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ORIGINAL PAPER

Oxidative stress, protein carbonylation, proteolysis and antioxidative defense system as a model for depicting water deficit tolerance in Indica rice seedlings

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Abstract Water deficit is an important constraint to rice (*Oryza sativa* L.) productivity. The present study was undertaken to investigate whether the level of oxidative stress, carbonylation of proteins, proteolysis and status of antioxidative defense could serve as a model to distinguish water deficit tolerant and sensitive rice cultivars. When 10-day-grown seedlings of two rice cultivars, Malviya-36 (drought-sensitive) and Brown Gora (drought-tolerant) were subjected to −1.0 and −2.1 MPa water deficit treatments for 24–72 h with polyethylene glycol 6000 in the medium, a greater decline in the growth of the seedlings and levels of leaf water potential, relative water content, Chl a, Chl b, carotenoids and greater increase in leaf water loss were observed in the sensitive cultivar than the tolerant. Under similar level of water deficit seedlings of sensitive cultivar showed higher level of superoxide anion generation, H_2O_2, lipid peroxidation and proteolysis in roots as well as shoots compared to the tolerant. Drought-tolerant cultivar had higher constitutive level of antioxidative enzymes superoxide dismutase and catalase and the activities of these two enzymes alongwith of guaiacol peroxidase showed greater increase in this cultivar under water deficit compared to the sensitive. A significant decline in the level of protein thiol and a higher increase in protein carbonyls content, also confirmed by protein gel blot analysis with an antibody against 2,4-dinitrophenylhydrazine was observed in the seedlings of drought sensitive *cv*. Malviya-36 compared to the tolerant *cv*. Brown Gora when subjected to similar level of water deficit. Seedlings of drought sensitive cultivar, under water deficit, showed higher proteolytic activity, higher number of in-gel activity stained proteolytic bands and higher expression of oxidized proteins in roots compared to the tolerant cultivar. Results suggest that poor capacity of antioxidative enzymes could be, at least partly, correlated with water deficit sensitivity of sensitive cultivar and that higher activity of antioxidative enzymes superoxide dismutase, catalase, guaiacol peroxidase, low proteolytic activity, lower level of protein carbonyls and protein thiolation could serve as a model to depict water deficit tolerance in Indica rice seedlings.

Keywords Oxidative stress · Protein carbonylation · Reactive oxygen species · Water deficit · Proteolysis · *Oryza sativa* L.

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Introduction

Rice (*Oryza sativa* L.) serves as an important food crop for the majority of world population. In arid and semiarid regions of the world, rice grown on arable soils with low moisture retention capacity is totally dependent for its growth on the availability of water. Drought or water deficit in the soil environment is one of the most important abiotic stresses that limit growth, development and productivity of the crops. Water deficit affects morphological, physiological, biochemical and molecular processes in plants resulting in growth inhibition, stomatal closure with consecutive reduction of transpiration, decrease in chlorophyll content, inhibition of photosynthesis and important protein modifications (Lawlor and Cornic 2002; Sikuku et al. 2010). Plants exposed to abiotic stresses often undergo oxidative damage due to overproduction of reactive oxygen species (ROS) such as superoxide radicals ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$), hydroxyl radicals ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), etc. (Sharma and Dubey 2005; Sharma et al. 2012). Increased concentrations

of lipid peroxidation end products as well as protein oxidation have been regarded as indicators of ROS derived oxidative damage in plants (Tuanhui et al. 2010). It has been shown that involvement of oxidative stress is an important component in expression of water deficit induced metabolic alterations in rice plants (Sharma and Dubey 2005).

In nature, plant systems have adapted protection from the cytotoxic effects of the ROS with antioxidants comprising of the antioxidative enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), peroxidase (POD, EC 1.11.1.9) as well as the non-enzymic antioxidants like glutathione, ascorbic acid, tocopherol, carotenoids, etc. (Sharma et al. 2012). These enzymic as well as non-enzymic antioxidants play crucial role in defense against overproduced ROS under abiotic stresses (Simova-Stoilova et al. 2008). The SOD scavenges $O_2^{\cdot-}$ to produce H_2O_2 and water. The enzyme POD reduces H_2O_2 to water using various substrates as electron donors, whereas CAT dismutates H_2O_2 to water and oxygen. The antioxidant protection system in plant cells is complex and highly compartmentalized: SODs represent a family of enzymes localized in organelles and in the cytosol, whereas CATs and PODs with broad specifities are located in vacuoles, cell walls, peroxisomes, mitochondria and cytosol (Simova-Stoilova et al. 2008; Gill and Tuteja 2010).

Formation of thiol components, glyoxidation adducts, nitration of tyrosine residues and carbonylation of specific amino acids can cause oxidative modification of proteins (Davies 2005). Protein thiolation involves the participation of sulfur containing amino acids which is a reversible process whereas most of the other protein oxidation processes are irreversible (Ghezzi and Bonetto 2003). Carbonylation is an irreversible oxidative process and can be determined by derivatization with 2,4-dinitrophenylhydrazine (DNPH). It is a good index of oxidative stress and the subsequent immunodetection of the resulting carbonylated hydrazone products can be done using monoclonal or polyclonal antibodies (Levine et al. 1994; Moller et al. 2007). Protein oxidation may result in modification of enzymes and their binding properties. Protein modification due to formation of protein bound carbonyl groups is selectively targeted and the sites and nature of oxidative modifications are still largely unknown (Job et al. 2005). Oxidized proteins undergo diverse structure and functional changes including change in their hydrophobicity which makes the proteins more susceptible to proteolysis (Pacifici and Davies 1990). Intracellular proteolysis may have an important role in the reorganization of plant metabolism under stress, as some experimental evidences suggest that drought sensitive species when subjected to stress have higher proteolytic activity as compared to the tolerant ones (Juszczuk et al. 2008).

Comparative analyses of the modulation of antioxidative defense system among sensitive and tolerant varieties of many plant species such as wheat (*Triticum aestivum* L.), maize (*Zea mays* L.), foxtail millet (*Setaria italica*) have been reported earlier under water stress (Simova-Stoilova et al. 2008; Moussa and Abdel-Aziz 2008; Abedi and Pakniyat 2010). It has been observed that a higher status of antioxidative defense system, especially higher activity levels of the antioxidative enzymes can be correlated with plant stress tolerance to abiotic stresses such as drought and salinity in certain crop species (Demiral and Turkan 2005; Wang et al. 2009; Hameed et al. 2011). In order to deduce a possible relationship of water deficit tolerance with antioxidant capacity in rice, the present study was aimed to examine the response of establishing Indica rice seedlings differing in water-deficit tolerance to progressive levels of polyethylene glycol (PEG-6000) induced water deficit on differential changes in generation of ROS, lipid peroxidation, carbonylation of proteins, proteolysis and status of antioxidative enzymes.

Materials and methods

Plant material and stress conditions

Seeds of two Indica rice (*Oryza sativa* L.) *cvs*. Malviya-36 (drought-sensitive) and Brown Gora (drought-tolerant), commonly grown in India were used in this study. Water-deficit tolerance and sensitivity of these two cultivars during seedling stage was ascertained based on morphological parameters using sand culture experiments. Seeds were surface sterilized with 0.1 % sodium hypochlorite solution for 10 min, rinsed with distilled water and imbibed for 24 h in water. Seedlings were then raised for 10 days in plastic pots containing purified quartz sea sand saturated with Yoshida nutrient solution (Yoshida et al. 1976). The pots were kept in a green house at 28 ± 1 °C under 80 % relative humidity and 12 h light/dark cycle with 190–200 µmol m^{-2} s^{-1} irradiance. Ten day grown seedlings were uprooted and subjected to water deficit treatments for 24, 48, and 72 h in fresh sand cultures using Yoshida nutrient solution supplemented with 15 and 30 % strengths of PEG-6000 to achieve osmotic potentials of −1.0 and −2.1 MPa (Michel and Kaufmann 1973) respectively. Yoshida nutrient solution served as control. All experimental studies were performed on the control and water deficit stressed seedlings in triplicate.

Measurement of seedling vigour, chlorophyll content and water status

At different hours of water deficit treatment, the length of root and shoot of the seedlings was determined based on 10 random samplings in triplicate. To determine chlorophylls and carotenoids, shoot tissues weighing 50 mg were homogenized in 5 mL of 5 % ethanol. After centrifugation at 16,000×*g* for

10 min, contents of chlorophyll a, chlorophyll b and total carotenoids were determined in the supernatant by recording absorbance at 664, 648 and 470 nm using spectrophotometer (Model SL 177, Elico Ltd. India) (Lichtenthaler 1987). Relative water content (RWC) was measured in roots and shoots according to the method of Weatherley (1950). RWC was calculated using the formula: RWC = (FW − DW)/(TW − DW) × 100 where FW = Fresh weight, DW = Dry weight and TW = Turgid weight. Leaf water potential (LWP) was measured using the dye methylene blue according to Knipling (1967) and leaf water loss (LWL) according to Xing et al. (2004).

Measurement of $O_2^{\cdot-}$ and H_2O_2

The rate of production of $O_2^{\cdot-}$ was measured in roots and shoots of control and water deficit stressed seedlings following the method of Mishra and Fridovich (1972). Small segments of root and shoot samples (2–5 mm), weighing 50 mg were placed in 2 mL reaction mixture consisting of 100 μM disodium EDTA, 20 μM NADH and 20 μM sodium phosphate buffer (pH 7.8). The reaction was initiated by adding 1.2 mM epinephrine. Increase in absorbance due to oxidation of epinephrine to adrenochrome was recorded at 1 min interval up to 10 min at 480 nm using a spectrophotometer (Model SL 177, ELICO Ltd., India). The rate of formation of $O_2^{\cdot-}$ was expressed as $\Delta A_{480}\,min^{-1}\,g^{-1}$ tissue fresh weight. The level of H_2O_2 in root and shoot samples was measured spectrophotometrically using titanium sulphate according to the method of Jana and Choudhuri (1981).The intensity of yellow colour developed was measured at 410 nm using spectrophotometer. The amount of H_2O_2 was calculated using extinction coefficient of 0.28 $\mu M^{-1}\,cm^{-1}$ and was expressed as nmol g^{-1} tissue fresh weight.

Determination of lipid peroxides

The method of Heath and Packer (1968) was followed for the measurement of lipid peroxidation products in terms of thiobarbituric acid reactive substances (TBARS). About 200 mg of root and shoot samples were ground in 5 mL of 10 % TCA containing 0.25 % thiobarbutiric acid and centrifuged at 10,000×*g* for 20 min. The supernatant was heated at 95°C for 25 min. It was then cooled in ice bath and centrifuged at 3,000×*g* for 15 min. The absorbance was then recorded at 532 and 600 nm (to correct unspecific turbidity by subtracting the absorbance). The concentration of lipid peroxides was expressed as nmol TBARS g^{-1} fresh weight of the tissues using an extinction coefficient of 155 $mM^{-1}\,cm^{-1}$.

