BIOLOGIA PLANTARUM 61 (3): 540-550, 2017 DOI: 10.1007/s10535-017-0727-6

Functional characterization of the antioxidant enzymes in rice plants exposed to salinity stress

I.L. VIGHI[1]*, L.C. BENITEZ[1], M.N. AMARAL[1], G.P. MORAES[1], P.A. AULER[1], G.S. RODRIGUES[1], S. DEUNER[1], L.C. MAIA[2], E.J.B. BRAGA[1]

Department of Plant Physiology, Federal University of Pelotas, Pelotas, 96160-000, RS, Brazil[1]
Plant Breeding Center, Federal University of Pelotas, Pelotas, 96160-000, RS, Brazil[2]

Abstract

The objective of this study was to relate the activation of enzymatic antioxidant system to the production of reactive oxygen species induced by salt stress. Rice (*Oryza sativa* L.) genotypes BRS Bojuru and BRS Pampa, tolerant and sensitive to salinity, respectively, were subjected to 150 mM NaCl for 0, 6, 24, 48, and 72 h. A significant increase of superoxide anion and H_2O_2 and a decrease in malondialdehyde (MDA) content were observed in the tolerant genotype, whereas in the sensitive genotype, there was no change in superoxide anion content, reduced H_2O_2 content, and increased MDA content. The superoxide dismutase (SOD) activity increased significantly in both genotypes, and increases in amounts of transcript were observed for *OsSOD3Cu/Zn* and *OsSODA1-Mn* in the tolerant genotype and for *OsSOD4-Cu/Zn*, *OsSOD3-Cu/Zn*, *OsSODCc1-Cu/Zn*, *OsSOD-Fe,* and *OsSODA1-Mn* in the sensitive genotype. The activities of catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) were not significantly and consistently changed, but *OsCATA*, *OsAPX2* and *OsGR1* were induced in both genotypes. *OsCATB* transcription was increased in the tolerant genotype and *OsCATC* and *OsAPX3* in the sensitive genotype under salinity. It is concluded that *OsAPX*3, *OsGR2*, *OsGR3*, and *OsSOD3-Cu/Zn* genes are the most suitable to distinguish tolerant from sensitive genotypes under salt stress.

Additional key words: ascorbate peroxidase, catalase, gene expression, glutathione reductase, NaCl, *Oryza sativa*, ROS, superoxide dismutase.

Introduction

Crop plants are often exposed to environmental stresses which interfere in a direct way in their growth and development. Among the cultivated species, rice (*Oryza sativa* L.) is the world´s most important food crop, however, it is quite vulnerable to adverse conditions, such as salinity, temperature extremes, drought and nutrient deficiency, which, together or separately, have an impact on its yield (Gao *et al.* 2007, Morison *et al.* 2007, Witcombe *et al*. 2008).

In many areas of agricultural production, the use of low quality water for irrigation and the excessive fertilizer application are some of the main reasons for the increase of soil salinity. Although salt stress is related to drought due to the decrease in water availability in the soil-plant-atmosphere continuum, the excessive presence of ions is also harmful for many metabolic processes. Thus, plants subjected to salinity face two parallel and simultaneous stresses, water stress and ionic stress (Dajic 2006).

Plant responses on phenotypic plasticity to adverse factors as salinity are complex and involve the activation of a signaling pathway and signal transduction which result in an increase of metabolites, such as abscisic acid, and proteins involved in cell protection and repair mechanisms, like enzymes specialized on the removal of reactive oxygen species (Ahmad *et al.* 2008, Bhatnagar-Mathur *et al.* 2008, Jaleel *et al.* 2009).

The production of reactive oxygen species (ROS) is a process that occurs naturally and is associated to both, respiratory and photosynthetic metabolism, representing a normal metabolic status of plant cells. However, under

Submitted 9 May 2016, *last revision* 14 September 2016, *accepted* 13 October 2016.
Abbreviations: APX - ascorbate peroxidase; CAT -catalase; GR - glutathione reductase; MDA - malondialdehyde; ROS - reactive oxygen species; SOD - superoxide dismutase; TBA - thiobarbituric acid; TCA - trichloroacetic acid.
Acknowledgements: Research supported by the following Brazilian funding agencies: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS).
* Corresponding author; e-mail: isavighi@hotmail.com

stressful situations, processes such as the electron transport in mitochondria and chloroplasts, fatty acid oxidation and detoxification reactions are reduced. This reduction causes an excessive increase in the production of ROS, such as superoxide anion, hydrogen peroxide, hydroxyl radical, and singlet oxygen, which are highly reactive and damage lipids, proteins, and nucleic acids (Mittler *et al.* 2004, Ahmad *et al.* 2010, Miller *et al.* 2010, Sharma *et al.* 2012).

Under these conditions, oxidative protection is dependent on the antioxidant systems, whose components are of enzymatic and non-enzymatic nature. The main antioxidant enzymes include superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.1.11.1), glutathione reductase (GR, EC 1.6.4.2), monodehydroascorbate reductase (MDHAR, EC 1.6.5.4), and dehydroascorbate reductase (DHAR, EC 1.8.5.1) (Ashraf 2009).

Antioxidant enzymes are encoded by multiple genes. For SOD enzyme, up to eight gene homologues have been identified so far in the rice genome, classified in accordance to the metal cofactor associated to the enzymes, *e.g*., copper/zinc (Cu/Zn-SOD), manganese (Mn-SOD), and iron (Fe-SOD), which are located in different cell compartments (Gill and Tuteja 2010). Catalase has a small multigene family, whose members in rice are called *OsCatA*, *OsCatB*, and *OsCatC* (Menezes-Benavente *et al.* 2004).

Ascorbate peroxidase is encoded by eight genes, including two cytosolic isoforms (encoded by *OsAPx1* and *OsAPx2*); two peroxisomal isoforms (encoded by *OsAPx3* and *OsAPx4*), and four chloroplastic isoforms (encoded by *OsAPx5*, *OsAPx6*, *OsAPx7*, and *OsAPx8*), and *OsAPx6* is also located in the mitochondria (Teixeira *et al.* 2004, 2006). The reductase has three genes: *OsGR2* (cytosolic), and *OsGR1* and *OsGR3* (chloroplastic isoforms) (Wu *et al.* 2013). According to Gill and Tuteja (2010), the activity of antioxidant enzymes in several plants results in their increased tolerance to abiotic stress due to their ROS scavenging capacity.

Thus, the objective of this study was to relate the production of ROS to the activation of the antioxidant enzyme system in rice plants induced by salt stress through the specific enzyme activity analysis of SOD, CAT, APX, and GR and the differential expression of genes encoding protein of each enzyme isoform.

Materials and methods

The experiment was conducted using two rice genotypes with contrasting response to salinity: BRS Pampa (subspecies *indica* - salt sensitive) and BRS Bojuru (subspecies *japonica* - salt tolerant). The seeds were germinated in paper rolls at a temperature of 25 ± 2 °C for 10 d in a *BOD*-type germination chamber (*Solab Cientifica*, Piracicaba, Brazil). Subsequently, the seedlings were transferred to a hydroponic floating type system in a greenhouse and kept at a temperature of 28 ± 2 °C, in trays containing 20 dm^3 of nutrient solution as described by Yoshida *et al.* (1976). We used three trays per genotype, each tray nursing 75 seedlings until achieving four fully expanded leaves (vegetative stage V_4). Following this stage, the Yoshida solution was modified according to Singh *et al*. (2010). NaH_2PO_4 was replaced by KH_2PO_4 and K_2HPO_4. Thus, the final NaCl concentration was 150 mM, and seedlings were exposed to it for periods of 0, 6, 24, 48, and 72 h. The experiment was laid in a completely randomized design (CRD) in a 2 × 5 factorial (2 rice genotypes × 5 stress periods), with three replicates per treatment, each replicate consisting of a bulk of 15 plants.

