

Water deficit and aluminum interactive effects on generation of reactive oxygen species and responses of antioxidative enzymes in the seedlings of two rice cultivars differing in stress tolerance

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Abstract Aluminum (Al) is a major constraint to crop productivity in acid soils, whereas water deficit severely limits crop production in arid and semi-arid regions of the world. The objective of the present study was to examine the effects of both stresses, Al excess and water deficit, individually and in combination on the production of the reactive oxygen species (ROS) superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical, and lipid peroxidation and the activity of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and guaiacol peroxidase (GPX) in the seedlings of two rice (*Oryza sativa* L.) cvs. Malviya-36 (sensitive to water deficit and Al) and Vandana (tolerant to water deficit and Al). When 15-day grown seedlings were exposed to water deficit (created with 15 % polyethylene glycol, PEG-6000) or Al (1 mM $AlCl_3$) treatment or both treatments together for 24–72 h, the lengths and fresh weights of root/shoot declined in the seedlings of the sensitive cultivar, whereas in the tolerant seedlings, either little or insignificant decline in these parameters was observed due to the treatments. Biochemical determinations and histochemical studies revealed that under a similar level of water deficit, Al, or combined treatment, seedlings of sensitive cultivar showed a higher level of production of $O_2^{\cdot-}$, H_2O_2 , hydroxyl radical, and lipid peroxides compared to the tolerant seedlings. Seedlings of tolerant cultivars, both in roots and shoots, had constitutively higher activity levels of antioxidative enzymes SOD, CAT, and GPX and showed a greater increase in activity under water deficit or

Al treatment alone or in combination compared to the similarly treated seedlings of sensitive cultivar. Our results suggest that a lower constitutive level of ROS and a high antioxidative enzyme capacity are associated with tolerance to both water deficit and Al excess in rice seedlings.

Keywords Aluminum toxicity · Water deficit · Oxidative stress · Antioxidative defense · *Oryza sativa* L

Introduction

Crop plants growing in the natural environment are often subjected to a wide range of stresses which cause severe loss in yield. The abiotic stresses of the environment cause a series of morphological, physiological, biochemical, and molecular changes in growing plants (Srivastava and Dubey 2012). Among the abiotic stresses, water deficit in the soil and excess levels of various metals in the soil have received considerable attention. The situation gets compounded, when plants face multiple stresses at a time in various combinations. The stressful factors either alone or in combination adversely affect germination of seeds, plant growth, or productivity (Farooq et al. 2009; Mahmood et al. 2012). Rice (*Oryza sativa* L.) is a prime cereal crop of the world as it serves as staple food for more than half of the global population. Rice is a semi-aquatic plant and it often suffers from the threat of water deficit in the soil. In acid soils, Al toxicity is a major constraint in cultivation of rice crop (Pandey et al. 2013). The enormity of challenge in rice production has significantly increased due to both water deficit and Al toxicity (Pandey et al. 2013).

Water deficit in the soil is one of the most frequently encountered environmental stresses by the plants in arid and semi-arid regions of the world. Growth and photosynthesis are the key processes which are severely affected due to water

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deficit, and both of them are the major causes of decrease in crop yield (Fatima et al. 2005). Water deficit in soil causes decrease in leaf water potential, turgor loss, stomatal closure, and decrease in cell elongation and expansion (Din et al. 2011). Nearly 30 % of the world's total land area and as much as 50 % of the world's potentially arable land suffers from the problem of soil acidity, where Al toxicity is a threat for cultivation of crops (Zheng 2010). Aluminum toxicity is prevalent in acid soils and it becomes particularly more severe at soil pH less than 5.0 (Poot-Poot and Hernandez-Sotomayor 2011). A typical and most sensitive symptom of Al toxicity is rapid inhibition of root elongation (Bhoomika et al. 2013). Root elongation is also inhibited under water deficit, but under water deficit, shoot growth is more adversely affected than root growth (Yamaguchi and Sharp 2010), whereas short- and medium-term Al excess may strongly reduce root growth without affecting shoot growth (Yang et al. 2009). Further, Al toxicity reduces cell elongation along the entire elongation zone (Horst et al. 2010), whereas under water deficit, cell elongation is inhibited only in the basal and central elongation zones (Yang et al. 2013). Since crop growth largely depends on the ability of roots to explore the soil and absorb water and nutrients, restriction of the development of the root system by Al will reduce crop yield if water and nutrients are limiting (Trachsel et al. 2010). Thus, in acid soils, where water is limiting, both Al and water deficit tolerance are required to achieve optimum productivity. Therefore, studies on individual and combined stress factors of Al and water deficit are important to unravel the opportunities and constraints in breeding for adaptation to these soils.