Determination of antioxidative enzyme activities

The activity of SOD was assayed following the method of Beauchamp and Fridovich (1971) based on the inhibition of p-nitro blue tetrazolium chloride (NBT) reduction by $O_2^{\cdot-}$ under light. Root and shoot samples weighing 200 mg were homogenized using a chilled mortar and pestle in 5 mL of 100 mM K-phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 0.1 % (v/v) Triton X-100 and 2 % (w/v) polyvinyl pyrrolidone (PVP). After centrifugation at 22,000×g for 10 min at 4 °C, supernatants were dialyzed in cellophane membrane tubings for 4 h against the extraction buffer in cold with 3–4 changes of the buffer. One unit of SOD activity is expressed as the amount of enzyme required to cause 50 % inhibition of the rate of NBT reduction measured at 560 nm. The extraction medium for CAT was similar to SOD except that 50 mM Tris–HCl buffer (pH 8.0) was used. The homogenate was centrifuged at 22,000×*g* for 10 min at 4 °C. In the supernatant, after dialysis CAT activity was assayed following the method of Beers and Sizer (1952). Reaction mixture in 1.5 mL volume contained 1,000 μL 100 mM K-phosphate buffer (pH 7.0), 400 μL 200 mM H_2O_2 and 100 μL enzyme. The decomposition of H_2O_2 was followed at 240 nm (extinction coefficient of 0.036 $mM^{-1}\,cm^{-1}$) by observing decrease in absorbance using a UV–VIS spectrophotometer (Perkin Elmer, LAMBDA EZ 201, USA). Enzyme specific activity is expressed as μmol H_2O_2 oxidized mg^{-1} protein min^{-1}. For extraction of GPX, root and shoot samples weighing 200 mg were homogenized using chilled mortar and pestle in 5 mL of 50 mM Na-phosphate buffer (pH 7.0). The homogenate was centrifuged at 22,000×*g* for 10 min at 4 °C and the supernatant after dialysis was used for enzyme assay according to Egley et al. (1983). Assay mixture in 5 mL volume contained 40 mM Na-phosphate buffer (pH 6.1), 2 mM H_2O_2, 9 mM guaiacol and 50 μL enzyme. Increase in absorbance due to formation of tetraguaiacohinone was measured at 420 nm (extinction coefficient of 26.6 $mM^{-1}\,cm^{-1}$) at 30 s intervals up to 2 min using spectrophotometer (Bausch and Lomb, Spectronic 20, USA). Enzyme specific activity is expressed as μmol H_2O_2 reduced mg^{-1} protein min^{-1}.

Determination of protein thiol and non protein thiol

Protein thiol content was determined in roots and shoots following the method of de Kok and Kuiper (1986). Samples weighing 150 mg were homogenized in 5 mL of 0.15 % (w/v) sodium ascorbate solution and the homogenate was centrifuged at 22,000×*g* for 10 min at 4 °C. To measure total thiol (–SH) content 0.5 mL supernatant was mixed with 1 mL of 0.2 M Tris–HCl (pH 8.0), 0.5 mL 8 % (w/v) SDS and 0.1 mL 10 mM DTNB (freshly prepared in 0.02 M potassium phosphate buffer, pH 7.0). After 15 min of incubation at 30 °C, yellow colour developed was measured at 415 nm and the correction was made for absorbance of the incubation mixture in the absence of DTNB (replaced with distilled water) and in absence of supernatant extract

(replaced with 0.15 % sodium ascorbate). To measure the non-protein thiol the homogenate was deproteinized by incubating it in a water bath at 100 °C for 4 min, followed by centrifugation at 22,000×*g* for 10 min. In 0.5 mL aliquot containing deproteinized extract -SH content was determined as earlier. The content of protein thiol was calculated by subtracting the content of non-protein thiol from total thiols and expressed in terms of nmol g^{-1} fresh weight of tissues using extinction coefficient of 13, 600 M^{-1} cm^{-1}.

Determination of protein bound carbonyls

The content of protein bound carbonyls was determined following the method of Levine et al. (1994). Root and shoot samples weighing 1 g were homogenized in 2.0 mL of 10 mM sodium phosphate buffer (pH 7.4) containing 1 mM EDTA, 2 mM dithiothreitol, 0.2 % (v/v) Triton x-100 and 1 mM phenylmethane sulphonyl fluoride (PMSF). The homogenate was centrifuged at 25,000×*g* for 30 min at 4 °C. Supernatants containing 500 µg protein were mixed with 1 % (w/v) streptomycin sulphate for 20 min to remove the nucleic acids. After centrifugation at 2,000×*g*, supernatants in 200 µL volumes were mixed with 300 µL of 10 mM 2,4-dinitrophenyl hydrazine (DNPH) (freshly prepared) in 2 M HCl. Individual blank samples were incubated in 2 M HCl. After 1 h incubation at room temperature (25 °C), proteins were precipitated with pre-cooled 10 % (w/v) trichloroacetic acid (TCA) and the pellets were washed three times with 500 µL of ethanol:ethylacetate (1:1). The pellets were then dissolved in 6 M guanidine hydrochloride in 20 mM potassium phosphate buffer (pH 2.3) and the absorbance was measured at 370 nm using UV–Vis spectrophotometer. Carbonyl content was calculated using a molar absorption coefficient for aliphatic hydrazones as 22,000 M^{-1} cm^{-1} and expressed in terms of nmol carbonyl mg^{-1} protein.

Protein gel-blot analysis of carbonyls

Protein carbonyls were immunochemically detected following the method of Romero-Puertas et al. (2002). Protein samples (0.5 mg) in 0.5 mL volumes were mixed with 200 µL of 10 mM DNPH (freshly prepared in 2 M HCl) and incubated at room temperature (25 °C) for 1 h. Samples were precipitated with pre cooled 10 % (w/v) TCA. After centrifugation at 5,000×*g* for 5 min, pellets were washed with ethanol:ethylacetate (1:1) three times. The pellets were re-suspended in 0.5 mL of 10 mM sodium phosphate buffer (pH 7.0). Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using Mini-Protean II slab cell (Bangalore Genei, India). Two gels were run simultaneously, one for protein staining with Coomassie Brilliant Blue R-250 and the other for immunochemical detection. The separated proteins were transferred onto polyvinylidene fluoride (PVDF) membranes using a Bangalore—Genei (India) Semi Dry Transfer Cell following the method of Corpas et al. (1998). Oxidized proteins were detected with antibodies against DNPH from Sigma-Aldrich Co. (St. Louis, MO, USA) (1:40,000 dilution) and using goat anti-rabbit IgG horseradish peroxidase as conjugate and 3,3′-diaminobenzidine (DAB) as substrate.

Determination of proteolytic activity

Proteolytic activity was measured in enzyme extracts prepared from root and shoot samples as described by Polge et al. (2009) with certain modifications. Tissues weighing 300 mg were homogenized using chilled mortar and pestle in 2 mL of 50 mM Tris–HCl buffer (pH 7.5) and centrifuged at 15,000×*g* for 15 min at 4 °C and supernatants were dialyzed in cellophane membrane tubings in cold against the extraction buffer with 3–4 changes of the buffer. Proteolytic activity was assayed in dialyzed enzyme (0.25 mL) by reaction with 0.25 mL of 2 % azocasein prepared in 0.1 M Tris–HCl (pH 8.4). After 6 h of incubation at 28°C the reaction was stopped by adding 1.2 mL of 10 % TCA. After placing on ice for 10 min, the contents were centrifuged at 3,000×*g* for 20 min. Then 1.0 mL of the resulting supernatant was mixed with 1.0 mL of 1.0 M NaOH. After 30 min, absorbance was recorded at 440 nm using spectrophotometer. The extinction coefficient of azocasein $\varepsilon_{1\%}$ in 1 M NaOH was taken as 37 L cm^{-1} g^{-1} to calculate the proteolytic activity. One unit of proteolytic activity is expressed as mg of azocasein degraded min^{-1} mg^{-1} protein.

In-gel staining of proteolytic activity

Proteolytic activities in enzyme preparations were resolved on 10 % SDS–gelatin polyacrylamide gels containing 0.10 % gelatin as substrate (Hellmich and Schauz 1988). Enzyme samples (50 µg protein) were mixed with equal volumes of non-reducing sample buffer (0.1 M Tris–HCl, pH 6.8; 2 % (w/v) SDS, 10 % (v/v) glycerol, 0.01 % bromophenol blue) and incubated at 37 °C for 30 min. After electrophoresis at room temperature the proteases were renatured by rinsing the gels in 50 mM Tris–HCl (pH 7.5) containing 2.5 % Triton X-100, 10 mM EDTA for 30 min with shaking. Gels were then incubated in a medium consisting of 10 mM Ca^{2+} and 10 mM Mg^{2+} in 50 mM Tris–HCl, pH 7.5 at 37 °C for 12–16 h in an incubator cum shaker. The gels were then stained with Coomassie Brilliant Blue R-250 (0.1 % CBB in 50 % methanol and10 % glacial acetic acid) and destained in a solution containing 50 % methanol and 10 % glacial acetic acid. Clear bands with proteolytic

activity were visible on a blue background representing the sites of proteolytic activity.

Protein determination

In all preparations protein concentration was determined following the method of Bradford (1976) using bovine serum albumin (BSA, Sigma) as standard.

Statistical analysis

All experiments were performed in triplicate. Values indicate mean ± S.D. based on three independent determinations. Differences among control and treatments were analyzed by one factorial ANOVA followed by Tukey's test. Asterisks (*) were used to identify the level of significance of the difference between control and water-deficit stress treatments as $*p \leq 0.05$ and $**p \leq 0.01$.

Results

Effect of water deficit on growth and relative water content in the seedlings

When seedlings of the rice cultivars, Malviya-36 (drought-sensitive) and Brown Gora (drought-tolerant) grown for 10 days in control conditions were subjected to progressive levels of water-deficit stress treatment of −0.1 MPa (mild stress) and −2.1 MPa (higher stress) for 24–72 h, an inhibition in growth and reduction in RWC of seedlings were observed. The data related to 48 h of water deficit stress treatment have been presented in Fig. 1.

As it is evident from the figure, water deficit treatment of 48 h caused a significant ($p \leq 0.05$) decline in length of roots and shoots and RWC in the seedlings of drought sensitive *cv*. Malviya-36 compared to controls, whereas in the seedlings of drought tolerant *cv*. Brown Gora, the decline was not significant. Seedlings of drought sensitive *cv*. Malviya-36 subjected to −1.0 and −2.1 MPa water deficit treatment for 48 h showed nearly 24–28 % decline in the length of roots and 13–26 % decline in the length of shoots compared to controls, whereas in the seedlings of drought tolerant rice *cv*. Brown Gora under similar level and duration of water deficit nearly 3–12 % decline in length of roots and 10–12 % decline in length of shoots was observed compared to controls. Similar to decline in length, 48 h water deficit treatment caused nearly 22–48 % (up to $p \leq 0.01$) decline in RWC in roots and 17–85 % (up to $p \leq 0.01$) decline in RWC of shoots in the seedlings of drought sensitive *cv*. Malviya-36, whereas in drought tolerant *cv*. Brown Gora under similar levels of water deficit an insignificant decline in RWC of 15–19 % was observed in roots and 3–24 % in shoots.