The content of superoxide radical ($O_2^{\bullet-}$) was determined according to Li *et al.* (2010). Leaf tissue (2 g) was macerated in 1.8 cm^3 of 65 mM phosphate buffer (pH 7.8) and centrifuged at 5 000 *g* for 10 min. Subsequently, 1.5 cm^3 of 130 mM phosphate buffer (pH 7.8) was added to the supernatant, and 50 mm^3 of 10 mM hydroxylamine hydrochloride for further incubation at 25 °C for 20 min. Then, 100 mm^3 of 17 mM sulfanilamide and 100 mm^3 of 7 mM α-napthylamine were added to the mixture. After 20 min at 25 °C, the solution absorbance was measured at 530 nm. A standard curve with nitrogen dioxide radical ($NO_2^{\bullet}$) was used to calculate the generation rate of $O_2^{\bullet-}$.

The content of H_2O_2 was determined according to Velikova (2000). Leaf samples (2 g) were macerated and homogenized in 2.0 cm^3 of trichloroacetic acid (TCA) (0.1 %, m/v) and centrifuged at 12 000 *g* for 15 min. After centrifugation, 0.8 cm^3 of 10 mM potassium phosphate buffer (pH 7.0) and 1.0 cm^3 of 1 M potassium iodide were added to the supernatant, spectrophotometer readings were performed at 390 nm, and H_2O_2 content was calculated by comparing the readings with a standard curve derived from different H_2O_2 concentrations.

Lipid peroxidation was determined by estimating the content of malondialdehyde (MDA) as described by Heath and Packer (1968). Approximately 2 g of fresh leaves were macerated in 2.0 cm^3 of 0.1% TCA and centrifuged at 12 000 *g* for 15 min. The supernatant was mixed with thiobarbituric acid (TBA; 0.5 %, m/v) plus 10 % TCA and heated in a water bath at 95 °C for 30 min. The reaction was stopped by rapid cooling in an ice bath for 10 min. Absorbance was determined at 535 and 600 nm. The concentration of the MDA-TBA complex was calculated by the equation: [MDA] = $(A_{535} - A_{600})/\xi$ where ξ (coefficient of absorbance) = 1.56 mM^{-1} cm^{-1}).

The enzyme extract was prepared by macerating 200 mg of leaf tissue in liquid N_2 and homogenized in 2.0 cm^3 of extraction buffer composed of 100 mM potassium phosphate (pH 7.0), 0.1 mM EDTA, and 10 mM ascorbic acid. The homogenate was centrifuged at 13 000 *g* and 4 °C for 15 min, and the resulting supernatant was used to

determine the activities of SOD, CAT, APX, and GR.

SOD activity was assessed according to its ability to inhibit the photoreduction of nitroblue tetrazolium (NBT) (Giannopolitis and Ries 1977) in a reaction medium containing 100 mM potassium phosphate (pH 7.8), 14 mM methionine, 0.1 µM EDTA, 75 µM NBT, 2 µM riboflavin, enzyme extract, and water to complete the final reaction volume to 2 cm^3. Readings were recorded at 560 nm. One unit of SOD equals the amount of enzyme capable of inhibiting NBT photoreduction by 50 %.

CAT activity determination, based on consumption of H_2O_2, was performed as described by Azevedo *et al.* (1998). The reaction medium was composed of 100 mM potassium phosphate buffer (pH 7.0), 12.5 mM H_2O_2, enzyme extract and water to complete the final reaction volume to 2 cm^3. The activity was monitored by the decrease in absorbance at 240 nm for 2 min (ξ = 39.4 mM^{-1} cm) and expressed as µmol(H_2O_2) mg^{-1}(protein) min^{-1}.

APX activity was determined according to Nakano and Asada (1981), considering the ascorbate oxidation rate at 290 nm (ξ = 2.8 mM cm^{-1}). The reaction medium, incubated at 28 °C, consisted of 100 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM H_2O_2, enzyme extract, and water to complete the final reaction volume to 2 cm^3. The activity was monitored by the decrease in absorbance at 290 nm for 2 min and expressed as µmol(ASC) mg^{-1}(protein) min^{-1}.

GR activity was calculated according to Cakmak *et al.* (1993), by decreasing absorbance at 340 nm due to NADPH oxidation (ξ = 6.2 mM cm^{-1}). The reaction consisted of 50 mM phosphate buffer (pH 7.8), 1 mM oxidized glutathione (GSSG), 75 uM NADPH, an aliquot of enzyme extract, and water to complete the final reaction volume to 2 cm^3. The values were expressed as µmol(NADPH) mg^{-1}(protein) min^{-1}.

All enzymatic activities were expressed on the basis of soluble protein, whose content was estimated according to Bradford (1976), using bovine serum albumin as standard and absorbances were determined using a spectrophotometer *Ultrospec™ 7000* (*GE Healthcare*, Little Chalfont, UK).

Data were submitted to analysis of variance ($P \leq 0.05$) in order to test the sources of variation and their possible interactions. Means were compared by Tukey test at 5 % probability, using *SAS 9.3* software package (*SAS Institute*, Cary, NC, USA).

Total RNA was extracted from 0.1 mg of leaf tissue using the *PureLink®* kit (*Invitrogen™*, Carlsbad, USA). To degrade DNA contaminants, samples were treated with *DNAse I* (*Invitrogen™*). The quality, quantity, and purity of total RNA was tested through 1 % agarose gel electrophoresis and the *NanoVue* (*GE Healthcare*) spectrophotometer. Using reverse transcription cDNA was obtained for each sample, using the commercial kit *SuperScript First-Strand* system for reverse transcription (RT)-PCR (*Invitrogen™*).

Primers were designed in the transcript region of rice gene isoforms SOD (*OsSOD4-Cu/Zn*, *OsSOD3-Cu/Zn*, *OsSOD2-Cu/Zn*, *OsSODCc1-Cu/Zn*, *OsSOD-Cu/Zn*, *OsSODB-Fe* , *OsSOD-Fe* and *OsSODA1-Mn*), CAT (*OsCATA*, *OsCATB* and *OsCATC*), APX (*OsAPX1*, *OsAPX2*, *OsAPX3*, *OsAPX4*, *OsAPX5*, *OsAPX6*, *OsAPX7* and *OsAPx8*) and GR (*OsGR1*, *OsGR2* and *OsGR3*) using the sequences available through the *RAP-DB* database and *PerlPrimer v.1.1.21*.

The stability of expression of *UBQ10* (AK101547) gene was evaluated, by our research group, as reference gene in rice leaves that were subjected to salt stress (150 mM) (Moraes *et al*. 2015) and was used as an internal normalizer for RT-qPCR reactions in this work. Only primers that had dissociation curves with a single peak and amplification efficiency close to 100 % were used in this study. Primers are listed in Table 1 Suppl.

The RT-qPCR reaction volume was 12 mm^3, of which 6.25 mm^3 of *FastStart Universal Master SYBR Green* (*Roche™*, Basel, Switzerland) fluorophore, 0.25 mm^3 (10 mM) of each primer (sense and antisense), 1 mm^3 of cDNA (1:5 dilution, previously defined), and 4.25 mm^3 of ultra-pure water. The reactions were conducted in a thermal cycler *Bio-Rad CFX* (Hercules, USA) using the following amplification parameters: 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 60 °C for 1 min with insertion of curve melt at 65 - 95 °C, with an increment of 5 °C for each fluorescence measurement. The relative quantification of the expression for each gene was obtained as described by Livak and Schmittgen (2001).

