Changes in growth, biochemical components and antioxidant genes expression in rice seedling (Oryza sativa L.) cultivar ‘IR64’ under salt stress

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Received: 25-11-2018 Accepted: 30-01-2019 DOI: 10.18805/IJARe.A-399

ABSTRACT

One of abiotic stresses that affects rice growth is salinity. Plant must develop adaptation process which includes morphological, biochemical and molecular changes. This research aimed to evaluate morpho-biochemical and molecular responses of rice ‘IR64’ to several levels of salinity stress at seedling stage. Seedlings of ‘IR64’ were grown in a hydroponic system and treated with different levels of salinity stress (4dSm−1, 6dSm−1 and 12dSm−1) for seven days. Responses were recorded on the final day of salt treatments. Gene expression analyses were done by semi-quantitative RT-PCR. RNA was extracted using RNase plant mini kit (Qiagen) and cDNA was synthesized using GoSript™ Reverse Transcription System (Promega). Results showed that shoot height and fresh weight decreased under salt stress. At plants treated with salt, the chlorophyll contents were lower than that of control plants, while MDA levels were higher in salt treated plants. Semi-quantitative PCR for MnSOD1 and cCu/ZnSOD1 revealed that MnSOD1 and cCu/ZnSOD1 expressions increased under salt stress which indicated oxidative stress defence, with the highest expression at 4dSm−1 and 6dSm−1 treatment, respectively.

Key words: Antioxidant genes, Morpho-biochemical, Rice, Salinity.

INTRODUCTION

Rice cultivar ‘IR64’ is one of the rice cultivars widely grown in Indonesia. It was released by IRRI in 1985 and classified as high quality rice. It is a lowland and irrigated rice cultivar. The period of this cultivar to reach maturity is 117 days. The yield reaches 5,965 kg/ha in dry season, while during rainy season the yield is around 3,852 kg/ha (Khush and Virk, 2005).

The productivity of rice is influenced by abiotic stress. Salinity is the second important stress condition that reduced productivity (Shrivastava and Kumar, 2015). The soil is categorized saline when it contains NaCl and soluble compound of other minerals such as Ca, Mg, K, Fe, B, SO4, CO3 and CH3O at high concentration (Szabolcs, 1989). Land salinization can be caused by improper drainage, use of salted irrigation water and climate change (Endo et al., 2011). Salinity stress triggers changes in morphological and biochemical characteristics of plants such as the increase of reactive oxygen species (ROS) production (Xu et al., 2011; Aref and Rad, 2012). The ROS production can be measured by accumulation of malondialdehyde (MDA) as a marker of oxidative stress due to membrane cell damage during salinity of drought stress (Xu et al., 2015).

Salt stress affected various stages of rice growth and development. The damages caused by high salt include the decrease of leaf area, rolled leaf, brown and dry leaf (Munns, 2005). High salt in soil environment causes limited availability of water to be absorbed by plant root. This condition leads to growth reduction, followed by salt accumulation in plant tissue. Salt poisoning induces tissue senescence and reduces growth (Munns, 2005). It was reported that, the agronomic traits that mostly affected by salinity in rice were grain yield and productive tillers per plant (Banumathy et al., 2018).

Plants adapt to salinity stress by several changes in their morphology, biochemical components and genes expression. Genes that have an important role in response to salinity stress are antioxidant genes. Superoxide dismutases (SODs) are genes that regulate antioxidant enzymes to combat oxidative stress. Oxidative stress can be induced by several factors including salinity stress (Wang et al., 2003, Xu et al., 2011). Plants that are tolerant to salt stress express a high level of antioxidant genes and have high antioxidant activities (Wang et al., 2003).

The aims of this study were to analyze growth response of rice cultivar IR64 to salinity stress at seedling stage and to evaluate SOD genes expression under salinity stress. This study provided information on morphological, biochemical and molecular changes in rice in response to various salinity stress levels.

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MATERIALS AND METHODS

Plant materials and salinity stress treatments: Seeds of rice cultivar ‘IR64’ were collected from a local farmer in Tabanan Regency, Bali, Indonesia. Seeds were surface sterilized in 10% commercial bleach for 15 min and soaked in water for two days. Seeds were then transferred to rockwool media for germination. One week seedlings were transferred to a hydroponic system containing ¼ x MS medium as a nutrient solution. After one week in a hydroponic system, seedlings were treated with NaCl at the concentration of 4dSm⁻¹, 6dSm⁻¹ and 12dSm⁻¹ in ¼ x MS medium for each week. Each treatment was done in three replicates.