Effect of water deficit on leaf water loss, leaf water potential and chlorophyll content

Corresponding to RWC, the water status in shoots of the seedlings of both the rice cultivars was further investigated by observing the LWL and LWP after 48 h of water deficit treatment. It was observed that the drought sensitive *cv*. Malviya-36 had significantly higher LWL of nearly 41–50 % ($p \leq 0.05$) compared to the tolerant *cv*. Brown Gora where a relatively lower LWL level of 35–39 % was observed under progressive level of −1.0 to 2.1 MPa of water deficit treatment (Table 1). Similarly, a more decline in LWP level of −1.20 and −1.40 MPa was noticed under −1.0 and −2.1 MPa of water deficit treatment levels respectively in seedlings of drought sensitive *cv*. Malviya-36 compared to the tolerant *cv*. Brown Gora where under similar level of water deficit treatment −0.98 and −1.02 MPa LWP level was observed. The level of Chl a, Chl b and carotenoids declined in the shoots of the seedlings of both the rice cultivars with increase in the level of water-deficit treatment and the decline was greater in the sensitive cultivar than the tolerant (Table 1). With imposition of water-deficit levels of −1.0 and −2.1 MPa for 48 h, seedlings of sensitive cultivar showed respectively 31.03 and 62.06 % ($p \leq 0.01$) decline in the level of Chl a, 20 % ($p \leq 0.05$) and 60 % ($p \leq 0.01$) decline in the level of Chl b and 50 and 75 % ($p \leq 0.01$) decline in the level of carotenoids in the leaves. Whereas with −2.0 MPa water deficit treatment level for 48 h in seedlings of tolerant cultivar the decline in the levels of Chl a, b and carotenoids was 28.8 % ($p \leq 0.05$), 20 and 30 % ($p \leq 0.01$) respectively compared to the controls.

Effects of water deficit on the levels of $O_2^{\cdot-}$ and H_2O_2

With increase in the level (−1.0 to −2.1 MPa) as well as duration (24–72 h) of water deficit treatment a consistent increase in the levels of $O_2^{\cdot-}$ and H_2O_2 was observed in roots and shoots of the seedlings of both sensitive as well as tolerant rice cultivars (Fig. 2). The extent of generation of both the ROS—$O_2^{\cdot-}$ and H_2O_2 on imposition of water deficit, was greater in the seedlings of drought sensitive *cv*. Malviya-36 compared to the tolerant *cv*. Brown Gora. Seedlings of drought sensitive *cv*. Malviya-36 subjected to water deficit treatment of −2.1 MPa for 72 h showed nearly 77 % ($p \leq 0.01$) increased $O_2^{\cdot-}$ level in roots and 283 % ($p \leq 0.01$) increased level in shoots, whereas under similar treatment conditions in drought tolerant *cv*. Brown Gora 50 % ($p \leq 0.01$) increased $O_2^{\cdot-}$ level was observed in roots and 200 % ($p \leq 0.01$) increased level in shoots compared to respective controls. Similarly, seedlings of sensitive *cv*. Malviya-36 subjected to −2.1 MPa water deficit treatment for 72 h showed 86 % ($p \leq 0.01$) increased H_2O_2 level in roots and 99 % ($p \leq 0.01$) increased level in shoots, whereas similarly stressed seedlings of tolerant *cv*. Brown Gora showed 48 % ($p \leq 0.01$) increased

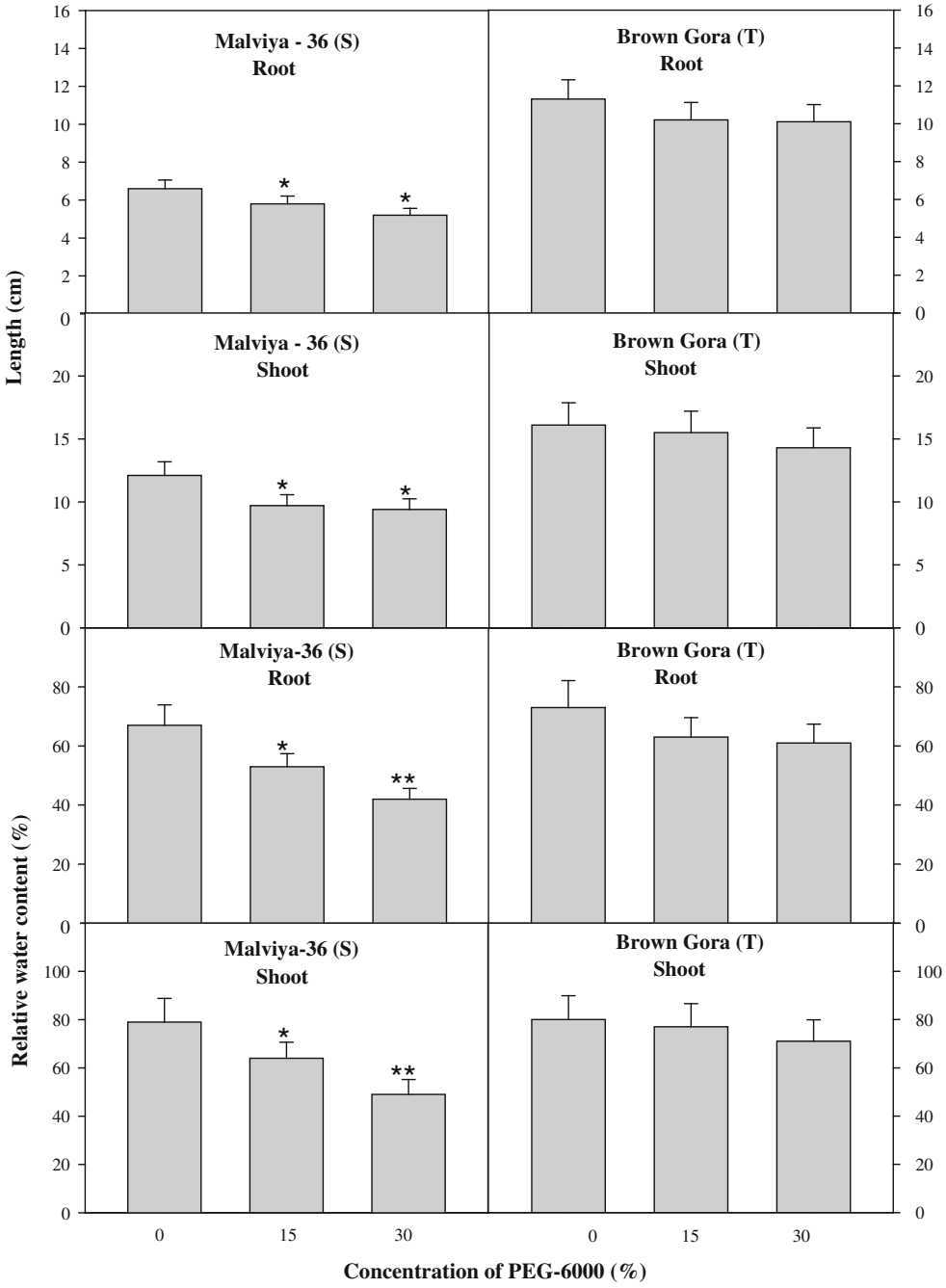

Fig. 1 Effect of increasing concentration of PEG-6000 in the growth medium on the length and relative water content of roots and shoots of 10-day-grown seedlings of rice *cvs.* Malviya-36 (drought-sensitive) and Brown Gora (drought-tolerant) after 48 h of water deficit treatment. 15 and 30 % concentrations of Polyethylene Glycol (PEG-6000) correspond to water deficit treatment levels of −1.0 and −2.1 MPa respectively. Values are mean ± SD based on three independent determinations and bars indicate standard deviations. * and **indicate values that differ significantly from controls at $p \leq 0.05$ and $p \leq 0.01$, respectively, according to Tukey's multiple range test

H_2O_2 level in roots and 46 % ($p \leq 0.01$) increased level in shoots compared to respective controls. Seedlings of drought sensitive cultivar always maintained higher level of $O_2^{\cdot-}$ and H_2O_2 compared to the seedlings of drought tolerant cultivar under both control and water deficit conditions.

Effect of water deficit on lipid peroxidation and SOD activity

Lipid peroxidation was measured in the seedlings in terms of TBARS production. With increase in the level and duration of water deficit treatment, content of TBARS increased in the seedlings with greater increase in the sensitive cultivar than the tolerant (Fig. 3). Seedlings of drought sensitive cultivar subjected to −2.1 MPa water deficit treatment for 72 h showed 150 % ($p \leq 0.01$) increased TBARS level in roots and 194 % ($p \leq 0.01$) increased level in shoots compared to the seedlings of tolerant cultivar which showed 100 % ($p \leq 0.01$) increased TBARS level in roots and 73 % ($p \leq 0.01$) increased level in shoots under similar level of water deficit. The activity of enzyme SOD increased significantly ($p \leq 0.01$) in roots as well as in shoots of the seedlings

Table 1 Effect of PEG-6000 on water parameters and photosynthetic pigments in rice seedlings

Rice cultivars	PEG treatment (%)	LWP (MPa)	LWL (%)	Chlorophyll a (mg g^{-1} fr wt)	Chlorophyll b (mg g^{-1} fr wt)	Total carotenoids (mg g^{-1} fr wt)
Malviya-36 (S)	0	−0.50 ± 0.035	36.20 ± 3.26	0.29 ± 0.026	0.05 ± 0.004	0.08 ± 0.006
	15	−1.20 ± 0.132*	41.90 ± 3.71*	0.20 ± 0.018**	0.04 ± 0.003*	0.04 ± 0.002**
	30	−1.40 ± 0.154*	50.34 ± 5.35*	0.11 ± 0.008**	0.02 ± 0.001**	0.02 ± 0.001**
Brown Gora (T)	0	−0.40 ± 0.033	32.10 ± 1.97	0.45 ± 0.049	0.10 ± 0.007	0.10 ± 0.007
	15	−0.98 ± 0.088*	35.80 ± 3.22*	0.35 ± 0.040	0.09 ± 0.010	0.08 ± 0.007*
	30	−1.02 ± 0.092*	39.80 ± 3.58*	0.32 ± 0.035*	0.08 ± 0.010	0.07 ± 0.006**

Effect of increasing concentration of PEG-6000 in the growth medium on the leaf water potential (LWP), leaf water loss (LWL) and contents of chlorophylls and carotenoids in shoots of 48 h water deficit stressed seedlings of rice *cvs.* Malviya-36 (drought-sensitive) and Brown Gora (drought-tolerant). 15 and 30 % concentrations of PEG-6000 correspond to water deficit treatment levels of −1.0 and −2.1 MPa respectively. Values are mean ± SD based on three independent determinations and bars indicate standard deviations. * and ** indicate values that differ significantly from controls at $p \leq 0.05$ and $p \leq 0.01$, respectively, according to Tukey's multiple range test

of both set of rice cultivars with increase in level and duration of water deficit treatment, however, the water deficit induced increase in SOD activity was greater in the roots, but not in the shoots of the drought-sensitive genotype compared to the tolerant one. A water deficit level of −2.1 MPa imposed for 72 h led to 75 % ($p \leq 0.01$) increase in SOD activity in roots and 30 % ($p \leq 0.05$) increased activity in shoots of seedlings of drought sensitive *cv.* Malviya-36, whereas in the tolerant cultivar at similar level and duration of stress treatment 42 % ($p \leq 0.01$) increased SOD activity was observed in roots and 85 % ($p \leq 0.01$) increased activity in shoots. It was pertinent to note that constitutive as well as water deficit inducible SOD activity level was always higher in drought tolerant seedlings compared to the sensitives.