The principal component analysis was based on the average of data from 29 variables, including expression of genes encoding for isoforms and activities of antioxidant enzymes, superoxide anion, hydrogen peroxide, and lipid peroxidation. Based on the principal components, a biplot graph was designed with the first and second principal components for each genotype. Data obtained for both genotypes were used for the selection of the principal components that contributed to the variability between them. Statistical analyses were performed using the *SAS 9.3* software package.

Results

Results from the analysis of variance showed that the content of superoxide anion ($O_2^{\bullet-}$), H_2O_2, and MDA was significantly influenced by the interaction between rice genotype and exposure time. In BRS Bojuru, a gradual increase in $O_2^{\bullet-}$ content was observed during the stress exposure, reaching its maximum at 72 h. However, for the BRS Pampa , $O_2^{\bullet-}$ content did not differ significantly across the different stress periods (Fig. 1*A*). In BRS Bojuru, H_2O_2 content also increased up to the stress period of 72 h, however, BRS Pampa showed the

maximum H_2O_2 content in the control treatment, differing only in the 24-h stress period (Fig. 1*B*). In BRS Bojuru, lipid peroxidation decreased over the days of exposure to salt stress when compared to the control treatment. However, BRS Pampa showed a different response pattern, significantly increasing lipid peroxidation during the stress period (Fig. 1*C*).

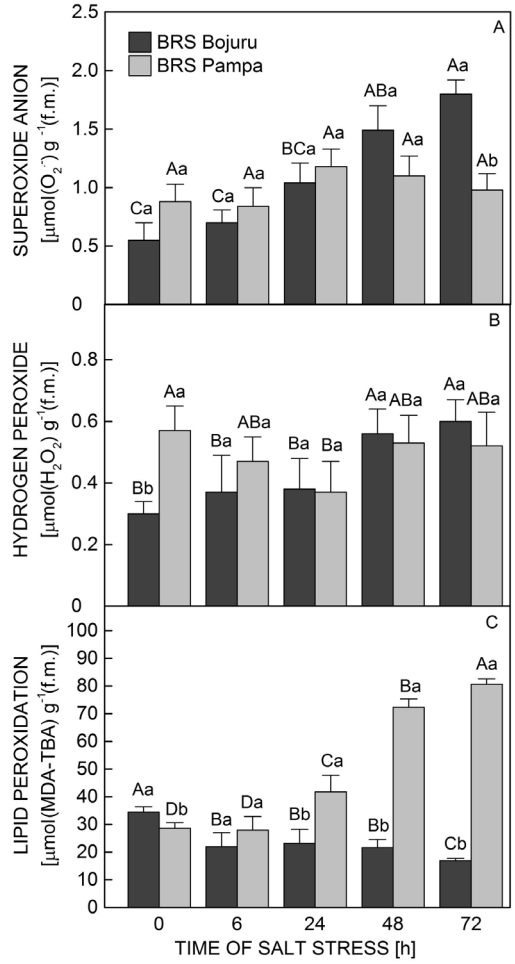

Fig. 1. Superoxide anion (*A*), hydrogen peroxide (*B*), and lipid peroxidation (C) in two rice genotypes (BRS Bojuru and BRS Pampa) as affected by salt stress (150 mM NaCl). Means ± SDs, *n* = 3. Different capital letters mark statistically significant differences among stress periods and different lowercase letters between genotypes (Tukey test at 0.05 level).

The results of analysis of variance indicated that the activities of SOD, CAT, and APX were affected by the interaction between genotype and stress exposure, while no significant effect on any of these variation sources was observed for the GR activity. The SOD activity in BRS Bojuru increased throughout the stress exposure periods compared to the control treatment, with the highest value recorded after 24 h. In BRS Pampa, the observed response trend was equal to that of the tolerant genotype, with the highest activity also after 24 h of stress (Fig. 2*A*).

No significant differences in CAT activities were observed for both rice genotypes at any stress period, but differences between genotypes were recorded for stress periods of 6 and 48 h, with the higher activities in BRS Pampa (Fig. 2*B*).

Similarly, no significant differences were observed in the APX activities in BRS Bojuru for all stress-exposure periods. In contrast, BRS Pampa showed an increase for all stress periods when compared to the control, although a significant difference was only observed for the 48-h stress period. When comparing both genotypes, it was noted that the sensitive genotype showed higher values of APX activity than the tolerant genotype (Fig. 2*C*).

Unlike the other enzymes studied in this work, no significant effects were determined for any of the factors on the activity of GR (Fig. 2D).

The analysis of the expression of different genes encoding for SOD, CAT, APX, and GR showed that they respond differently to salinity, according to the genotype and the time of exposure to stress. In the tolerant genotype BRS Bojuru, the highest values for the expression of the gene family encoding for SOD were observed at the 72-h stress period for all of the eight genes tested. Gene homologues *OsSOD3Cu/Zn* [relative quantification (RQ) = 27.16], *OsSOD-Cu/Zn* (RQ = 8.39), and *OsSODA1-Mn* (RQ = 9.51) showed the highest response, while the other genes exhibited minor changes in their expression under salinity (Fig. 3). In the sensitive genotype, increases in gene expression for all stress periods were observed for *OsSOD4-Cu/Zn*, *OsSOD3-Cu/Zn*, *OsSOD-Fe*, and *OsSODA1-Mn*. The highest expression exhibited *OsSODA1-Mn* (RQ = 8.67), whereas the expression of *OsSOD2-Cu/Zn* decreased in all stress levels compared to control treatment (Fig. 3).

The genes *OsCATA*, *OsCATB*, and *OsCATC* in BRS Bojuru differed in their responses. The *OsCATA* was the gene most responsible to salt stress, increasing its expression at stress periods 6 and 24 h (RQ = 6.65 and 3.03, respectively), followed by reduction in the subsequent times. For gene *OsCATC*, the observed values were lower than those of the control treatment, except for the 48-h stress period, suggesting that this gene had the lowest impact on responses to salt stress in the salinity-tolerant genotype (Fig. 4). Similarly, BRS Pampa showed the highest expression for gene *OsCATA* at 6 and 24 h (RQ = 15.7 and 17.23, respectively). Moreover, *OsCATC* had higher values than that of the control treatment through all stress exposure periods, differing from the tolerant genotype, while *OsCATB* was modulated in a similar fashion in both genotypes (Fig. 4).

Among the eight genes encoding for APX, *OsAPX1* and *OsAPx8* maintained expressions similar under stress and in the control treatment in both genotypes, whereas *OsAPX2* gene was the most induced by salinity, reaching peak expression at 24 h of stress period (QR = 22.44 for BRS Bojuru and QR = 26.29 for BRS Pampa). The largest contrasting response between genotypes was observed for the gene encoding for the isoform *OsAPX3*. It was slightly induced in the tolerant genotype and

positively modulated in the sensitive genotype with QR = 12.77 after 48 h of exposure, which makes it a possible molecular marker for the different response to salinity between contrasting genotypes (Fig. 5).

The tolerant genotype showed increased expression of three genes encoding different GRs in all stress periods, when compared to the control treatment. In *OsGR1*, the expression reached QR = 15.11 at 24 h of stress and maintained this pattern during the following stress periods. The *OsGR2* and *OsGR3* were significantly induced by salt stress, both peaking their expression after 48 h of stress (RQ = 87.58 and 66.22, respectively). In the sensitive genotype, an increase in the expression of gene *OsGR1* was observed in all stress treatments, with the highest value recorded after 24 h (RQ = 2.97), while the expressions of genes *OsGR2* and *OsGR3* decreased under salinity stress, indicating a contrasting response between genotypes (Fig. 6).