Growth and biochemical observation: Seedlings were harvested on final day of treatment. Shoot height and weight were measured. Chlorophyll was extracted at final day of salt treatments using 80% acetone. Chlorophyll content was analyzed using spectrophotometer at 645 nm and 663 nm and total chlorophyll was calculated according to formulas:

\[
\text{Chl a (mg g}^{-1}\text{leaf) = }\frac{(12.7 \times \text{A663}) - (2.6 \times \text{A645})}{\text{ml acetone}} \times \text{ml acetone}
\]

\[
\text{Chl b (mg g}^{-1}\text{leaf) = }\frac{(22.9 \times \text{A645}) - (4.68 \times \text{A663})}{\text{ml acetone}} \times \text{ml acetone}
\]

\[
\text{Total Chl = Chl a + Chl b (mg g}^{-1}\text{leaf) = }\frac{20.2 (A645) + 8.02 (A663)}{\text{ml acetone}} \times \text{ml acetone}
\]

Data were analyzed using ANOVA.

Malonyaldehyde (MDA) was estimated according to Xu et al. (2015) with modification. As much as 0.25 g shoot were homogenised in 3 ml of 0.1% (w/v) trichloroacetic acid followed by centrifugation at 15,000 rpm for 20 min at 4°C. Supernatant (0.5 ml) was transferred to a test tube and 2 ml of 0.5% thioarbituricacidin 20% trichloroacetic acid was added. Test tube was heated at 95°C water bath for 30 min and immediately cooled in ice and centrifuged at 15,000 rpm, 4°C for 20 min. The absorbance was measured at 532 nm and 600 nm and 0.5% thioarbituric acid in 20% trichloroacetic acid was used as blanko.

Gene expression: The expression of manganese superoxide dismutase (MnSOD) and cytosolic copper/zinc superoxide dismutase cCu/ZnSOD under salt stress were analysed using semi-quantitative RT-PCR. RNA was extracted from rice shoot using RNase mini kit (Qiagen) following company instruction. First strand cDNA was synthesized using GoSript™ Reverse Transcription System (Promega) using 100 ng of total RNA in 20 μl reaction mixture.

Amplification of MnSOD1 was done using forward primer 5’GGAGGCGCTGATCATTTTCC3’ and reverse primer 5’CACAAGTGCCAGGCAAAG (Kim et al., 2004). The cCu/ZnSOD1 amplification was conducted using primer pair GAGATTCCAACGAGCCAAG (forward primer) and TTGTAGTGCGCCAGTTGA as reverse primer (Kim et al., 2004). As control expression, Actin gene was used with forward primer 5’ATGCTCTCCCCCAGCTATC3’ and reverse primer 5’TCTTCTTGTGCTCATCCTGTC3’ (Hong et al., 2007). The PCR reaction consisted of 1 × PCR buffer, 0.2 mM dNTP, 2 mM MgCl₂, 1 U Taq polymerase, 1.5 μM of each primer and 1 μl cDNA in 25 μl reactions. The cycles of thermal reaction were initial denaturation 1 × at 95°C for 5 min, followed by 40 × 1 min at 95°C for, 1 min at 57°C (MnSOD1) or 50°C (cCu/ZnSOD1) or 58°C (Actin), 1 min at 72°C for. Final extension was done 1 × at 72°C for 10 min.

PCR products were analyzed using 1.5% agarose gel electrophoresis in TAE buffer and stained with ethidium bromide. The electrophoresis was done for 45 min using 100V and the gel was visualized using UV-transilluminator. Quantitative analysis of PCR products was conducted using Image J software (Schneider et al., 2012).

RESULTS AND DISCUSSION

Salinity stress caused reduction of rice ‘IR64’ growth. Salt stress significantly reduced shoot height and weight (Table 1). The highest salt concentration tested (12dSm⁻¹) had the highest reduction in shoot length and weight. Seedling leaf turned yellow under salinity treatments. Yellow leaves became dominant at higher salt concentration. Fig 1 shows changes in leaf colour of rice IR64 seedlings as affected by salt stress. Chlorophyll content (chlorophyll a, b and total chlorophyll) decreased in NaCl treated seedlings. Table 2 shows chlorophyll content in control seedlings and in salt treated seedlings. The ratio of chlorophyll a/b decreased at salt concentration of 12dSm⁻¹, but the ratio was not affected at lower salt concentration.

Salinity inhibited seedlings growth of rice ‘IR64’. The reduction of growth under salt stress could be due to reduction in photosynthesis which reduces carbohydrate supply for plant growth (Dhanyalakshmi et al., 2013). High concentration of NaCl induces ion toxicity which affected growth. Salt stress significantly reduced shoot height and weight. Seedling leaf turned yellow under salinity treatments. Table 1 shows shoot length and seedling weight of rice ‘IR64’ under salt stress.

| Salinity Level | Shoot Length (cm) | Shoot Weight (g) |
|----------------|-------------------|------------------|
| Control        | 24.85             | 0.162            |
| 4dSm⁻¹         | 24.17             | 0.146            |
| 6dSm⁻¹         | 23.36             | 0.132            |
| 12dSm⁻¹        | 21.40             | 0.104            |

Numbers are means from three replicates. Same letters following means in the same column are not significantly different at P=0.05.