The effects of various environmental stresses in plants are known to be mediated at least in part, due to the increased production of reactive oxygen species (ROS) (Sharma and Dubey 2011). These ROS, which include superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$), are produced as a result of successive reduction of molecular oxygen in the cells. Overproduced ROS are potentially toxic and cause oxidative damage to cellular lipids, proteins, nucleic acids, etc. Both water deficit and Al toxicity, due to overproduced ROS, pose a threat to cells by causing peroxidation of lipids, oxidation of proteins, damage to nucleic acids, enzyme inhibition, and activation of the programmed cell death (PCD) pathway ultimately leading to cell death (Sharma et al. 2012). These events ultimately cause decrease in plant growth and decline in yield (Gratão et al. 2005). To scavenge ROS and to protect the cells against oxidative damage, plants possess non-enzymic and enzymic antioxidants which play an important role in regulating the level of ROS. Among ROS scavenging enzymes, superoxide dismutase (SOD) dismutates $O_2^{\cdot-}$ to H_2O_2 and is present in cytosol and different cellular organelles. SODs are classified on the basis of metal present in catalytic site, i.e., Cu/Zn-SOD localized in cytosol and chloroplasts, Mn-SOD in mitochondrial

matrix, and peroxisomes and Fe-SOD in chloroplasts (Fernandez-Ocana et al. 2011). Catalases (CAT) eliminate H_2O_2 by breaking it down to H_2O and O_2 and do not require any reducing equivalent (Sharma et al. 2012). Peroxidases, which are located in the cytosol, vacuole, as well as extracellular space, scavenge H_2O_2 by oxidation of substrates. Guaiacol peroxidases (GPX), which include all peroxidases which can utilize guaiacol as electron donor, are important ROS scavengers because of their broader substrate specificity and strong affinity for H_2O_2 than catalase (Balakhnina and Borkowska 2013). GPXs decompose H_2O_2 to H_2O (Yadegari et al. 2013).

As oxidative stress is an important contributor to Al toxicity as well as water deficit induced damages in rice plants, and these two stresses are often experienced simultaneously in acid upland soils where soil pH is below 5.0 and water availability is limiting, the analysis of oxidative stress parameters and activities of antioxidative enzymes in sensitive and tolerant cultivars of rice will help in targeting the components of antioxidative defense system associated with both Al and water deficit tolerance in rice. Identification of such tolerance mechanisms would be helpful in breeding programs for selection of both Al- and water deficit-tolerant rice varieties.

Therefore, the present study was undertaken using two Indica rice cultivars differing in Al/water deficit tolerance with the objectives of examining the production of ROS in tissues, induction of oxidative stress, and behaviors of key antioxidant enzymes SOD, CAT, and GPX in the seedlings exposed to Al and water deficit treatment either individually or in combination.

Materials and methods

Plant material and treatment levels

Seeds of two Indica rice (*O. sativa* L.) cvs. Malviya-36 (Al/drought sensitive) and Vandana (Al/drought tolerant), commonly grown in India, were used in this study. Al/water deficit tolerance and sensitivity of these two cultivars during seedling stage were ascertained as described earlier in our laboratory based on morphological and other growth parameters in sand culture experiments (Pyngrupe et al. 2013; Bhoomika et al. 2013; Pandey et al. 2013). Seeds imbibed for 24 h in distilled water were spread on a filter paper kept in Petri plates and moistened with 0.5 mM $CaCl_2$ solution. Petri plates were kept for germination of seeds for 5 days at 28 ± 1 °C with 80 % relative humidity in humidity cum B.O.D. incubator (York Scientific Industries, New Delhi, India). Seedlings were then raised in sand cultures for another 10 days in plastic pots containing purified quartz sea sand saturated with Yoshida nutrient solution (Yoshida et al. 1976), as described earlier (Pandey et al. 2013). The pots for sand cultures were kept in

a greenhouse at 28 ± 1 °C under 80 % relative humidity and 12 h light/dark cycle with $190\text{--}200 \mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance. Seedlings thus raised for a total period of 15 days were uprooted and subjected to water deficit and Al toxicity treatments individually or in combination. For this, seedlings were exposed to either Al (1 mM) or polyethylene glycol (PEG, 15 %) treatments or in combination of both for 24, 48, and 72 h in fresh sand cultures using Yoshida nutrient solution containing 0.5 mM CaCl_2 and maintained at pH 4.5. Yoshida nutrient solution served as control. All the experiments were performed in triplicate using three replicates for each treatment.