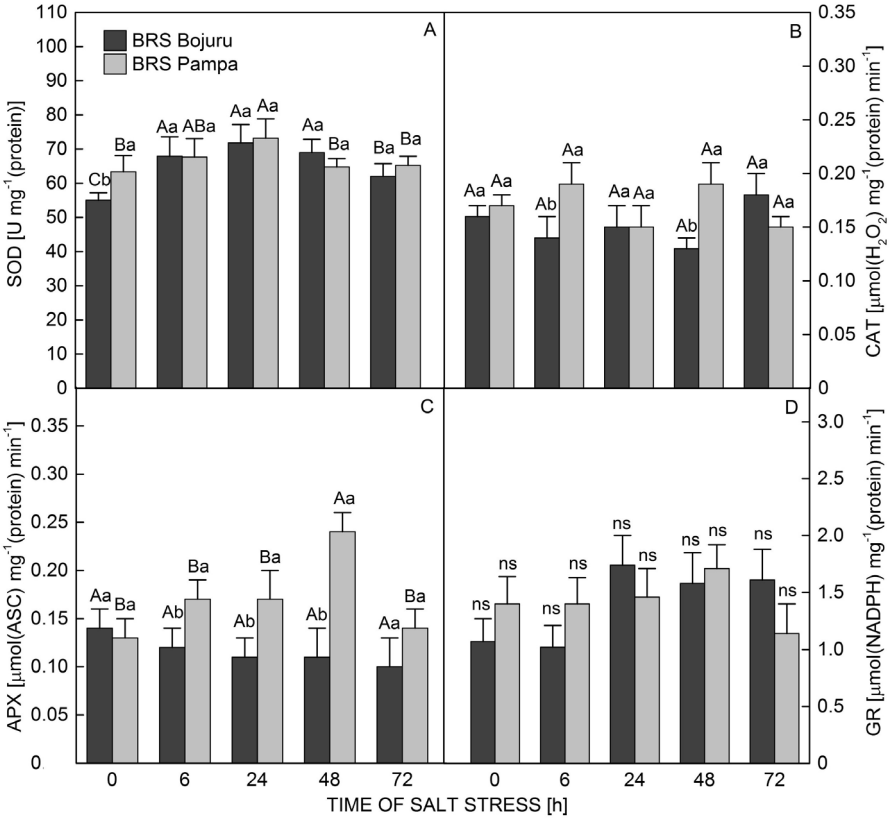

Fig. 2. Activities of antioxidant enzymes SOD (*A*), CAT (*B*), APX (*C*), and GR (*D*) in two rice genotypes (BRS Bojuru and BRS Pampa) as affected by salt stress (150 mM NaCl). Means ± SDs, n = 3. Different capital letters mark statistically significant differences among stress periods and different lowercase letters between genotypes (Tukey test at 0.05 level; ns - not significant).

The principal component analysis (PCA), based on the mean of data from the 29 variables studied in this work, provides the eigenvalues (variance) of each principal component, the percentage of variance, and the cumulative values. A similar procedure was used for both genotypes, choosing the first two principal components that explained 89 and 80 % of the variation for BRS Bojuru and BRS Pampa, respectively, when subjected to salt stress (Table 2 Suppl.).

The traits with greater weight in the principal component 1 for BRS Bojuru were *OsCATA* (-0.21), *OsCATB* (-0.23), *OsSOD3-Cu/Zn* (0.22), and the content of superoxide anion (0.23), while for BRS Pampa the most relevant traits were *OsCATA* (0.27), *OsGR1* (0.20), and *OsSODA1-Mn* (0.24).

Principal component 2 in BRS Bojuru showed the expression of genes *OsAPX2* (0.23), *OsGR2* (0.30), and enzyme activity of GR (0.24) as the variables with the greatest weight on stress response, while in BRS Pampa these variables corresponded to the expression of genes *OsAPX2* (0.26), *OsCATC* (0.21), *OsAPX5* (-0.28), *OsSODCc1-Cu/Zn* (0.27), and hydrogen peroxide content (-0.23).

In the biplot graph, in which major components 1 and 2 were plotted, quadrant I contains, for BRS Bojuru, the variables $O_2^{\bullet-}$, gene *OsCATC OsAPX2*, *OsGR2* expressions and GR activity, whose eigenvectors ranged from 0.23 to 0.30. In quadrant IV, we observed the expression of genes encoding for all isoforms of SOD as well as CAT activity (Fig. 1 Suppl.). As for BRS Pampa, the expression of genes encoding isoforms of SOD and APX were distributed throughout all four quadrants, highlighting the difference in response between both genotypes (Fig. 1 Suppl.). Data obtained for both

genotypes were used simultaneously for the selection of the principal components that contributed the most to the variability between them. The traits with greater weight (eigenvectors) in the principal component 1 were the expression of genes *OsAPX3* (-0.24), *OsGR2* (0.25), *OsGR3* (0.26), and *OsSOD3-Cu/Zn* (0.21). Hydrogen peroxide (0.28) was considered to be one of the most relevant factors contributing to the differences between genotypes. Variables with lower impact on differences between both rice genotypes were *OsAPX4* (0.07), *OsAPx8* (-0.05), and *OsAPX2* (-0.05), which turns them of low importance to distinguish between tolerant and sensitive genotypes under the experimental conditions in this work.

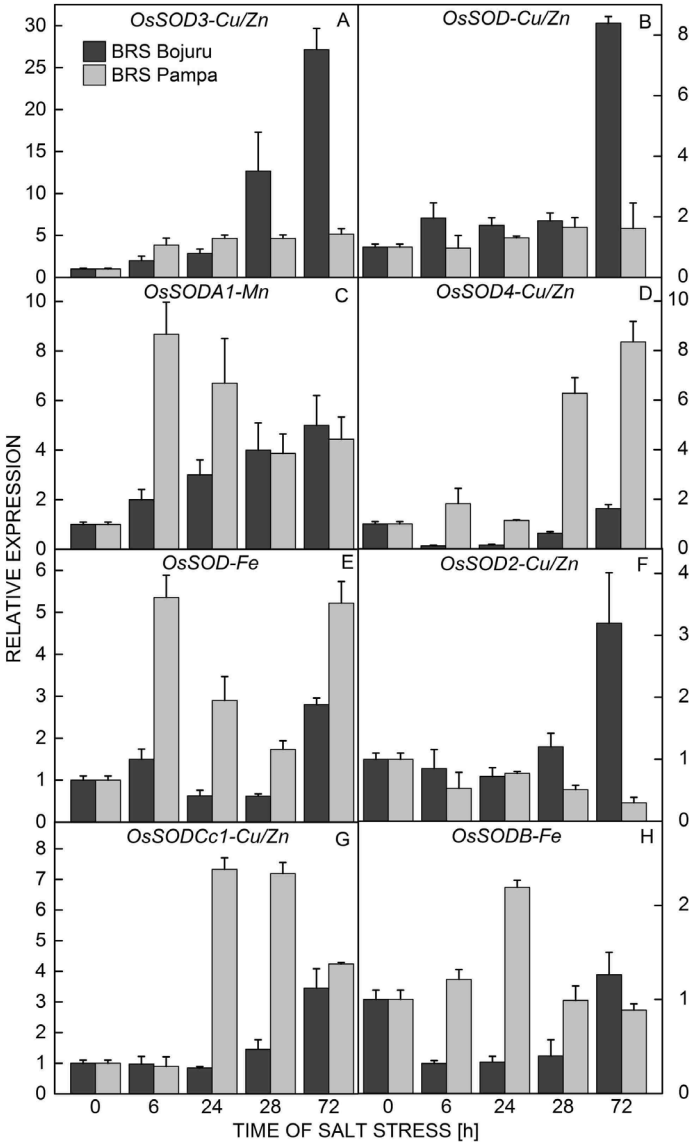

Fig. 3. Relative expression of superoxide dismutase genes in rice genotypes BRS Bojuru and BRS Pampa under salt stress (150 mM NaCl). Means ± SDs, $n = 3$.

