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Research article

Combined herbicide and saline stress differentially modulates hormonal regulation and antioxidant defense system in *Oryza sativa* cultivars

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ABSTRACT

Plants are simultaneously exposed to a combination of biotic and abiotic stresses in field conditions. Crops respond to the combined stress in a unique way which cannot be understood by extrapolating the results of individual stress. In the present study, effects of individual and combined stress of herbicide (2,4-dichlorophenoxyacetic acid) and salinity (NaCl) on two *Oryza sativa* cultivars (ZJ 88 and XS 134) were investigated. Both herbicide and saline stress affected the plant growth differentially and produced oxidative stress in rice cultivars. Interestingly, the combination of herbicide and salinity showed a significant protection to both rice cultivars by reducing ROS (H_2O_2, $O_2^{\cdot-}$) and lipid peroxidation through modulation of enzymatic (SOD, POD, CAT and APX) and non-enzymatic (TSP, sugars, phenolic and proline) antioxidants. In addition, active regulation of transcript levels of genes encoding Na^+ and K^+ (*OsHKT1;5, OsLti6a,b, OsHKT2;1, OsSOS1, OsCNGC1, OsNHX1* and *OsAKT1*) transporter proteins reduced sodium and enhanced potassium accumulation under combined stress, resulted a better growth and ionic homeostasis in both rice cultivars. The production of ABA and IAA was significantly higher in cultivar XS 134 compared to cultivar ZJ 88 under control conditions. However, combined herbicide and saline stress enhanced the accumulation of phytohormones (IAA and ABA) and transcription of ethylene in cultivar ZJ 88, which might be one of the factors responsible for poor salt tolerance in sensitive cultivar. These findings indicated that herbicide application under saline stress confers tolerance to salinity in rice cultivars, likely by reducing oxidative damage, modulating mineral absorption, upgradation of antioxidant defense and by dynamic regulation of key genes involved in Na^+ and K^+ homeostasis in plants.

1. Introduction

Among abiotic stress, salinity is one of the most important environmental stress factors, limiting the plant growth and agricultural productivity worldwide (Islam et al., 2015a). Plant growth related effects of saline stress are associated with nutritional toxicity, water stress and accumulation of excess sodium ions (Na^+) (Islam et al., 2015b). Moreover, salt stress affects photosynthesis/carbohydrate metabolism (Chaves et al., 2009) which in turn increase the generation of reactive oxygen species (ROS) and cause oxidative stress in plant (Gill and Tuteja, 2010). On the other hand, herbicides are the most widely used chemicals against the weeds in farmland to increase crop productivity. Prolonged use of herbicides over vast areas results in the evolution of weeds resistance (Duke,

Abbreviations: ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; A.I, acid equivalent; APX, ascorbate peroxidase; CAT, catalase; DAB, Diaminobenzidine tetrahydrochloride; IAA, Indole acetic acid; GABA T, GABA-transaminase; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; NBT, nitro-blue tetrazolium; $O_2^{\cdot-}$, superoxide; POD, peroxidase; P5CS, pyrroline-5-carboxylate synthase; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, Thiobarbituric acid; TCA, Trichloroacetic acid; TPC, phenolic content; XS 134, Xiushui 134; EL, electrolyte leakage; GSSG, oxidize glutathione content; GSH, reduced glutathione; ZJ 88, Zhejing 88; 2,4-D, 2,4-dichlorophenoxyacetic acid.

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2012). To deal with resistant weed, relatively higher amount of herbicide are often used multiple times in a growing season (Duke, 2012) to achieve high and lasting efficacy. When crop fields are sprayed with sub-lethal doses of herbicides, toxic effects on crop plant or sensitive species can be observed (Boutin et al., 2014). Herbicide, like 2,4-dichlorophenoxyacetic acid (2,4-D), mimic of natural plant hormone auxin, is extensively used around the world. Nearly, 46 million pounds per year of 2,4-D was used in USA during 1992–2000 (EPA, 2005). 2,4-D causes epiastic deformations, senescence, ROS production and inhibition of roots and shoot growth in sensitive plants. The reductions in stomatal aperture, change in carbon assimilation, transpiration, membrane damage and localized cell death are associated toxicity features of 2,4-D in plants (Bukowska, 2006; Wafa et al., 2012). Natural and synthetic auxins can increase the activity of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase; result in the induction of ethylene, which activates the biosynthesis of another plant hormone, abscisic acid (ABA) (Pazmino et al., 2011). ABA accumulation causes stomatal closure, which limits biomass production and can induce overproduction of reactive oxygen species (ROS), the main factor responsible for the oxidative damage and cell death (Pazmiño et al., 2012). In this way, herbicide (2,4-D) acts as stress factor and different studies have demonstrated its toxic effects on plants growth and biochemical processes (Pazmiño et al., 2012; Boutin et al., 2014).