Effect of water deficit on CAT and GPX activity

When rice seedlings were subjected to water deficit treatment of −1.0 and −2.1 MPa for 24–72 h, in sensitive cultivar CAT activity increased in roots with increase in the level as well as duration of water deficit treatment (Fig. 4). However, in the shoots the activity increased compared to controls at water deficit treatment level of −1.0 MPa but it declined when the seedlings were subjected to a higher water deficit level of −2.1 MPa for 72 h. In seedlings of drought tolerant *cv.* Brown Gora, a consistent increase in CAT activity was observed in both roots and shoots with increase in the level and duration of water deficit treatment. Both constitutive as well as water deficit inducible CAT activity levels were higher in the seedlings of tolerant cultivar compared to the sensitive. The activity of GPX increased in both sensitive and tolerant rice seedlings under water deficit and the extent of increase was greater in the tolerant seedlings than the sensitives (Fig. 4). Seedlings of sensitive cultivar subjected to −2.1 MPa water deficit treatment for 72 h showed 13 % ($p \leq 0.05$) increased GPX activity in roots and 77 % ($p \leq 0.01$) increased activity in shoots, whereas similarly stressed seedlings of tolerant cultivar showed 34 % ($p \leq 0.01$) increased enzyme activity in roots and 97 % ($p \leq 0.01$) increased activity in shoots compared to respective controls.

Effect of water deficit on protein and non-protein thiol content

When level of protein thiol was determined in rice seedlings undergoing water deficit treatment, an insignificant decline in the level of protein thiol was observed in the seedlings of tolerant *cv.* Brown Gora due to water deficit compared to unstressed controls (Fig. 5). In seedlings of drought sensitive *cv.* Malviya-36, however, with −2.1 MPa water deficit treatment for 48–72 h a significant ($p \leq 0.05$–0.01) decline in protein thiol level was observed in roots whereas in shoots the decline was not significant. Seedlings of sensitive cultivar subjected −2.1 MPa water-deficit for 72 h showed 21 % ($p \leq 0.01$) decline in protein thiol level in roots compared to controls. No definite pattern of alteration in the level of non-protein thiol could be observed in the seedlings with water deficit, except in the seedlings of tolerant cultivar with 24 h water deficit treatment, where a significant decline in non-protein thiol level was observed. Seedlings of tolerant *cv.* Brown Gora, when water deficit stressed with −2.1 MPa for 24 h showed 31.03 % ($p \leq 0.05$) decline in non-protein thiol level in roots and 38.4 % ($p \leq 0.01$) decline in the level in shoots compared to respective controls.

Effect of water deficit on protein bound carbonyls and proteolytic activity

With increase in the level and duration of water deficit, the content of protein bound carbonyls increased in the seedlings of both sensitive and tolerant rice cultivars, with

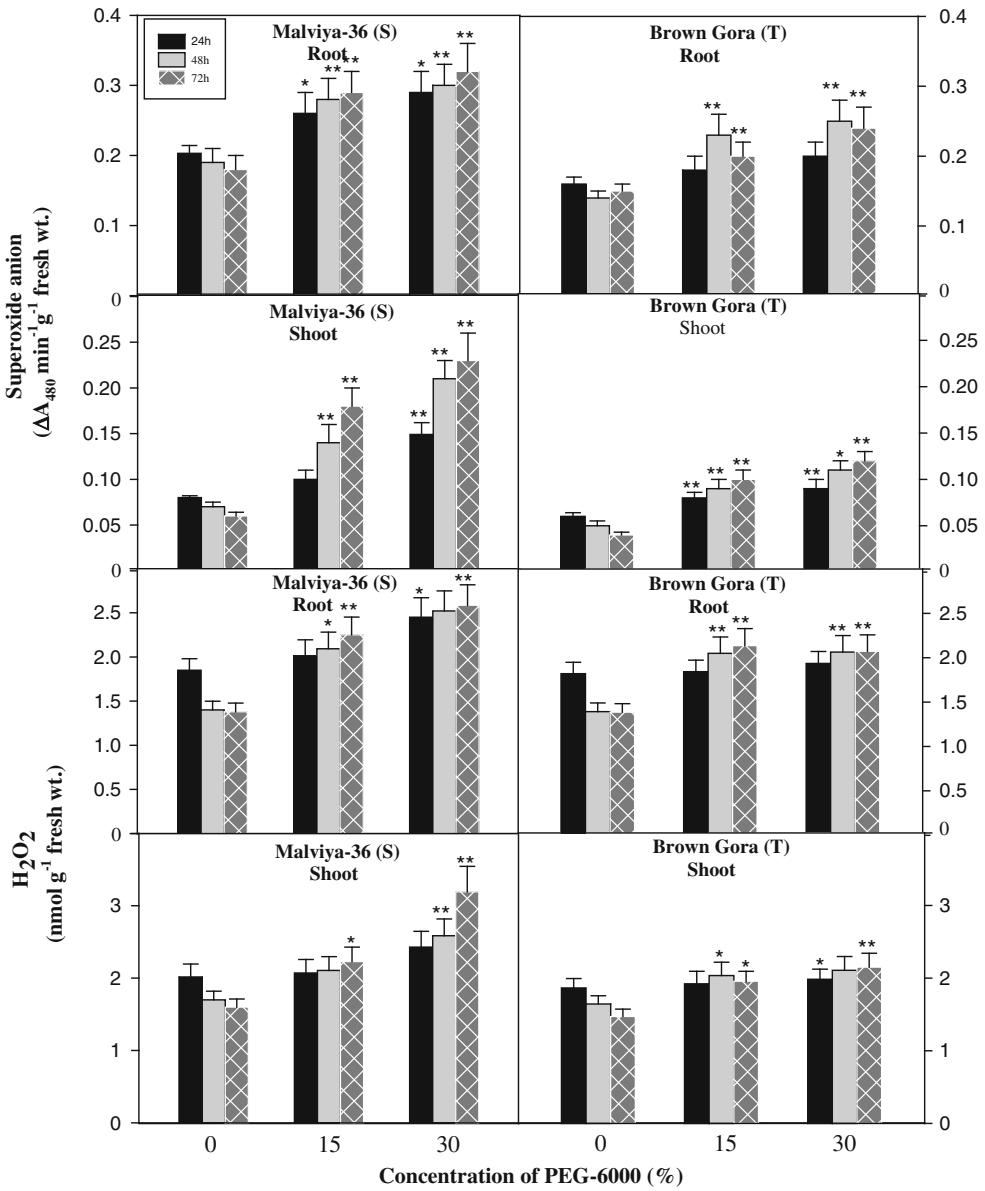

Fig. 2 Effect of increasing concentration of PEG-6000 in the growth medium on the root and shoot contents of superoxide anion and H_2O_2 in rice seedlings at 24, 48 and 72 h of water deficit treatment. S and T in parentheses indicate drought sensitive and tolerant rice cultivars whereas 15 and 30 % concentrations of PEG-6000 correspond to water deficit treatment levels of −1.0 and −2.1 MPa respectively. Values are mean ± SD based on three independent determinations and bars indicate standard deviations. * and **indicate values that differ significantly from controls at $p \leq 0.05$ and $p \leq 0.01$, respectively, according to Tukey's multiple range test

greater increase in the sensitive than the tolerant (Fig. 6). Seedlings of drought sensitive *cv*. Malviya-36 stressed with −2.1 MPa water deficit for 72 h showed 43 % ($p \leq 0.01$) increase in protein carbonyls in roots and 78 % ($p \leq 0.01$) increase in shoots compared to respective controls. Similarly, stressed seedlings of tolerant cultivar showed 7 % increased protein carbonyl level in roots and 14 % increased level in shoots.

Similar to increase in the content of protein bound carbonyls, the proteolytic activity increased in the seedlings of both set of rice cultivars with increase in the level and duration of water deficit treatment (Fig. 6). However, the extent of proteolysis under water deficit was greater in the seedlings of sensitive cultivar than the tolerant. Seedlings of drought sensitive *cv*. Malviya-36 subjected to water deficit of −2.1 MPa for 72 h showed 200 % ($p \leq 0.01$) increased proteolytic activity in roots and 85 % ($p \leq 0.01$) increased activity in shoots. Whereas under similar level and duration of water deficit, seedlings of drought tolerant *cv*. Brown Gora showed 38 % ($p \leq 0.05$) increased proteolytic activity in roots and 40 % ($p \leq 0.05$) increased activity in shoots.

Effect of water deficit on in-gel proteolytic activity and protein gel blot analysis of carbonylated proteins

When enzyme extracts prepared from root and shoot samples of control and water deficit stressed seedlings of both sensitive and tolerant cultivar were electrophoresed on gelatin-polyacrylamide gels and proteolytic activities were resolved in gels with CBB staining, it was observed that due to water deficit the intensities of enzymic bands corresponding to proteolytic

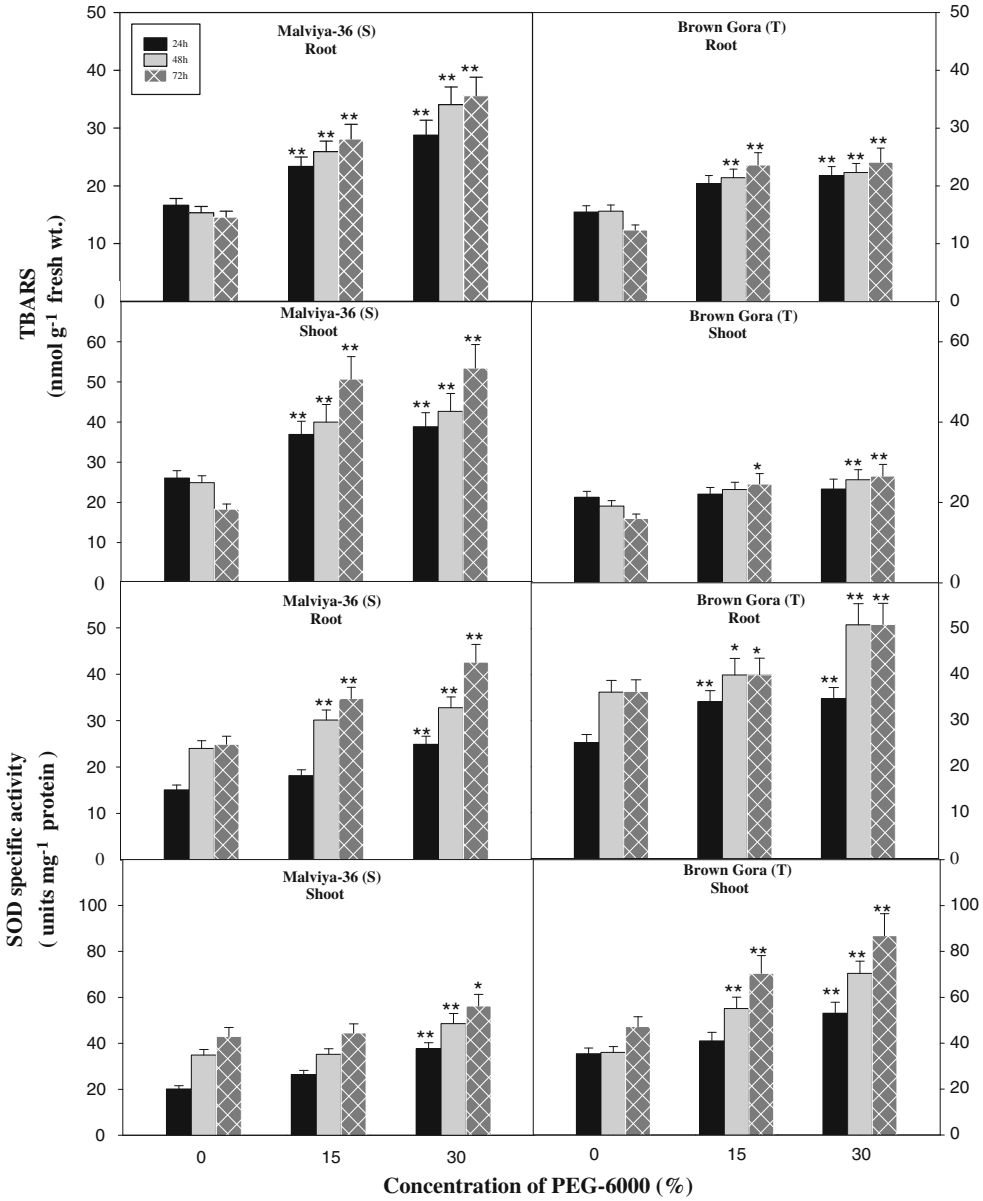

Fig. 3 Effect of increasing concentration of PEG-6000 in the growth medium on the root and shoot contents of thiobarbituric acid reactive substances (TBARS) and activity of superoxide dismutase (SOD) in rice seedlings after 24, 48 and 72 h of water deficit treatment. S and T in parentheses indicate drought sensitive and tolerant rice cultivars whereas 15 and 30 % concentrations of PEG-6000 correspond to water deficit levels of −1.0 and −2.1 MPa respectively. Values are mean ± SD based on three independent determinations and bars indicate standard deviations. * and **indicate values that differ significantly from controls at $p \leq 0.05$ and $p \leq 0.01$, respectively, according to Tukey's multiple range test