Discussion

The increase in production of ROS is a metabolic alteration indicative of plants subjected to biotic or abiotic stresses (Foyer and Noctor, 2005, Asada 2006). Superoxide radical is the first to be produced by adding one electron to the molecular oxygen. However, superoxide cannot traverse biological membranes and is readily dismuted to H_2O_2 by the superoxide dismutase. Subsequently, H_2O_2 can be eliminated by catalase, preferably in peroxisomes, as well as by ascorbate peroxidase, acting in different cell components, through ascorbate-glutathione cycle (Aydin *et al.* 2013).

The results of this study showed different responses in

the production of ROS between both rice genotypes. Increases in the salt stress exposure time caused significant increases of $O_2^{\bullet -}$ and H_2O_2 content in leaves of the tolerant genotype, even though a decrease in MDA content was observed. The H_2O_2 increase can be explained partly by the increased activity of SOD; however, the decrease in MDA content cannot be attributed to the CAT, APX, and GR activities, since they were not statistically different throughout all salt stress periods (Fig. 2*A*,*B*,*C*). Thus, it is believed that H_2O_2 produced during salt stress was not in a direct relation to lipid peroxidation. It is suggested that H_2O_2 could be directed to other processes, such as stomata closure, peroxisome biogenesis, formation of transverse connections in the cell wall, and inactivation of the enzymes of Calvin Cycle (Neill *et al.* 2002, Foyer and Noctor 2003).

A sensitive to salt stress cv.BRS Pampa did not show changes in $O_2^{\bullet -}$ content, but there was a reduction in H_2O_2 content and an increase in MDA content with increasing duration of the stress treatments. In this case, a decreased H_2O_2 content could not be explained by changes in CAT, APX, and GR activities, because their increase was not statistically significant except for APX in the 48-h treatment. According to Courtney *et al*. (2016), the peroxidase (POD) is also capable of the elimination of H_2O_2. As concerns the increased lipid peroxidation, it might be caused by toxic molecules other than H_2O_2, such as hydroperoxides (HO_2^-), which are formed from $O_2^{\bullet -}$ and are capable of traversing membranes and take away hydrogen atoms from polyunsaturated fatty acids and lipid hydroperoxides, thus starting lipid self-oxidation (Neill *et al.* 2002). Similar results were found in rice plants subjected to stresses, where salt stress induced an increase in MDA content in the salt-sensitive genotype (Demiral and Türkan 2005).

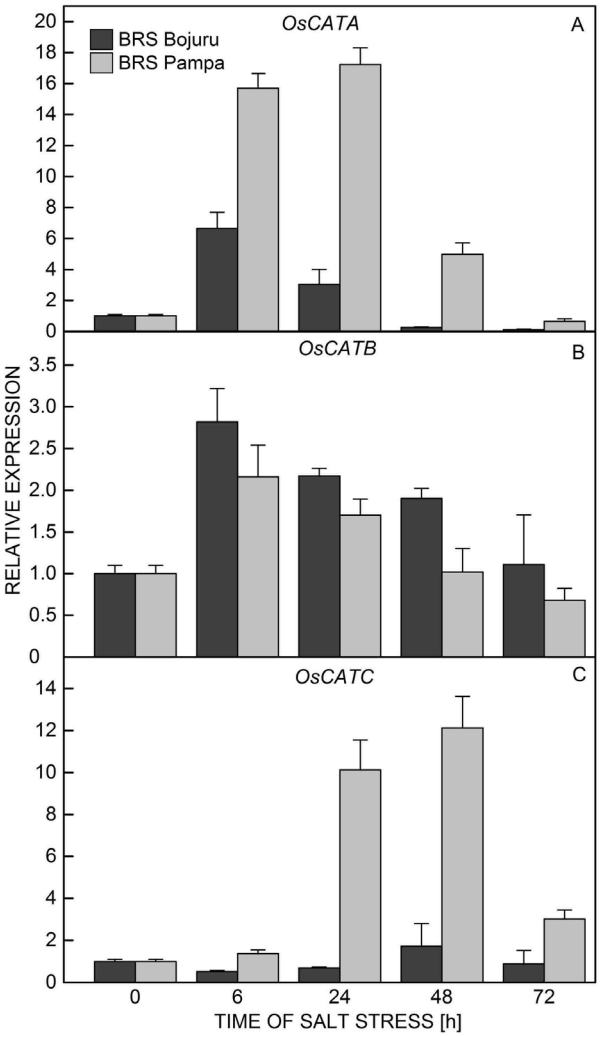

Fig. 4. Relative expression of catalase genes in rice genotypes BRS Bojuru and BRS Pampa under salt stress (150 mM NaCl). Means ± SDs, $n = 3$.

In this work, both rice genotypes showed increased activity of SOD (Fig. 2*A*), which was also observed by Turan and Tripathy *et al.* (2013) and Khare *et al.* (2014) in rice plants under salt stress. Among the genes encoding SOD, the most significant increases were observed for homologues *OsSOD3Cu/Zn* and *OsSODA1-Mn* in the tolerant genotype, suggesting that most of SOD activity was due to these genes (Fig. 3). We observed increases in the expression of genes *OsSOD4-Cu/Zn*, *OsSOD3-Cu/Zn*, *OsSODCc1-Cu/Zn*, *OsSOD-Fe* and *OsSODA1-Mn* in the sensitive genotype, indicating that chloroplasts and the cytosol may be important locations for superoxide cleaning, since the products of these genes act in these organelles (Fig. 3). Kaminaka *et al*. (1999) observed the differential gene expressions of rice SOD isoforms under environmental stresses. The expressions of abscisic acid (ABA)-inducible genes, *Mn-SOD* gene and one of the cytosolic *Cu/Zn-SOD* genes, were strongly induced by drought and salinity. The *Fe-SOD* gene and the cytosolic Cu/Zn-SOD gene (*sodCc1*) were also induced by ABA.

The CAT activity did not differ among treatments in both genotypes; however, the highest activity was observed after 72 h of salt stress in the tolerant genotype and after 48 h in the sensitive genotype. Similarly, Simova-Stoilova *et al.* (2010) reported increased CAT activity in wheat cultivars sensitive to drought stress. Regarding genes encoding catalase isoforms, *OsCATA* gene was the most responsive in both genotypes, whereas gene *OsCATC* was modulated by salt stress only in the sensitive genotype. Khare *et al.* (2014) reported an increase in *OsCATA* expression in rice plants tolerant to salinity and a decrease in the sensitive ones. The results obtained by Yamane *et al.* (2010) suggest that the increase in CAT enzyme activity in rice leaves of plants tolerant to salt stress is related to the expression of the *OsCATB* and *OsCATC* genes. However, Menezes-Benavente *et al.* (2004) report that salt stress in rice plants does not alter the expression of *OsCATA* and *OsCATC* genes, however, increases the expression of *OsCAT*, indicating that a single gene can be induced or repressed depending on the genotype. According to Lee *et al.* (2002), the interaction of *cis*-elements, presented in the promoter region of each genotype, leads to the

activation or repression of gene expression. Thus, the presence and/or absence of specific *cis*-elements within a genotype may modulatee in a positive or negative way the expression of these genes in response to salt stress.

