzed the ameliorating role of salicylic acid (SA) on quinclorac toxicity in rice plants. The results revealed that exogenous application of SA significantly increased plant biomass and total chlorophyll contents in herbicide stressed plants. The lipid peroxidation and ROS (H_2O_2, $O_2^{-}\cdot$, ^{-}OH) production were significantly increased in roots and leaves of both rice cultivars under quinclorac stress, demonstrating an oxidative burst in rice plants. Whereas, application of SA significantly lowered ROS contents under quinclorac stress. Further, exogenous SA treatment significantly modulated antioxidant enzymes and enhanced GSH concentration in stress plants. Anatomical observations of leaf and root revealed that herbicide affected internal structures, while SA played a vital role in protection from toxic effects. Expression analysis of stress hormone ABA genes (*OsABA8oxs*, *OsNCEDs*) revealed that quinclorac application enhanced stress condition in cultivar ZJ 88, while SA treatment downregulated ABA genes more in cultivar XS 134, which correlated with the enhanced tolerance to quinclorac induced oxidative stress in this cultivar. The present study delineated that SA played a critical role under quinclorac stress in both rice cultivars by regulating antioxidant defense system, reducing ROS formation and preventing the degradation of internal cell organelles.

1. Introduction

Rice (*Oryza sativa* L.) plays a dominant role in food production in the world. In China, it accounts for one third of the total area of cereal crops, approaching 30 million ha. In recent decades, with the increasing population, food production becomes a profound issue all over the world. Crop production is challenged by many biotic and abiotic stresses, among which, weeds are considered as a serious threat that impairs the production and quality of crops (Kudsk and Streibig, 2003).

The quinolinecarboxylic acid quinclorac (3,7-dichloro-8-quinolinecarboxylic acid), is a new class of highly selective auxin herbicides that have been used as pre- and post-application in transplanted and directly seeded rice. It is effective to control dicotyledonous and monocotyledonous weeds, particularly barnyardgrass (*Echinochloa crus-galli*) in paddy fields (Grossmann, 1998). Quinclorac herbicide causes the symptoms of epinasty, chlorosis, necrosis, growth inhibition under high concentration in susceptible dicots. Its mode of action occurs through the induction of ethylene biosynthesis in barnyardgrass. Overproduction of ABA appears to be a crucial factor in growth inhibition and phytotoxic response to herbicide (Grossmann, 2003). The key step regulating ABA biosynthesis is catalysed by the plastid enzyme NCED encoded by a family of NCED genes (Schwartz et al., 2003). The level of ABA is catabolized by ABA 8′-hydroxylase, a cytochrome P450 monooxygenase which isomerizes ABA into phaseic acid (PA) (Krochko et al., 1998). Although, rice has relative higher resistance to this herbicide, excessive use of herbicides can cause higher risks to the growth of rice at early stage.

Quinclorac herbicide is widely used for the control of barnyardgrass in China since 1990. Rice farmers started reporting the

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failure of barnyardgrass control in 2000. Quinclorac-resistant barnyardgrass is now a problem in some rice farming areas of China (Li et al., 2016) and farmers apply quinclorac more than the recommended dose to control barnyardgrass. Recently, Resgalla et al. (2007) detected quinclorac as most frequent agrochemical residue in five of seven hydrographic basins of State of Santa Catarina (Brazil), additionally it was also detected in rivers flowing through irrigated paddy field areas. Therefore, the investigation of quinclorac induced toxic effects and its tolerance in rice plants under such conditions are necessary to suggest resistant cultivar under high dosage application areas. The normal metabolism of herbicides detoxification includes several stages; i) Oxidation by P450s and hydrolyses by carboxylesterases, ii) Bonds with molecules such as reduced glutathione (GSH) or glucose, catalyzation by glutathione transferases (GSTs), iii) Conjugates transportation into the vacuole, and iv) Processing of conjugates involving partial degradation, secondary conjugations and incorporation into cell wall components (Riechers et al., 2010).

Besides these reactions, reactive oxygen species (ROS) have been found associated with herbicide toxicity. At high concentrations, quinclorac induces oxidative injuries in plants. Previously, antioxidant enzyme activities i.e. superoxide dismutase (SOD), catalase (CAT), ascorbic acid peroxidase (APX), and glutathione reductase (GR) were studied in tolerant rice (*Oryza sativa* L. cv. Nipponbare) and a particularly susceptible grass weed, *Echinochloa oryzicola* Vasing (Sunohara and Matsumoto, 2004).

Salicylic acid (SA) has been found to play a vital role in plant defense system. It can induce heat acclimation, improve drought and chilling tolerance and increase chlorophyll contents (Horváth et al., 2007). SA induces basic β-1,3-glucanase (Glu2) and chitinase isozymes effectively in sugar beet (Burketová et al., 2003). SA as a signaling molecule also mediates oxidative accumulation which leads to a hypersensitive response and cell death (Horváth et al., 2007). In addition, according to the study of Sunohara and Matsumoto (2008), Dayan et al. (2010) and Grossmann (2010), quinclorac induced phytotoxicity in susceptible grass weeds such as *Echinochloa, Digitaria*, and *Setaria*, through ethylene and its biosynthetic pathway-related substances including cyanide, while in crop plants quinclorac-induced cell death is due to the ROS accumulation and lipid peroxidation. Therefore, the objective of this study was to investigate the ameliorating role of SA on morphological, physio-biochemical processes and ABA pathway related gene expression under quinclorac stress, in order to understand the mechanisms regarding the alleviation of SA-induced herbicide stress tolerance in two rice cultivars.