The investigations concerning the mechanism of plant adaptations to abiotic stresses are mainly carried out in response to single stress factors, which is incompatible with the actual field conditions. Plants normally exposed to a combination of several abiotic stresses in the environment (Colmenero-Flores and Rosales, 2014). Previous studies have revealed that plant's respond to the combined stress (like saline and heat, saline and drought, drought and heat) in a unique way, which cannot be generalized from exposure to single stress factor (Rivero et al., 2014). Stress combination instead of individual stress is considered as realistic threats faced by the plants. Hence, more attention is needed to understand the plant responses to combination of stresses for crop development with better adaptation under field conditions (Pandey et al., 2015). Furthermore, to best of our knowledge, there is little information concerning the mechanism underlying the acclimation of rice plants to combination of herbicide and saline stress conditions.

Rice is predicted to become the world's most important crop, in terms of human food supply. Additionally, this crop grows well in arid and semiarid areas, making it an excellent model to study the key physiological/biochemical mechanisms involved in plant tolerance to multiple abiotic stresses such as application of herbicide and salinity. In the present study, our aim was to investigate the effect of the recommended dose of 2,4-D on the saline stress rice cultivars to determine individual and combined stress responses by measuring the changes in plant growth, photosynthesis, chloroplast ultrastructure, ROS (H_2O_2, $O_2^{\cdot-}$) production, variation in enzymatic (SOD, POD, CAT and APX) and non-enzymatic antioxidant (proline, TPS, soluble sugar, phenolic), micronutrients uptake, and regulation of key genes involved in hormonal (IAA, ABA, ET), Na^+ and K^+ homeostasis in two contrasting rice cultivars.

2. Materials and methods

2.1. Plant material

The healthy and uniform seeds of two rice (*Oryza sativa* L.) cultivars i.e. Zhejing 88 (ZJ 88) and Xiushui 134 (XS 134) were surfaced sterilized in 0.1% NaClO for 15 min, then rinsed and soaked with distilled water for further 20 min. The seeds were germinated on moistened filter paper kept in darkness for 48 h, and then in a growth chamber with day/night temperatures of 24/16 °C, a 16-hr photoperiod, irradiance of 300 μmoL m^{-2} s^{-1}, and relative humidity of 60–70%. Ten days old uniform seedlings were transferred to greenhouse in plastic pots containing 5 L of half strength nutrient solution. After two weeks of acclimatization, plants were treated with different concentrations of NaCl as determined by preliminary experiment (data not shown) and sprayed with a recommended dose of 2, 4-dichlorophenoxyacetic acid (0.8 kg a.i. ha^{-1}). Usually salinity is measured in units of electrical conductivity EC, and according to the International Rice Research Institute (IRRI) salinity beyond ECe ~ 4 dS m^{-1} is considered as moderate salinity while more than 8 dS m^{-1} becomes high for rice plants (http://www.knowledgebank.irri.org/ricebreedingcourse/Breeding_for_salt_tolerance.htm). In the present experiment, the selected EC concentrations of NaCl solutions were suitable to study the saline stress on these rice cultivars (ZJ 88 and XS 134) without causing their death during the experiment. The experiment was comprised of following treatments: T_1 (control with EC 1.2 dS m^{-1}), T_2 (4 dS m^{-1}), T_3 (8 dS m^{-1}), T_4 (spray of recommended dose of 2,4-D), T_5 (4 dS m^{-1} + spray of recommended dose of 2,4-D), T_6 (8 dS m^{-1} + spray of recommended dose of 2,4-D). Each treatment was replicated four times. The nutrient solution was renewed every five days. Eighteen days after treatment, samples for morphological and biochemical analysis were collected as described below.

2.2. Morphological parameters

After 15 days of treatment, plants were harvested. Fresh weight (FW) of plants was measured immediately after harvesting, while for dry biomass, plants were placed in an oven at 80 ± 1.50 °C and weighed after 5 days (Momoh and Zhou, 2001).

2.3. Chlorophyll pigments

Leaf photosynthetic pigments i.e. chlorophyll contents were measured according to the method of Arnon (1949).

2.4. Determination of malondialdehyde and reactive oxygen species contents

Lipid peroxidation was determined in terms of malondialdehyde (MDA) contents by following the method of Zhou and Leul (1998). Hydrogen peroxide (H_2O_2) contents were measured according to the method of Velikova et al. (2000). Briefly, fresh leaves (0.5 g) were extracted with 0.1% (w/v) TCA (5.0 mL) in an ice bath and the extraction was centrifuged for 15 min at 12,000 rpm. The supernatant (1.5 mL) was collected after the centrifugation and mixed with 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 M KI (1 mL). The H_2O_2 contents were calculated by using a standard curve after getting the absorbance of the samples at 390 nm. The superoxide anion ($O_2^{\cdot-}$) levels were determined spectro-photometrically by measuring the reduction of nitro blue tetrazolium (NBT) according to the method of Doke (1983).

2.5. Electrolyte leakage estimation

Electrolyte leakage was measured using electrical conductivity meter as described by Lutts et al. (1996) by following the formula;

EL = EC1/EC2*100