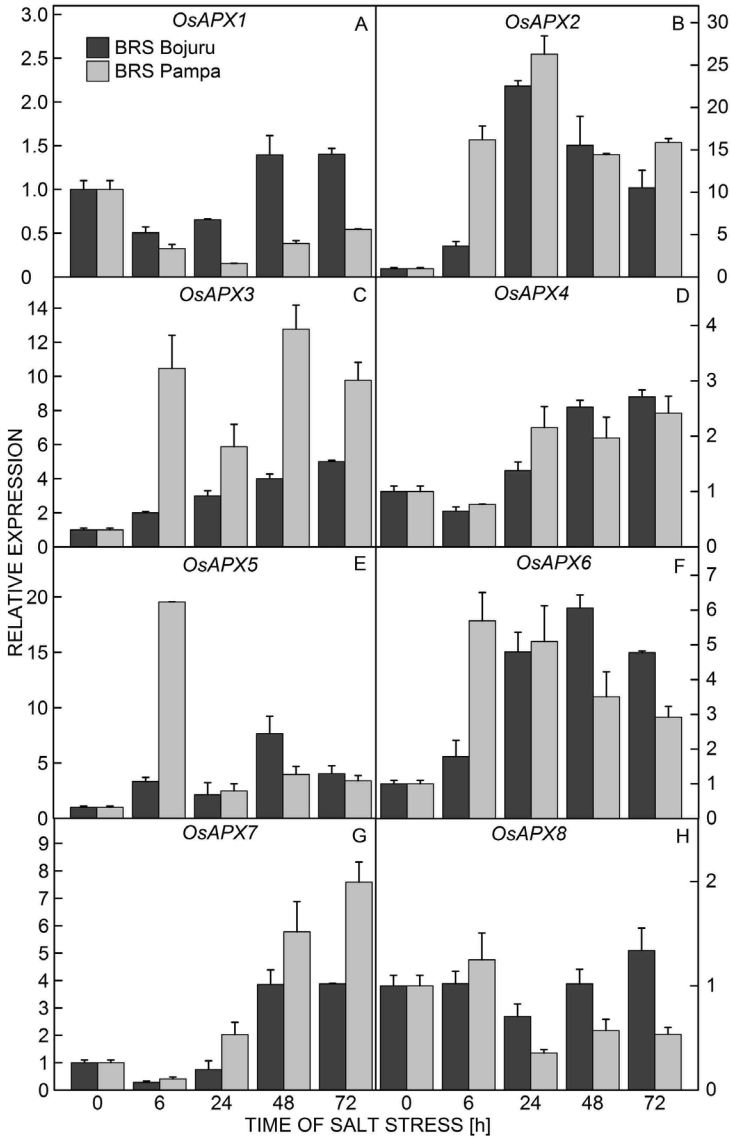

Fig. 5. Relative expression of ascorbate peroxidase genes in rice genotypes BRS Bojuru and BRS Pampa as affected by salt stress (150 mM NaCl). Means ± SDs, $n = 3$.

The first enzyme of the ascorbate-glutathione cycle, APX, plays a vital role in the elimination of H_2O_2. APX has higher affinity to H_2O_2 when compared to CAT, being capable of eliminating it even at low concentrations (Gill and Tuteja 2010). Research on the activity by APX in soybean and rice salt-tolerant genotypes (Moradi and Ismail 2007, Hakeem *et al.* 2012, Mishra *et al.* 2013), show increased activity of this enzyme when compared to susceptible genotypes. However, in our study, the sensitive genotype showed the highest activity of APX, although it differed only at 48 h compared to the other stress periods (Fig. 2*C*). The results of this study are in agreement with Turan and Tripathy (2013) and Khare *et al.* (2014), who reported an increase in APX activity in rice plants sensitive to salinity. The analysis of the expression profile of APX genes showed that the expression of *OsAPX2* was the most responsive to salt stress in both genotypes (Fig. 5). However, the increased expression had no effect on the enzyme activity, especially in the tolerant genotype, suggesting that APX inhibition might occur during the stress periods. One reason for this inhibition may be irreversible inactivation of APX in the absence of reduced ascorbate (Shikanai *et al.* 1998, Asada 1999). Morita *et al.* (2011) reported correlation between the increases in the expression of genes *OsAPX2* and *OsAPX1* and the increase of APX activity in rice plants subjected to drought stress. Turan and Tripathy (2013), studying the response of contrasting

rice genotypes to salt stress, have found that an increase in the expression of gene *OsAPX2* is correlated with the enzyme activity.

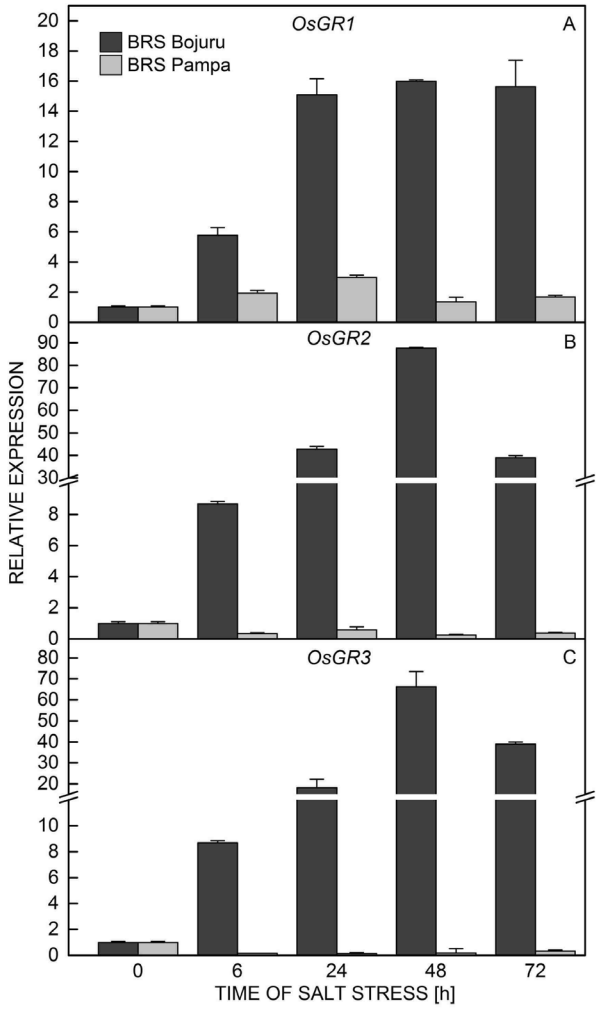

Fig. 6. Relative expression of glutathione reductase genes in rice genotypes BRS Bojuru and BRS Pampa as affected by salt stress (150 mM NaCl). Means ± SD, $n = 3$.

The increase in the GR activity under different stresses conditions was observed in many plant species, *e.g.*, rice, wheat, peanut, maize, and tobacco (Guo *et al.* 2006, Tang *et al.* 2010a,b, Tan *et al.* 2011). However, in this study there was no difference in the GR enzyme activity in any of the rice genotypes (Fig. 2*D*). However, the three genes encoding GR isoforms showed an increase in the expression patterns in the tolerant genotype, *OsGR2* and *OsGR3* being the most induced under salt stress (Fig. 6). The lack of observed correlation between enzyme activity and gene expression can be explained by post-transcriptional and post-translational modifications and existing protein-protein interactions, which result in the non-functional enzyme. Moreover, Hong *et al.* (2009), working with rice roots reported that the increased expressions of genes *OsGR2* and *OsGR3* is associated with increased GR activity, and that this increase is significantly induced by salinity and mediated by H_2O_2. In this work, H_2O_2 content increased in the tolerant genotype, indicating that it may act as an inducer of the expression of the three GR gene homologues. In rice cv. BRS Pampa, increase in gene expression was observed only for *OsGR1*, whereas *OsGR2* and *OsGR3* decreased their expression in the presence of salt stress (Fig. 6). Turan and Tripathy *et al.* (2013), working with rice plants under salt stress, reported increased expression of the gene *OsGR2* in both tolerant and sensitive genotypes.