activity increased in the both set of seedlings (Fig. 7). Eight proteolytic activity bands ranging between molecular weight 24–225 kDa were observed in roots of control grown seedlings of drought sensitive cv. Malviya-36 and the intensities of most of these bands increased further under water deficit. In shoots of this cultivar, however, only 4 bands were observed in controls and their intensities further increased due to water deficit. In control grown seedlings of drought tolerant cultivar only 5 bands corresponding to proteolytic activity were observed in roots, the intensities of which increased due to water deficit whereas in shoots, only 2 bands were observed in controls and their intensities also increased under water deficit. Seedlings of drought sensitive cultivar were characterized by possession of high number of proteolytic activity bands in roots compared to the tolerant cultivar.

To determine whether water deficit caused selective modification of proteins in rice seedlings, carbonylated proteins from the two sets of rice seedlings were detected by immunochemical methods using antibody against DNPH. As it is evident, in homogenates prepared from control and water deficit treated seedlings of both the rice cultivars, carbonylated proteins appeared in the molecular mass range of 12–80 kDa (Fig. 8). With increase in the level of water deficit treatment, the intensity of cross reacting bands increased in homogenates from both roots and shoots, however, the intensity of bands of oxidized proteins was much higher in homogenates from seedlings of drought sensitive *cv*. Malviya-36 compared to the tolerant *cv*. Brown Gora. A higher expression of oxidized proteins was observed in roots compared to the shoots.

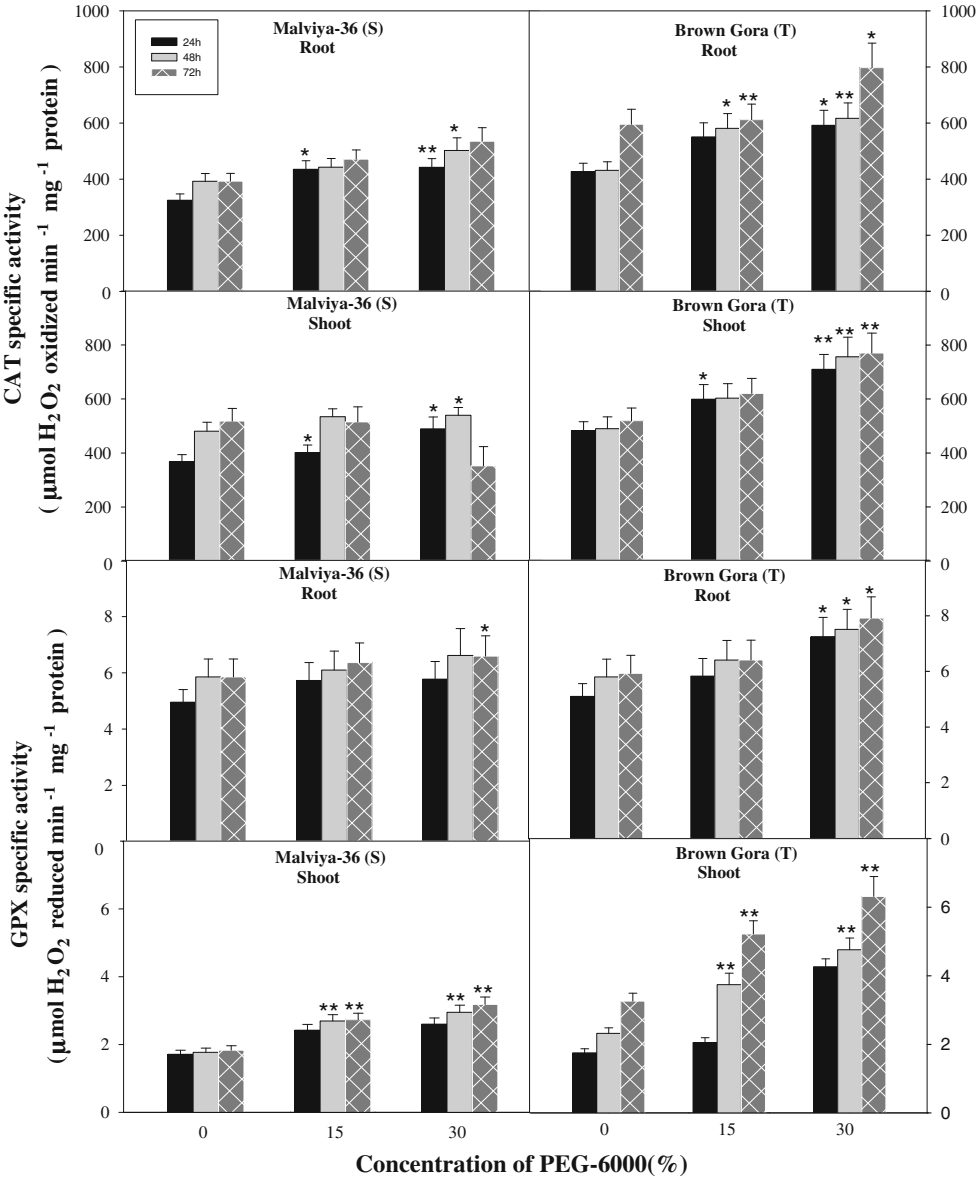

Fig. 4 Effect of increasing concentration of PEG-6000 in the growth medium on the root and shoot activities of catalase (CAT) and guaiacol peroxidase (GPX) in rice seedlings after 24, 48 and 72 h of water deficit treatment. S and T in parentheses indicate drought sensitive and tolerant rice cultivars, whereas 15 and 30 % concentrations of PEG-6000 correspond to water deficit levels of −1.0 and −2.1 MPa respectively. Values are mean ± SD based on three independent determinations and bars indicate standard deviations. * and **indicate values that differ significantly from controls at $p \leq 0.05$ and $p \leq 0.01$, respectively, according to Tukey's multiple range test

Discussion

An important requirement for understanding stress tolerance in plants is a proper comparison of biochemical parameters among the varieties differing in stress tolerance, when subjected to similar levels of water deficit (Simova-Stoilova et al. 2008). Drought tolerance or sensitivity of a plant has often been correlated with its antioxidant response, as the activities of antioxidative enzymes often get modulated when plants are exposed to drought (Wang et al. 2009). Many studies conducted by various groups of investigators have shown that plant species tolerant to drought or salinity possess better antioxidant capacity to protect themselves from oxidative damage caused due to stressful conditions compared to sensitive plants and show enhanced activity of antioxidative enzymes when subjected to stresses (Sekmen et al. 2007; Mishra et al. 2012). In the present study we conducted the experiments on seedlings of two rice cultivars differing in drought tolerance i.e., Malviya-36 (drought-sensitive) and Brown Gora (drought-tolerant) to investigate the correlations of ROS production, lipid peroxidation, oxidative modifications of proteins, extent of proteolysis and the antioxidant capacity of the plants with their ability to withstand water deficit stress. Water deficit stress was imposed in sand culture experiments by PEG-6000 in the growth medium which is an inert, water binding polymer, with non-ionic impermeable long chain that accurately mimics water deficit stress in the fields (Couper and Eley 1984). We observed varying level of ROS production, lipid peroxidation and different pattern of response of antioxidative enzyme activities in the seedlings of sensitive and tolerant rice cultivars when

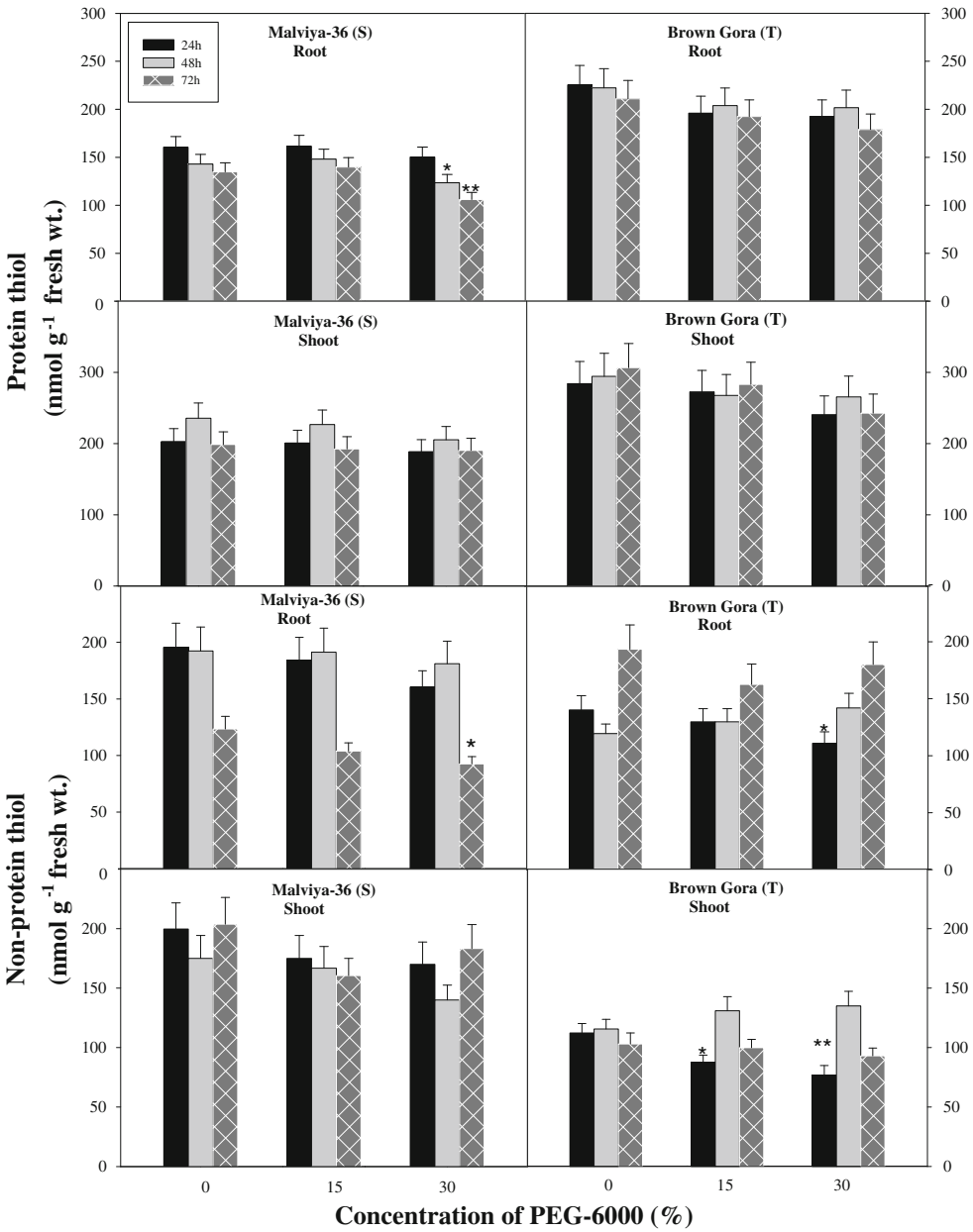

Fig. 5 Effect of increasing concentration of PEG-6000 in the growth medium on the root and shoot contents of protein thiol and non-protein thiol in rice seedlings after 24, 48 and 72 h of water deficit treatment. S and T in parentheses indicate drought sensitive and tolerant rice cultivars whereas 15 and 30 % concentrations of PEG-6000 correspond to water deficit treatment levels of −1.0 and −2.1 MPa respectively. Values are mean ± SD based on three independent determinations and bars indicate standard deviations. * and **indicate values that differ significantly from controls at $p \leq 0.05$ and $p \leq 0.01$, respectively, according to Tukey's multiple range test

subjected to increasing duration and intensity of water deficit.