Table 2: Chlorophyll content in rice ‘IR64’ under salt stress.

| Salinity Level | Chl a (mg g⁻¹) | Chl b (mg g⁻¹) | Total Chl (mg g⁻¹) | Chl a/b ratio |
|----------------|---------------|---------------|--------------------|--------------|
| Control        | 1.425         | 0.396         | 17.937             | 4.047        |
| 4dSm⁻¹         | 1.139         | 0.381         | 15.525             | 3.279        |
| 6dSm⁻¹         | 1.063         | 0.391         | 15.139             | 2.796        |
| 12dSm⁻¹        | 0.536         | 0.299         | 9.533              | 1.786        |

Chl=chlorophyll. Numbers are means from three replicates. Means followed by the same letters in the same column are not significantly different at P=0.05.
Table 3: MDA content in shoot of rice ‘IR64’ under salt stress.

| Salinity Level | MDA Content (µmol/g FW) |
|---------------|-------------------------|
| Control       | 30.66                   |
| 4dSm⁻¹        | 41.28*                  |
| 6dSm⁻¹        | 43.23*                  |
| 12dSm⁻¹       | 63.12*                  |

Numbers are means from three replicates. Means followed by the same letters in the same column are not significantly different at P=0.05.

cell metabolism and inhibits cell division (Wang et al., 2003). In saline condition, high salt leads to membrane disorganization and modification of metabolites activation of cell wall. As a result, deposition of materials occurs and limits the elasticity of cell wall and disrupts cell expansion (Ali et al., 2004). High salt changes the activity of enzymes and causes premature senescence (Reddy et al., 2017) leading to growth reduction.

Photosynthesis depends on chlorophyll content of leaf, therefore, low chlorophyll content leads to a reduction of photosynthesis (Amirjani, 2011). Chlorophyll content affected by NaCl is inversely proportional to concentration of sodium, therefore chlorophyll content can be used as index of salt tolerance (Lutts et al., 1996). Salt tolerant rice has higher chlorophyll content than salt sensitive rice (Lutts et al., 1996). In this study, a higher concentration of NaCl (12dSm⁻¹) decreased the ratio of chlorophyll a/b. The ratio of chlorophyll a/b has been used as an indicator of salt tolerance (Kura-Hotta et al., 1987) leading to growth reduction.

Malondialdehyde (MDA) is an indicator of oxidative stress. As can be seen in Table 3, the concentrations of MDA increased in shoot of seedlings treated with salt. Higher salt concentration induced higher level of oxidative stress as indicated by high MDA content. According to Dhanyalakshmi et al. (2013), low MDA levels contributed for salt tolerance in rice.

In this study, it was found that the expression of antioxidant gene MnSOD1 and Cu/ZnSOD1 in rice ‘IR64’ increased under salt stress (Fig 2a and b). Fig 2c shows control gene expression using Actin where there were no changes in Actin gene expression under salt stress. Fig 2d is a quantitative expression of MnSOD1 and Cu/ZnSOD1 as estimated using Image J software and expressed as relative intensity of RNA level.

Salt stress induces accumulation of reactive oxygen species (Xu et al., 2011). Certain genes will be induced if plant undergoes salinity stress, therefore plant can increase tolerance to salt stress. Manganese superoxide dismutase gene (MnSOD) and cytosolic copper/zinc superoxide dismutase (cCu/ZnSOD) are two genes among various antioxidant genes responded to abiotic stress. It has been demonstrated that MnSOD can be used as a candidate for improving salt tolerant in plants (Gill and Tuteja, 2010).

The expression of MnSOD1 and Cu/ZnSOD1 in ‘IR64’ seedling increased after salt treatments. The increase of expression of these two genes under salinity stress has also been reported in local rice of Indonesia, ‘Cempo Ireng’ seedling (Refli and Purwestri, 2016).

The expression of MnSOD1 increased highly at 4dSm⁻¹ salt stress, and moderately increased at 6dSm⁻¹, while the highest expression of Cu/ZnSOD1 was at 6dSm⁻¹. At severe salt stress, both genes had only slight increase in its expression as compared to control. This may indicate that at an extreme concentration of salt, there is only little oxidative defence. Transgenic plants over expressed SOD gene have an increase in their resistance to abiotic stress (Baek and Skinner, 2010).
CONCLUSION
Salinity induced the decrease of growth characteristics in rice ‘IR64’ seedling. The decrease included shoot length and weight and chlorophyll content. High salinity induced oxidative stress showed by high levels of MDA in shoot of salt treated seedlings. There were changes in antioxidant gene expression under salt stress, generally the expression of antioxidant genes increased as a response to salinity.

ACKNOWLEDGEMENT
This study was funded by Ministry of Research, Technology and Higher Education Republic of Indonesia. The authors thank I Wayan Tika for providing rice seeds.

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