The PCA allows the identification of compounds exhibiting the greatest variation within a population and determines the closely related compounds (Kim *et al.* 2010). Furthermore, in possession of the PCA results, elimination of redundant and difficult-to-measure characteristics is possible, thus reducing the time and cost of experimentation (Paiva *et al.* 2010). This study allowed us the observation that genes *OsAPX3*, *OsGR2*, *OsGR3*, and *OsSOD3-Cu/Zn* contributed the most to the variability between genotypes, indicating their potential for use as references to differentiate between tolerant and sensitive genotypes under salt stress conditions (Fig. 1 Suppl.).

Conclusions

The presented results from this work allow to conclude that there are differences in the response to salt stress between tolerant and sensitive rice genotypes, especially in the production of ROS and lipid peroxidation. As concerns antioxidative enzymes, only SOD activity significantly increased under salt stress in both genotypes. The expressions of genes *OsSOD3Cu/Zn*, *OsSODA1-Mn*, *OsCATA*, *OsCATB*, and *OsAPX2* in tolerant genotype and *OsSOD4-Cu/Zn*, *OsSOD3-Cu/Zn*, *OsSOD-Fe*, *OsSODCc1-Cu/Zn*, *OsCATA*, *OsCATC*, *OsAPX2*, and *OsAPX3* in the sensitive genotype were positively regulated under salt stress, although the increase in the amount of transcripts did not result in a significant increase in the CAT and APX activities. It is suggested that *OsAPX*3, *OsGR2*, *OsGR3*, and *OsSOD3-Cu/Zn* genes are the most suitable to distinguish tolerant from sensitive genotypes under salt stress conditions.

References

Ahmad, P., Sarwat, M., Sharma, S.: Reactive oxygen species, antioxidants and signaling in plants. - J. Plant Biol. **51**: 167-173, 2008.

Ahmad, P., Jaleel, C.A., Salem, M.A., Nabi, G., Sharma, S.: Roles of enzymatic and nonenzymatic antioxidants in plants during abiotic stress. - Crit. Rev. Biotechnol. **30**: 161-175, 2010.

Asada, K.: The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. - Annu. Rev. Plant Physiol. Plant. mol. Biol. **50**: 601-639, 1999.

Asada, K.: Production and scavenging of reactive oxygen species in chloroplasts and their functions. - Plant Physiol. **141**: 391-396, 2006.

Ashraf, M.: Biotechnological approach of improving plant salt tolerance using antioxidants as markers. - Biotechnol. Adv. **27**: 84-93, 2009.

Azevedo, R.A., Alas, R.M., Smith R.J., Lea, P.J.: Response of antioxidant enzymes to transfer from elevated carbon dioxide to air and ozone fumigation, in the leaves and roots of wild-type and a catalase-deficient mutant of barley. - Physiol. Plant. **104**: 280-29, 1998.

Aydin, S.S., Büyük, I., Aras, S.: Relationships among lipid peroxidation, SOD enzyme activity, and SOD gene expression profile in *Lycopersicum esculentum* L. exposed to cold stress. - Genet. mol. Res. **12**: 3220-3229, 2013.

Bhatnagar-Mathur, P., Valdez, V., Sharma K.K.: Transgenic approaches for abiotic stress tolerance in plants: retrospect and prospects. - Plant Cell Rep. **27**: 411- 424, 2008.

Bradford, M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Cakmak, I., Strbac, D., Marschner, H.: Activities of hydrogen peroxide-scavenging enzymes in germination wheat seeds. - J. exp. Bot. **44**: 127-132, 1993.

Courtney, A.J., Xu, J., Xu, Y.: Responses of growth, antioxidants and gene expression in smooth cordgrass (*Spartina alterniflora*) to various levels of salinity. - Plant Physiol. Biochem. **99**: 162-170, 2016.

Dajic Z.: Salt stress. In: Madhava, R.K.V., Raghavendra, A.S., Janardhan, R.K. (ed.): Physiology and Molecular Biology of Stress Tolerance in Plants. Vol. 1. Pp. 41-100. Springer, Dordrecht 2006.

Demiral, T, Turkan, I.: Comparative lipid peroxidation, antioxidant defense systems and proline content in roots of two rice cultivars differing in salt tolerance. - Environ. exp. Bot. **53**: 247-257, 2005.

Foyer, C.H., Noctor, G.: Redox sensing and signaling associated with reactive oxygen in chloroplast, peroxisomes and mitochondria. - Physiol. Plant. **119**: 355-364, 2003.

Foyer, C.H., Noctor, G.: Oxidant and antioxidant signaling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. - Plant Cell Environ. **28**: 1056-1071, 2005.

Gao, J.P., Chao, D.Y., Lin, H.X.: Understanding abiotic stress tolerance mechanisms: recent studies on stress response in rice. - J. integr. Plant Biol. **49**: 742-750, 2007.

Giannopolitis, C.N., Ries, S.K.: Superoxide dismutase. I. Occurrence in higher plants. - Plant Physiol. **59**: 309-314, 1977.

Gill, S.S., Tuteja, N.: Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. - Plant Physiol. Biochem. **48**: 909-930, 2010.

Guo, Z., Ou, W., Lu, S., Zhong, Q.: Differential responses of antioxidative system to chilling and drought in four rice cultivars differing in sensitivity. - Plant Physiol. Biochem. **44**: 828-836, 2006.

Hakeem, K.R., Khan, F., Chandna, R., Siddiqui, T.O., Iqbal, M.: Genotypic variability among soybean genotypes under NaCl stress and proteome analysis of salt-tolerant genotype. - Appl. Biochem. Biotechnol. **168**: 2309-2329, 2012.

Heath, R.L., Packer, L.: Photoperoxidation in isolated chloroplast. I. Kinetics and stoichiometry of fatty acid peroxidation. - Arch. Biochem. Biophys. **125**: 189-198, 1968.

Hong, C.Y., Chao, Y.Y., Yang, M.Y., Cheng, S.Y., Cho, S.C., Kao, C.H.: NaCl-induced expression of glutathione reductase in roots of rice (*Oryza sativa* L.) seedlings is mediated through hydrogen peroxide but not abscisic acid. - Plant Soil **320**: 103-115, 2009.

Jaleel, C.A., Riadh, K., Gopi, R., Manivannan, P., Inès, J., Al-Juburi, H.J., Zhao, C.-X., Shao, H.-B., Panneerselvam, R.: Antioxidant defense responses: physiological plasticity in higher plants under abiotic constraints. - Acta Physiol. Plant. **31**: 427-436, 2009.

Kaminaka, H., Morita, S., Tokumoto, M., Tanaka, K.: Differential gene expression of rice superoxide dismutase isoforms to oxidative and environmental stresses. - Free Radicals Res. **31**: 219-225, 1999.

Khare, T., Kumar, V., Kishor, K.P.B.: Na^+ and Cl^- ions show additive effects under NaCl stress on induction of oxidative stress and the responsive antioxidative defense in rice. - Protoplasma. **252**: 1149-65, 2014.