In our studies, *cv*. Malviya-36 appeared to be water deficit sensitive due to a more decline in length, RWC, LWP and increase in LWL in the seedlings of this cultivar compared to *cv*. Brown Gora when exposed to increasing levels of water deficit. The *cv*. Brown Gora was regarded as water deficit tolerant due to an insignificant decline in length, RWC and lesser decline in LWP and lesser increase in LWL compared to similarly stressed seedlings of *cv*. Malviya-36. These observations suggest an enhanced capacity in the seedlings of *cv*. Brown Gora for withstanding the damage caused due to water deficit. Root and shoot growth have been widely used as a physiological trait to evaluate sensitivity to drought or acclimation to water deficit. Decrease in the RWC of leaves of rice and tomato has been observed earlier with imposition of water stress (Hsu et al. 2003; Zgallai et al. 2005).

All abiotic stressful conditions of the environment including water deficit, cause oxidative damage to the tissues of growing plants due to overproduction of ROS. A greater decline in the level of chlorophylls and carotenoids due to water deficit in drought-sensitive *cv*. Malviya-36 as compared to *cv*. Brown Gora (drought-tolerant), could be possibly due to a direct or indirect effect of increasingly produced ROS in the sensitive cultivar leading to increased lipid peroxidation and consequently increased destruction of chlorophylls and carotenoids in this cultivar

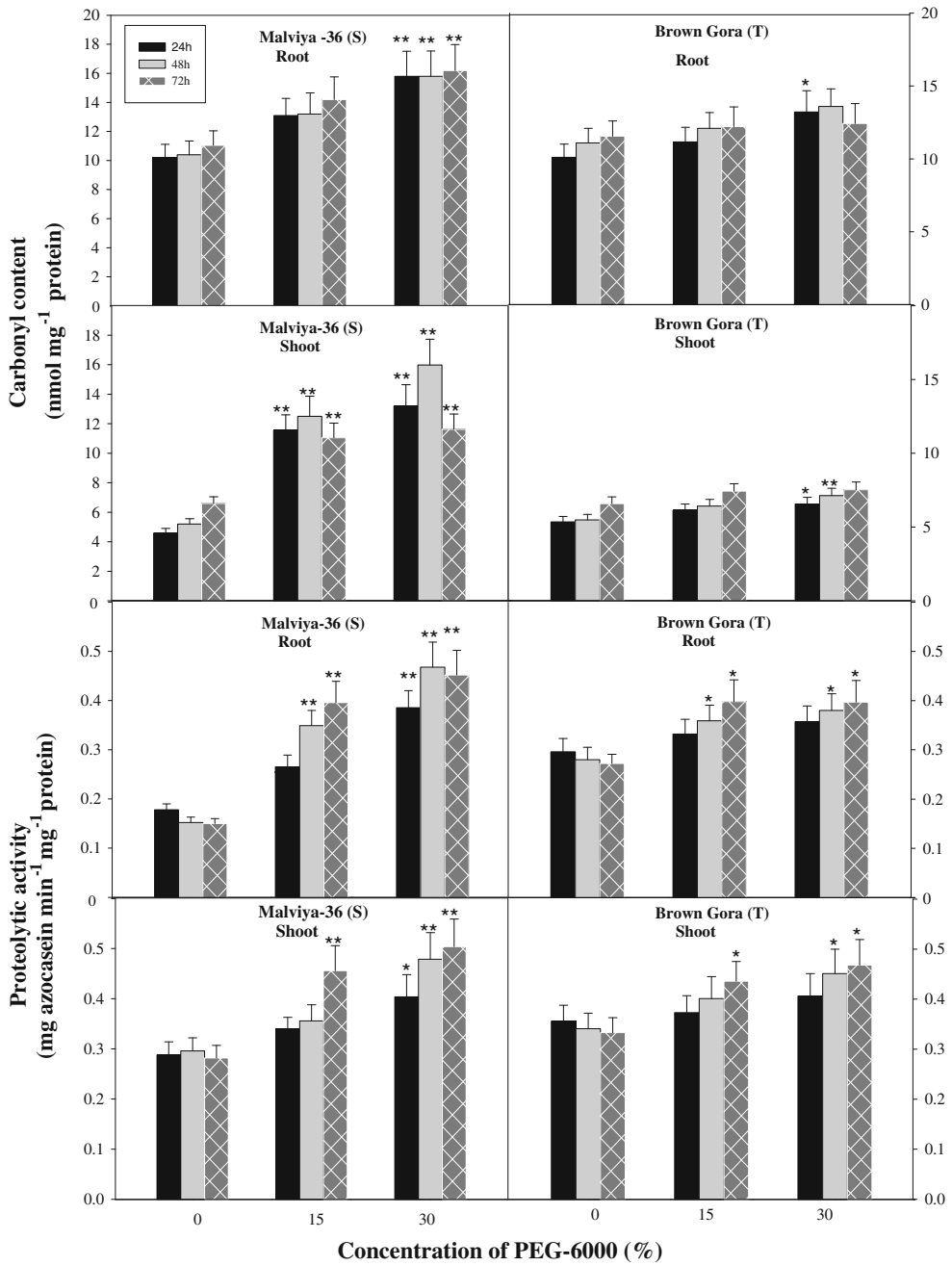

Fig. 6 Effect of increasing concentration of PEG-6000 in the growth medium on the root and shoot protein carbonyls and proteolytic activity in rice seedlings after 24, 48 and 72 h of water deficit treatment. S and T in parentheses indicate drought sensitive and drought tolerant rice cultivars whereas 15 and 30 % concentrations of PEG-6000 correspond to water deficit treatment levels of −1.0 and −2.1 MPa respectively. Values are mean ± SD based on three independent determinations and bars indicate standard deviations. * and **indicate values that differ significantly from controls at $p \leq 0.05$ and $p \leq 0.01$, respectively, according to Tukey's multiple range test

(Ravi et al. 2011). Water deficit or drought stress is inevitably associated with oxidative stress in the tissues due to enhanced accumulation of ROS particularly $O_2^{\cdot-}$ and H_2O_2 in chloroplasts, mitochondria and peroxisomes (Foyer and Noctor 2003). Enhanced oxidative injury, indicated by increased lipid peroxidation is common in plant cells exposed to water deficit (Sharma and Dubey 2005). In our studies lower levels of $O_2^{\cdot-}$, H_2O_2 and lipid peroxidation were observed in both roots and shoots of seedlings of drought tolerant *cv*. Brown Gora compared to sensitive *cv*. Malviya-36 when subjected to similar levels of water deficit. This suggests that drought tolerance in rice

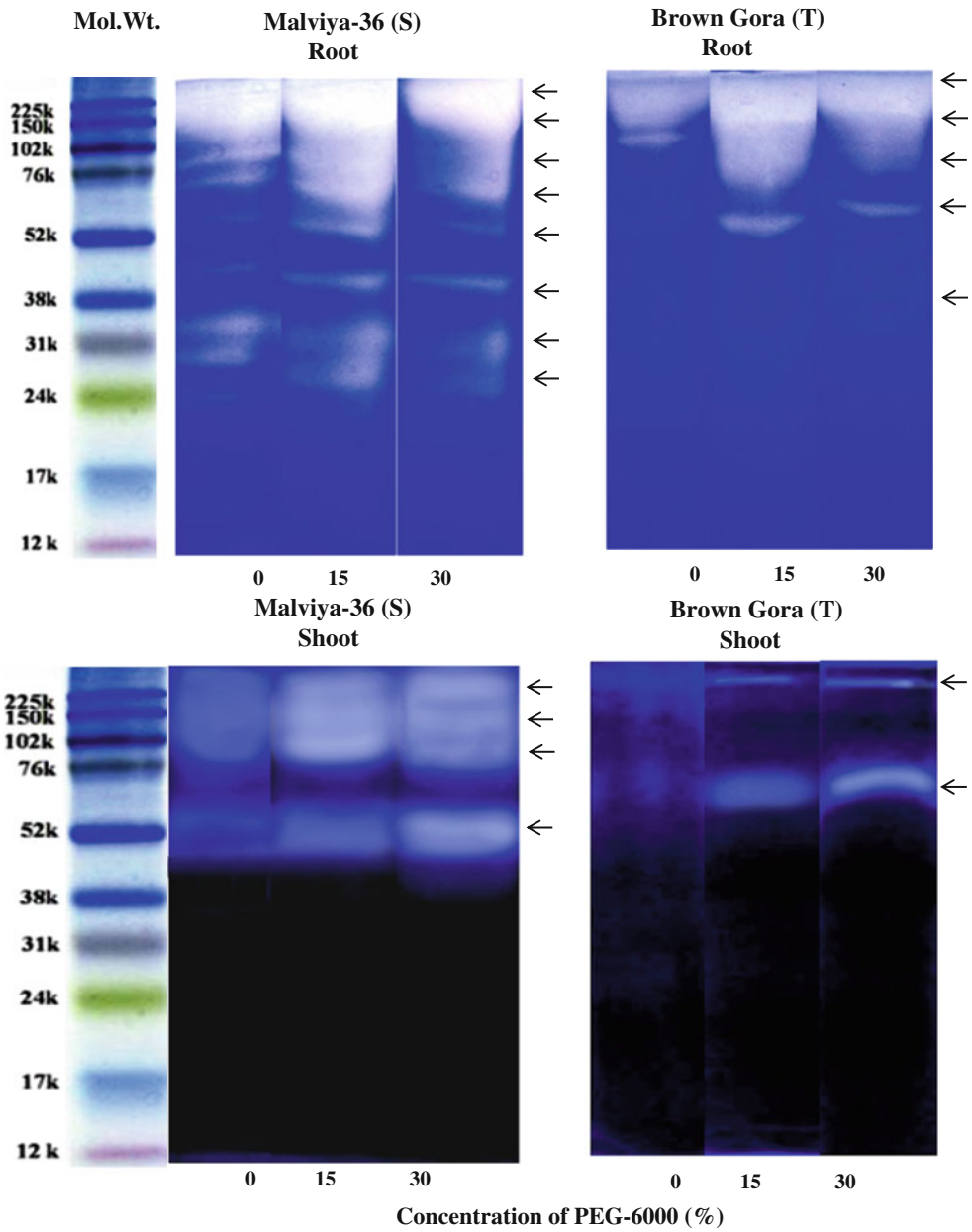

Fig. 7 In-gel proteolytic activity in enzyme preparations from roots and shoots of 48 h water-deficit stressed rice seedlings. 15 and 30 % concentrations of PEG-6000 were used to create water deficit levels −1.0 and −2.1 MPa respectively. *Arrows* represent bands corresponding to proteolytic activities. S and T in parentheses indicate drought sensitive and tolerant rice cultivar

seedlings is associated with lower level of ROS production and oxidative stress as compared to sensitive seedlings, when subjected to similar levels of water deficit. Similar findings were reported earlier in *Medicago sativa*, *Medicago truncatula* and rice plants tolerant to the abiotic stresses such as drought, salinity and heavy metals (Naya et al. 2007; Zhou et al. 2007; Mishra et al. 2012).