2. Materials and methods

2.1. Plant materials and treatments

Two Japonica rice (*Oryza sativa* L.) cultivars i.e. Xiushui 134 (XS 134) and Zhejing 88 (ZJ 88) that are widely used in southeast China were selected for the present study. Mature and healthy seeds were surface-sterilized in 0.1% NaClO for 15 min, then rinsed and soaked with distilled water for further 20 min. Seeds were sown in plastic germination boxes (18 × 12 × 10 cm) with double filter papers wetted by half-strength Hoagland solution. Under dark for two days, germinated seeds with uniform size radicals were selected and cultured in a growth chamber under the following conditions: 300 μmol m^{-2} s^{-1} active photon flux density, 25/20 °C (day/night) temperature, 70–80% relative humidity, and 14/10 h (light/dark) photoperiod. The solution was renewed after every 5 days with half-strength Hoagland solution. Experimental design was completely randomized with three replications. When the height of the seedlings reached 10–12 cm, uniform size plants were transferred into full-strength Hoagland solution. 20 days later, the quinclorac herbicide (with the concentration of 10%, in wet powder form) was applied in a solution at different concentrations (0, 0.1, 0.5 g/L) at four-leaf stage. The treatment concentrations were based on pre-experimental studies, in which several lower and higher levels of herbicide were used, i.e., 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 g/L of quinclorac. The herbicide quinclorac at 0.1 g/L showed a little damage on plant growth and 0.5 g/L imposed a significant damage to plant growth, while those higher than 0.5 g/L were too toxic for plant growth. SA treatment at 10 mg/L was applied in a solution two days before quinclorac treatment. Ten days after treatment, plants were harvested and separated into shoots and roots to carry out the morphological, biochemical and ultrastructural examinations.

2.2. Determination of morphological changes

Five plants were taken for the measurement of plant height and biomass 10 days after treatment. Chlorophyll contents in leaves were determined according to the method of Porra et al. (1989).

2.3. Analysis of lipid peroxidation and reactive oxygen species (ROS)

Malondialdehyde (MDA) content was determined by 2-thiobarbituric acid (TBA) reactive metabolites to express the level of peroxidation of polyunsaturated fatty acids (Zhou and Leul, 1999). For the analysis of hydrogen peroxide (H_2O_2), leaves and roots (0.5 g) were homogenized in 5 mL trichloroacetic acid (TCA) (0.1%, w/v) in an ice bath and centrifuged at 12,000*g* for 15 min (Velikova et al., 2000). Then 0.5 mL supernatant was added into the reaction mixture with 0.5 mL 10 mM potassium phosphate buffer (pH 7.0) and 1 mL 1 M KI. The absorbance was measured at 390 nm and the H_2O_2 level was calculated by the standard curve. Superoxide radical ($O_2^{-\cdot}$) was assayed according to Jiang and Zhang (2001). The fresh tissues (0.5 g) were homogenized in 3 mL 65 mM potassium phosphate buffer (pH 7.8) and then centrifuged at 5000*g* for 10 min at 4 °C. 1 mL supernatant was extracted to mix with 0.9 mL of 65 mM potassium phosphate buffer (pH 7.8) and 0.1 mL of 10 mM hydroxylamine hydrochloride, and then incubated at 25 °C for 24 h. After the incubation, 1 mL sulphanilamide (17 mM) and 1 mL a-naphthylamine (7 mM) were mixed in 1 mL solution for further 20 min at 25 °C. After that, *n*-butanol in the same volume was added and centrifuged at 1500 g for 5 min. The absorbance in the supernatant was read at 530 nm. Standard curve was used to calculate the generation rate of O_2^- (Jiang and Zhang, 2001).

For estimation of extra-cellular hydroxyl radicals ($^{\cdot}OH$), fresh samples (0.1 g) were homogenized in 1 mL 10 mM Na-phosphate buffer (pH 7.4) consisting of 15 mM 2-deoxy-D-ribose (SRL, Mumbai) at 37 °C for 2 h (Halliwell and Gutteridge, 1990). Following incubation, an aliquot of 0.7 mL from the above mixture was added to reaction mixture containing 3 mL 0.5% (w/v) thiobirbuteric acid (TBA) (Hi Media, Mumbai, 1% stock solution made in 5 mM NaOH) and 1 mL glacial acetic acid, heated at 100 °C in a water bath for 30 min and cooled down to 41 °C for 10 min before measurement. The absorbance was measured at 550 nm.

2.4. GSH and oxidized glutathione (GSSG) contents

GSSG and GSH were measured according to Law et al. (1983) with some modifications. Fresh leaves and roots (0.3 g) were ground in 5 mL 10% (w/v) TCA and centrifuged at 15,000*g* for 15 min 1 mL reaction mixture comprised of 150 μL supernatant, 100 μL of 6 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), 50 μL of glutathione reductase (10 units mL^{-1}), and 700 μL of 0.3 mM NADPH was used to evaluate the total glutathione. All of the reagents were kept in 125 mM NaH_2PO_4 buffer, containing 6.3 mM

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