Kim, J.K., Lee, S.Y., Chu, S.M., Lim, S.H., Suh, S.-C., Lee, Y.T., Cho, H.S., Ha, S.H.: Variation and correlation analysis of flavonoids and carotenoids in Korean pigmented rice (*Oryza sativa* L.) cultivars. - J. Agr. Food Chem. **58**: 12804-12809, 2010.

Lee, T., Rinaldi, N.J., Robert, F., Odom, D.T., Bar-Joseph, Z., Gerber, G.K., Hannett, N.M., Harbison, C.T., Thompson, C.M., Simon, I.: Transcriptional regulatory networks in *Saccharomyces cerevisiae*. - Science **298**: 799-804, 2002.

Li, C., Bai, T., Maa, F., Hana, M.: Hypoxia tolerance and adaptation of anaerobic respiration to hypoxia stress in two *Malus* species. - Sci. Hort. **124**: 274-279, 2010.

Livak K.J., Schmittgen, T.D.: Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. - Methods **25**: 402-408, 2001.

Menezes-Benavente, L., Teixeira, F.K., Kamei C.L.A., Margis-Pinheiro, M.: Salt stress induces altered expression of genes encoding antioxidant enzymes in seedlings of a Brazilian indica rice (*Oryza sativa* L.). - Plant Sci. **166**: 323-331, 2004.

Miller, G., Suzuki, N., Ciftci-Yilmaz, S., Mittler, R.: Reactive oxygen species homeostasis and signalling during drought and salinity stresses. - Plant Cell Environ. **33**: 453-467, 2010.

Mishra, P., Bhoomika, K., Dubey, R.S.: Differential responses of antioxidative defense system to prolonged salinity stress in salt-tolerant and salt-sensitive indica rice (*Oryza sativa* L.) seedlings. - Protoplasma **250**: 3-19, 2013.

Mittler, R., Vanderauwera, S., Gollery, M., Van-Breusegem, F.: Reactive oxygen gene network of plants. - Trends Plant Sci. **9**: 490-498, 2004.

Moradi, F., Ismail, A.M.: Responses of photosynthesis, chlorophyll fluorescence and ROS-scavenging systems to salt stress during seedling and reproductive stages in rice. -

Ann. Bot. **99**: 1161-1173, 2007.

Moraes, G.P., Benitez, L.C., Amaral Do, M.N., Vighi, I.L., Auler, P.A., Maia Da, L.C., Bianchi, V.J., Braga, E.J.B.: Evaluation of reference genes for RT-qPCR studies in the leaves of rice seedlings under salt stress. - Genet. mol. Res. **14**: 2384-2398, 2015.

Morison, J.I.L., Baker, N.R., Mullineaux, P.M., Davies, W.J.: Improving water use in crop production. - Phil. Trans. roy. Soc. London B. Biol. Sci. **363**: 639-658, 2007.

Morita, S., Nakatani, S., Koshiba, T., Masumura, T., Ogihara, Y., Tanaka, K.: Differential expressions of two cytosolic ascorbate peroxidase and two superoxide dismutase genes in response to abiotic stress in rice. - Rice Sci. **18**: 157-166, 2011.

Nakano, Y., Asada, K.: Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. - Plant Cell Physiol. **22**: 867-880, 1981.

Neill, S.J., Desikan, R., Clarke, A., Hurst, R.D., Hancock, J.T.: Hydrogen peroxide and nitric oxide as signaling molecules in plants. - J. exp. Bot. **53**: 1237-1247, 2002.

Paiva, A.L.C., Teixeira, R.B., Yamaki, M., Menezes, G.R.O., Leite, C.D.S., Torres, R. A.: Principal component analysis in laying hen production traits. - Roy. Bras. Zootech. **39**: 285- 288, 2010.

Sharma, P., Jai, A.B., Dubey, R.S., Pessarakli, M.: Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. - J. Bot. **1**: 1-26, 2012.

Shikanai, T., Takeda, T., Yamauchi, H., Sano, S., Tomizawa, K., Yokota, A., Shigeoka, S.: Inhibition of ascorbate peroxidase under oxidative stress in tobacco having bacterial catalase in chloroplasts. - FEBS Lett. **428**: 47-51, 1998.

Simova-Stoilova, L., Vaseva, I., Grigorova, B., Demirevska, K., Feller, U.: Proteolytic activity and cysteine protease expression in wheat leaves under severe soil drought and recovery. - Plant Physiol. Biochem. **48**: 200-206, 2010.

Singh, R.K., Redoña, E., Refuerzo, L.: Varietal improvement for abiotic stress tolerance in crop plants: special reference to salinity in rice. - In: Pareek, A., Sopory, S.K., Bohnert, H., Govindjee (ed.): Abiotic Stress Adaptation in Plants: Physiological, Molecular and Genomics Foundation. Vol. 1. Pp. 387-415. Springer, New York 2010.

Tan, W., Brestic, M., Olsovska, K., Yang, X.: Photosynthesis is improved by exogenous calcium in heat-stressed tobacco plants. - J. Plant Physiol. **168**: 2063-2071, 2011.

Tang, B., Xu, S-Z., Zou, X-L., Zheng, Y-L., Qiu, F-Z.: Changes of antioxidative enzymes and lipid peroxidation in leaves and roots of waterlogging-tolerant and waterlogging-sensitive maize genotypes at seedling stage. - Agr. Sci. China **9**: 651-661, 2010a.

Tang, K., Zhan, J-C., Yang, H-R., HuanG, W-D.: Changes of resveratrol and antioxidant enzymes during UV-induced plant defense response in peanut seedlings. - J. mol. Evol. **167**: 95-102, 2010b.

Teixeira, F.K., Menezes-Benavente, L., Galvão, V.C., Margis, R., Margis-Pinheiro, M.: Rice ascorbate peroxidase gene family encodes functionally diverse isoforms localized in different subcellular compartments. - Planta **224**: 300-314, 2006.

Teixeira, F.K., Menezes-Benavente, L., Margis, R., Margis-pinheiro, M.: Analysis of the molecular evolutionary history of the ascorbate peroxidase gene family: inferences from the rice genome. - J. mol. Evol. **59**: 761-770, 2004.

Turan, S., Tripathy, B.C.: Salt and genotype impact on antioxidative enzymes and lipid peroxidation in two rice cultivars during de-etiolation. - Protoplasma **250**: 209-222, 2013.

Velikova, V., Yordanov, I., Edreva, A.: Oxidative stress and some antioxidant systems in acid rain-treated bean plants. - Plant Sci. **151**: 59-66, 2000.

Witcombe, J.R., Hollington, P.A., Howarth, C.J., Reader, S., Steele, K.A.: Breeding for abiotic stresses for sustainable agriculture. - Trans. roy. Soc. London B. Biol. Sci. **363**: 703-716, 2008.

Wu, T.M., Lin, W.R., Kao, Y.T., Hsu, Y.T., Yeh, C.H., Hong, C.Y., Kao, C.H.: Identification and characterization of a novel chloroplast/mitochondria co-localized glutathione reductase 3 involved in salt stress response in rice. - Plant mol. Biol. **83**: 379-390, 2013.

Yamane, K., Mitsuya, S., Taniguchi, M., Miyake, H.: Transcription profiles of genes encoding catalase and ascorbate peroxidase in the rice leaf tissues under salinity. - Plant Prod. Sci. **13**: 164-168, 2010.

Yoshida, S., Forno, D.A., Cock, J.H., Gomez, K.A. (ed.): Laboratory Manual for Physiological Studies of Rice. - International Rice Research Institute, Manila 1976.