Protein modifications due to overproduced ROS may occur as a result of direct oxidation of an amino acid side chain leading to formation of carbonyl groups or oxidation of protein thiol groups (Hameed et al. 2011). Protein oxidation has often been considered as a significant marker of oxidative damage under stressful conditions (Moller et al. 2007) since carbonyl groups formation requires stringent conditions than the reversible oxidation of protein thiols (Moller and Kristensen 2004). Our results showed a significant decline in the level of root protein thiol and a higher increase in carbonyl content in the seedlings of drought sensitive *cv*. Malviya-36 compared to *cv*. Brown Gora when subjected to similar level of water deficit. A higher level of carbonyl content in water deficit stressed seedlings of sensitive *cv*. Malviya-36 compared to the tolerant *cv*. Brown Gora was also confirmed by gel blot analysis of proteins from stressed seedlings of the two rice cultivars. Oxidative modification of proteins characterized by production of carbonyl groups is a common feature associated with oxidative stress. These groups can be derivatized with DNPH and their immunochemical

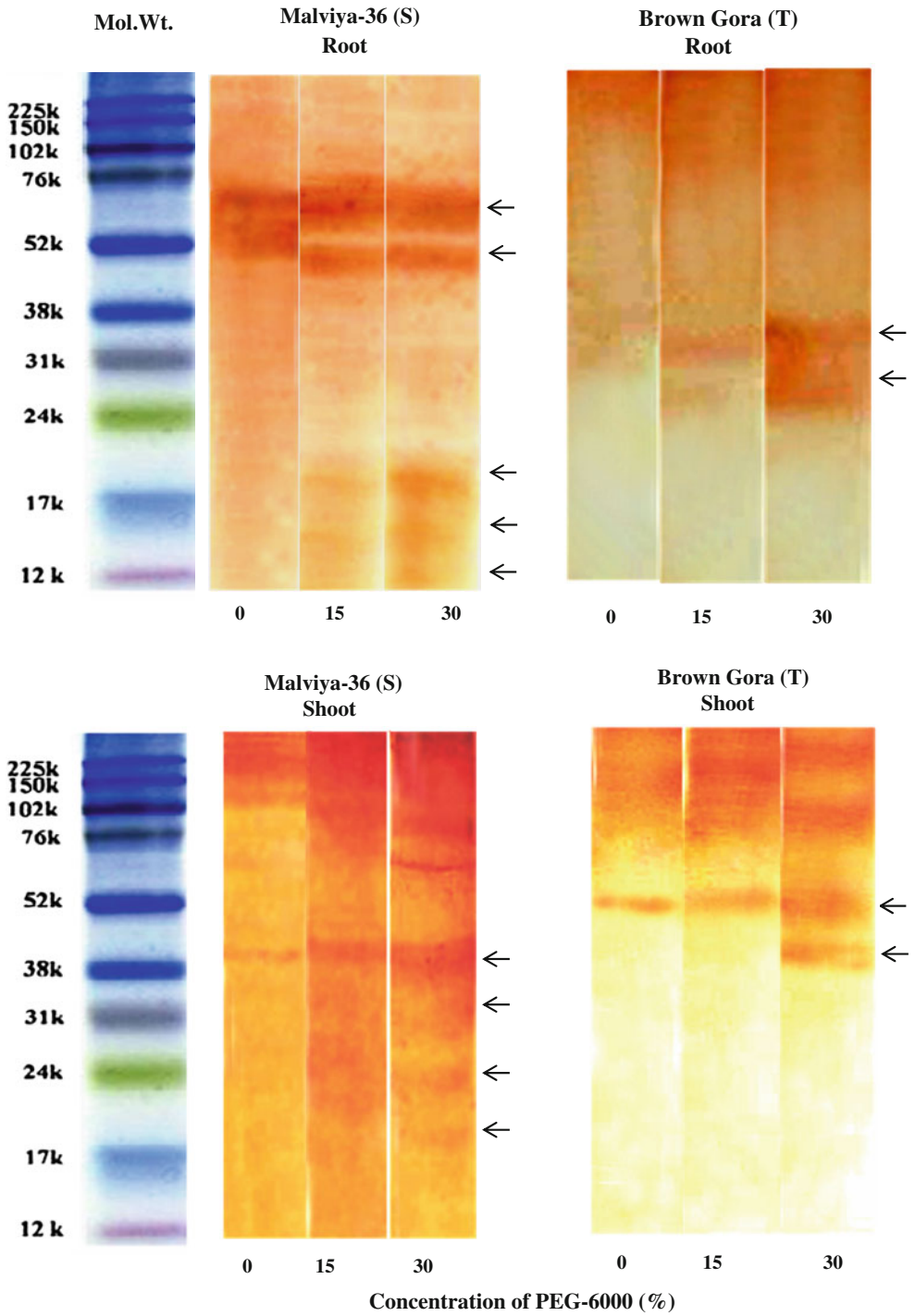

Fig. 8 Gel-blot analysis of protein-bound carbonyls in proteins extracted from roots and shoots of the rice seedlings after 48 h of water deficit treatment. S and T in parentheses indicate drought sensitive and drought tolerant rice cultivars whereas 15 and 30 % concentrations of PEG-6000 were used to create water deficit treatment levels of −1.0 and −2.1 MPa respectively. *Arrows* represent bands corresponding to protein-bound carbonyls

detection can be done with an antibody against DNPH (Romero-Puertas et al. 2002). By using this measurement as an index of water deficit induced oxidative injury in rice tissues, it can be concluded that water deficit promotes protein carbonylation in rice and that drought tolerant seedlings are characterized by a lesser degree of carbonylation compared to the sensitive under similar level of water deficit. Increased carbonylation of proteins has been

shown under water stress and oxidative stress in wheat and under Cd toxicity in pea plants (Romero-Puertas et al. 2002; Bartoli et al. 2004). In chloroplasts isolated from water-stressed wheat leaves increased carbonylation was detected using anti-DNPH antibody and the carbonylation was more evident in 41–68 kDa region (Tambussi et al. 2000). In our studies carbonylation under water deficit was more pronounced for the proteins in mol. wt. region of 17–52 kDa.

Our results of proteolytic activity measurements as well as in-gel activity staining revealed a higher degree of proteolysis in water deficit stressed seedlings of drought sensitive *cv*. Malviya-36, compared to the tolerant cv. Brown Gora. In drought sensitive genotype a direct correlation appears to exist between increase in the level of water deficit treatment and carbonylation of proteins as well as proteolytic activity. The increased carbonylation together with a higher proteolysis in water deficit stressed seedlings of sensitive *cv*. Malviya-36, unlike *cv*. Brown Gora, suggests that in the sensitive cultivar increasingly produced ROS under water deficit cause enhanced oxidative damage to proteins making the proteins more susceptible to proteolysis. It has been shown that carbonylated proteins when accumulate in the cells become toxic and also become more susceptible to proteolysis due to unfolding of target protein domains (Polge et al. 2009). In Cd exposed pea and *Arabidopsis* plants oxidized proteins were more efficiently degraded due to enhanced proteolytic activity (Romero-Puertas et al. 2002).

When rice seedlings were exposed to −2.1 MPa water deficit for 48–72 h a significant decline in the level of protein thiol was observed in roots of the seedlings of sensitive *cv*. Malviya-36, but not in the tolerant *cv*. Brown Gora. This suggests that prolonged water deficit causes oxidation of –SH groups of proteins in the roots of sensitive rice seedlings but not in the tolerant. Earlier studies have shown decline in protein thiol level due to oxidation of –SH groups in sensitive varieties of Indica rice seedlings exposed to Mn toxicity or salt stress (Srivastava and Dubey 2011; Mishra et al. 2012). Thiol groups of amino acid side chains are major target of attack due to ROS under stressful conditions (Dat et al. 2000). In drought tolerant *cv*. Brown Gora especially at early hours (24 h) of water deficit non protein thiol level declined but it increased further under prolonged water deficit. In rice, the pool of non protein thiol comprises of cysteine, γ-glutamyl cysteine, glutathione, etc. Among these, glutathione serves as an important component of antioxidative defense system and is involved in acclimation and tolerance of the plants against environmental stresses (Tausz et al. 2004; Srivastava and Dubey 2011). Rice seedlings differing in salinity tolerance, when exposed to prolonged salinity stress, however, did not show any definite pattern of alteration in the level of non-protein thiol (Mishra et al. 2012).

An important determinant of tolerance of plants towards stressful conditions could be its high ROS scavenging capacity due to efficient antioxidant defense system that includes the activity levels of antioxidative enzymes SOD, CAT and GPX. Our observations showed constitutive higher activity levels of all these enzymes examined, in the seedlings of drought tolerant *cv*. Brown Gora and a further greater increase in activity under prolonged water deficit compared to the seedlings of sensitive *cv*. Malviya-36. This suggests that tolerant rice seedlings possess a better $O_2^{\cdot-}$ and H_2O_2 scavenging capacity compared to sensitive seedlings. This agrees with other reports showing increased SOD activity in salt-tolerant cultivars of pea (Hernandez et al. 2001), rice (Mishra et al. 2012), tomato (Koca et al. 2006) and drought-tolerant cultivars of maize (Jagtap and Bhargava 1995) and common bean plants (Turkan et al. 2005) compared to activity levels in sensitive plants when subjected to respective stresses. SOD catalyzes the conversion of $O_2^{\cdot-}$ to H_2O_2 and O_2. Accumulation of H_2O_2 is toxic for the tissues and therefore it needs to be eliminated from the cell in reactions catalyzed by the enzymes CAT and GPX. A constitutive higher level of CAT activity and GPX and its further enhancement under water deficit in drought-tolerant *cv*. Brown Gora seedlings provides higher capacity to this cultivar for the decomposition of H_2O_2, compared to the sensitive cultivar, thus warding off the irreversible damaging effects caused due to H_2O_2 at the cellular and subcellular levels. Our studies showed a decline in CAT activity in shoots of seedlings of drought sensitive *cv*. Malviya-36 after prolonged duration (72 h) of −2.1 MPa water deficit. This suggest that in drought sensitive cultivar, the H_2O_2 scavenging mechanism by CAT is less effective under prolonged water deficit compared to the tolerant cultivar. This is supported by a much higher level of H_2O_2 observed in water deficit stressed seedlings of sensitive cultivar compared to tolerant. A decline in CAT activity in sensitive cultivar under prolonged water deficit could be attributed either due to inactivation of enzyme with its direct interaction with ROS (Dat et al. 2000), its decreased synthesis or impaired protein assembly (Ushimaru et al. 1999). A marked decline in CAT activity has been observed earlier in drought sensitive rice (Sharma and Dubey 2005) and wheat plants (Simova-Stoilova et al. 2010) subjected to water deficit.

Our findings thus suggest that coordinated activity of antioxidative enzymes SOD, CAT and GPX play important role in scavenging ROS and mitigating oxidative stress under water deficit in rice seedlings and that a constitutive higher status of the enzymes SOD, CAT and GPX and their further enhancement under water deficit, low level of proteolysis and reduced level of protein carbonyls in water deficit stressed plant parts can be regarded as a model for

depicting water deficit tolerance in Indica rice seedlings. The two genotypes of rice seedlings with different drought tolerance could be distinguished by the investigated physiological and biochemical parameters, however, experiments with more number of genotypes need to be conducted to generalize this conclusion.

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References

Abedi T, Pakniyat H (2010) Antioxidant enzyme changes in response to drought stress in ten cultivars of oilseed rape (*Brassica napus* L.). Czech J Genet Plant Breed 46:27–34

Bartoli CG, Gomez F, Martinez DE, Guiamet JJ (2004) Mitochondria are the main target for oxidative damage in leaves of wheat (*Triticum aestivum* L.). J Exp Bot 55:1663–1669

Beauchamp CO, Fridovich I (1971) Superoxide dismutase: improved assay and an assay applicable to acrylamide gels. Anal Biochem 44:276–287

Beers RF, Sizer IW (1952) Colorimetric method for estimation of catalase. J Biol Chem 195:133–139

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Corpas FJ, Barraso JB, Sandalio LM, Distefano S, Palma JM, Lupianez JA, del Rio LA (1998) A dehydrogenase mediated recycling system of NADPH in plant peroxisomes. Biochemical J 330:777–784

Couper A, Eley D (1984) Surface tension of polyethylene glycol solutions. J Polym Sci 3:345–349

Dat J, Vandenbeele S, Vranova E, Van Montagu M, Inze D, Van Breusegm F (2000) Dual action of the active oxygen species during plant stress responses. Cell Mol Life Sci 57:779–795

Davies MJ (2005) The oxidative environment and protein damage. Biochim Biophy Acta 1703:93–109

de Kok LJ, Kuiper PJC (1986) Effect of short-term dark incubation with chloride and selenate on the glutathione content of spinach leaf discs. Physiol Plant 68:477–482

Demiral T, Turkan I (2005) Comparative lipid peroxidation, antioxidants defense systems and proline content in roots of two rice cultivars differing in salt tolerance. Environ Exp Bot 53:247–257

Egley GH, Paul RN, Vaughn KC, Duke SO (1983) Role of peroxidase in the development of water impermeable seed coats in *Sida spinosa* L. Planta 157:224–232

Foyer C, Noctor G (2003) Redox sensing and signaling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. Physiol Plant 119:355–364

Ghezzi P, Bonetto V (2003) Redox proteomics: identification of oxidatively modified proteins. Proteomics 3:1145–1153

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem 48:909–930

Hameed A, Bibi N, Akhter J, Iqbal N (2011) Differential changes in antioxidants, proteases, and lipid peroxidation in flag leaves of wheat genotypes under different levels of water deficit conditions. Plant Physiol Biochem 49:178–185

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125:189–198

Hellmich S, Schauz K (1988) Production of extracellular alkaline and neutral proteases of *Ustilago maydis*. Exp Mycol 12:223–232

Hernandez JA, Ferrer MA, Jimenez A, Barcelo AR, Sevilla F (2001) Antioxidant systems and $O_2^{\bullet -}/H_2O_2$ production in the apoplast of pea leaves—its relation with salt-induced necrotic lesions in minor veins. Plant Physiol 127:817–831

Hsu SY, Hsu YT, Kao CH (2003) The effect of polyethylene glycol on proline accumulation in rice leaves. Biol Plant 46:73–78

Jagtap V, Bhargava S (1995) Variation in antioxidant metabolism of drought-tolerant and drought-susceptible varieties of *Sorghum bicolor* (L.) Moench exposed to high light, low water and high temperature stress. J Plant Physiol 145:195–197

Jana S, Choudhuri MA (1981) Glycolate metabolism of three submerged aquatic angiosperms during aging. Aquat Bot 12: 345–354

Job C, Rajjou L, Lovigny Y, Belghazi M, Job D (2005) Patterns of protein oxidation in *Arabidopsis* seeds during germination. Plant Physiol 138:790–802

Juszczuk IM, Tybura A, Rychter AM (2008) Protein oxidation in the leaves and roots of cucumber plants (*Cucumbis sativus* L.), mutant MSC 16 and wild type. J Plant Physiol 165:355–365

Knipling EB (1967) Effects of leaf aging on water deficit—water potential relationships of dogwood leaves growing in two environments. Physiol Plant 20:65–72

Koca H, Ozdemir F, Turkan I (2006) Effect of salt stress on lipid peroxidation and superoxide dismutase and peroxidase activities of *Lycopersicon esculentum* and L. *pennellii*. Biol Plant 50:745–758

Lawlor DW, Cornic G (2002) Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. Plant, Cell Environ 25:275–294

Levine RL, Williams J, Stadtman ER, Shacter E (1994) Carbonyl assays for determination of oxidatively modified proteins. Methods Enzymol 233:346–357

Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol 148:350–382

Michel BE, Kaufmann MR (1973) The osmotic potential of polyethylene glycol 6000. Plant Physiol 51:914–916

Mishra HP, Fridovich I (1972) The role of superoxide anion in autooxidation of the epinephrine and sample assay for SOD. J Biol Chem 247:3170–3175

Mishra P, Bhoomika K, Dubey RS (2012) Differential responses of antioxidative defense system to prolonged salinity stress in salt-tolerant and salt-sensitive Indica rice (*Oryza sativa* L.) seedlings. Protoplasma. doi:10.1007/s00709-011-0365-3

Moller IM, Kristensen BK (2004) Protein oxidation in plant mitochondria as a stress indicator. Photochem Photobiol Sci 3: 730–735

Moller IM, Jensen PE, Hansson A (2007) Oxidative modifications to cellular components in plants. Annu Rev Plant Biol 58:459–481

Moussa HR, Abdel-Aziz SM (2008) Comparative response of drought tolerant and drought sensitive maize genotypes to water stress. Aust J Crop Sci 1:31–36

Naya L, Ladrera R, Ramos J, Gonzalez EM, Arrese-Igor C, Minchin FR, Becana M (2007) The response of carbon metabolism and antioxidant defenses of alfalfa nodules to drought stress and to the subsequent recovery of plants. Plant Physiol 144:1104–1114

Pacifici RE, Davies KJA (1990) Protein degradation as an index of oxidative stress. Methods Enzymol 186:485–502

Sharma P, Jha AB, Dubey RS, Pessarakli, M (2012) Reactive oxygen species, oxidative damage and antioxidative defense mechanism in plants under stressful conditions. J Bot, Article ID 217037, 26. doi:10.1155/2012/217037

Polge C, Jaquinod M, Holzer F, Bourguignon J, Walling L (2009) Evidences for the existence in *Arabidopsis thaliana* of the proteasome proteolytic pathway activation in response to cadmium. J Biol Chem Mol Biol 284:35412–35424

Ravi RK, Krishna K, Naik GR (2011) Effect of polyethylene glycol induced water stress on physiological and biochemical responses in Pigeonpea (*Cajanus cajan* L. *Millsp*.). Rec Res Sci Tech 3: 148–152

Romero-Puertas MC, Gomez JM, del Rio A, Sandalio LM (2002) Cadmium causes the oxidative modification of proteins in pea plants. Plant, Cell Environ 25:677–686

Sekmen AH, Turkan I, Takio S (2007) Differential responses of antioxidative enzymes and lipid peroxidation to salt stress in salt-tolerant *Plantago maritima* and salt-sensitive *Plantago media*. Physiol Plant 131:399–411

Sharma P, Dubey RS (2005) Drought induces oxidative stress and enhances the activities of antioxidant enzymes in growing rice seedlings. Plant Growth Regul 46:209–221

Sikuku PA, Netondo GW, Onyango JC, Musyimi DM (2010) Chlorophyll fluorescence, protein and chlorophyll content of three nerica rainfed rice varieties under varying irrigation regimes. ARPN J Agric Biol Sci 5:19–25

Simova-Stoilova L, Demirevska K, Petrova T, Tsenov N, Feller U (2008) Antioxidative protection in wheat varieties under severe recoverable drought at seedling stage. Plant Soil Environ 54: 529–536

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

Srivastava S, Dubey RS (2011) Manganese-excess induces oxidative stress, lowers the pool of antioxidants and elevates activities of key antioxidative enzymes in rice seedlings. Plant Growth Regul 64:1–16

Tambussi EA, Bartoli CG, Beltrano J, Guiamet JJ, Araus JL (2000) Oxidative damage to thylakoid proteins in water-stressed leaves of wheat (*Triticum aestivum*). Physiol Plant 108:398–404

Tausz M, Sircelj H, Grill D (2004) The glutathione system as a stress marker in plant ecophysiology: is a stress-response concept valid? J Exp Bot 55:1955–1962

Tuanhui B, Cuiying L, Fengwang M, Fengjuan F, Huairui S (2010) Responses of growth and antioxidant system to root-zone hypoxia stress in two *Malus* sp. Plant Soil 327:95–105

Turkan I, Bor M, Zdemir F, Koca H (2005) Differential responses of lipid peroxidation and antioxidants in the leaves of drought-tolerant *P. acutifolius* and drought-sensitive *P. vulgaris* subjected to polyethylene glycol mediated water stress. Plant Sci 168:223–231

Ushimaru T, Kanematsu S, Shibasaka M, Tsuji H (1999) Effect of hypoxia on the antioxidative enzymes in aerobically grown rice (*Oryza sativa*) seedlings. Physiol Plant 107:181–187

Wang H, Liu RL, Jin JY (2009) Effects of zinc and soil moisture on photosynthetic rate and chlorophyll fluorescence parameters of maize. Biol Plant 53:191–194

Weatherley PE (1950) Studies in the water relation of cotton plant 1. The field measurement of water deficits in leaves. New Phytol 49:81–97

Xing H, Tan LL, An LZ, Zhao ZG, Wang SM, Zhang CL (2004) Evidence for the involvement of nitric oxide and reactive oxygen species in osmotic stress tolerance of wheat seedlings: inverse correlation between leaf abscisic acid accumulation and leaf water loss. Plant Growth Regul 42:61–68

Yoshida S, Forno DA, Cock JH, Gomez KA (1976) Laboratory manual for physiological studies of rice. IRRI Philippines, Manila

Zgallai H, Steppe K, Lemeur R (2005) Photosynthetic, physiological and biochemical responses of tomato plants to polyethylene glycol-induced water deficit. J Integr Plant Biol 47:1470–1478

Zhou ZS, Huang SQ, Guo K, Kant Mehta S, Zhang PC, Yang ZM (2007) Metabolic adaptations to mercury-induced oxidative stress in roots of *Medicago sativa* L. J Inorg Biochem 101:1–9