Measurement of the reactive oxygen species $\text{O}_2^{\cdot-}$, H_2O_2 , and $\cdot\text{OH}$

The rate of production of $\text{O}_2^{\cdot-}$ was measured in roots and shoots of control and treated 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 a 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 (Spectronic 20D+, Thermo Scientific, USA). The rate of formation of $\text{O}_2^{\cdot-}$ was expressed as ΔA_{480} per minute per gram tissue fresh weight. The level of H_2O_2 in root and shoot samples was measured spectrophotometrically using titanium sulfate according to the method of Jana and Choudhuri (1981). The intensity of the yellow color developed was measured at 410 nm using a spectrophotometer. The amount of H_2O_2 was calculated using extinction coefficient of $0.28 \mu\text{M}^{-1} \text{cm}^{-1}$ and was expressed as nanomole per gram tissue fresh weight. To determine the production of $\cdot\text{OH}$, a deoxyribose assay was performed according to Halliwell and Gutteridge (1981). Fresh root/shoot samples weighing 100 mg were homogenized in 2 ml of 50 mM sodium phosphate buffer (pH 7.0) and centrifuged at $22,000 \times g$ for 10 min at 4 °C. A reaction mixture containing 1 mL of supernatant, 0.8 ml of 2.5 mM 2-deoxyribose, and 200 μL of 2 mM FeSO_4 was incubated for 1 h in the dark. This was followed by addition of 1 mL of 0.25 % thiobarbituric acid prepared in 10 % TCA. The contents were then boiled for 20 min and cooled immediately for 10 min in ice bath. After centrifugation at $3,000 \times g$ for 10 min, absorbance was read at 532 nm in the spectrophotometer.

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). Root and shoot samples weighing 200 mg were ground in 5 mL of 10 % TCA containing 0.25 % thiobarbituric acid and centrifuged at $10,000 \times g$ for 20 min. The supernatant was heated at 95 °C for 25 min and then cooled in ice bath and centrifuged at $3000 \times g$ 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 nanomole TBARS per gram fresh weight of the tissues using an extinction coefficient of $155 \text{mM}^{-1} \text{cm}^{-1}$.

Histochemical localization of $\text{O}_2^{\cdot-}$, H_2O_2 , and lipid peroxidation

Localization of $\text{O}_2^{\cdot-}$ in situ was detected in leaves of control and treated seedlings using nitrobluetetrazolium (NBT) as substrate following the method of Frahy and Schopfer (2001). Leaf discs (3–5 mm) were immersed in 6 mM NBT (prepared in 10 mM Na-citrate buffer, pH 6.0) at 25 °C for 8 h under light. NBT reacts with $\text{O}_2^{\cdot-}$ and dark blue insoluble formazan spots appear in the leaves. The leaves were then immersed in boiling ethanol (95 %) for 10 min to decolorize the leaves except the dark blue insoluble formazan deposits produced by the reaction of NBT with $\text{O}_2^{\cdot-}$. After cooling, leaf discs were photographed. Histochemical detection of H_2O_2 in situ was done according to Thordal-Christensen et al. (1997). Leaf discs, measuring 3–5 mm were incubated in a solution containing 1 mg mL^{-1} DAB (3,3'-diaminobenzidine) prepared in HCl acidified (pH 3.8) water. After 8 h of incubation at 25 °C, leaf discs were kept in boiling 95 % ethanol for 10 min and were then cleared in saturated chloral hydrate. Leaf discs were then examined under a light microscope. Reddish brown colored spots appeared characteristic of the reaction of DAB with H_2O_2 . Histochemical detection of lipid peroxides was done in roots as described by Pompella et al. (1987). The roots were stained with Schiff's reagent for 20 min, which detects aldehydes that originate from lipid peroxidation. After the reaction with Schiff's reagent, roots were rinsed with sulfite solution (0.5 % (w/v) $\text{K}_2\text{S}_2\text{O}_5$ in 0.05 M HCl). The stained roots were kept in sulfite solution to retain the staining color. Stained roots were then examined under a light microscope.

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*-nitrobluetetrazolium chloride (NBT) reduction by $\text{O}_2^{\cdot-}$ 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 $22,000 \times g$ 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 defined as the amount of enzyme required to cause 50 % inhibition of the rate of NBT reduction measured at 560 nm, and the activity was expressed as SOD units per milligram protein. 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 1000 µL 100 mM K-phosphate buffer (pH 7.0), 400 µL 200 mM H₂O₂, and 100 µL enzyme. The decomposition of H₂O₂ was followed at 240 nm (extinction coefficient of 0.036 mM⁻¹ cm⁻¹) by observing the decrease in absorbance using a UV–VIS spectrophotometer (PerkinElmer, LAMBDA EZ 201, USA). Enzyme activity is expressed as micromole H₂O₂ oxidized per milligram protein per minute. 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 contained 40 mM Na-phosphate buffer (pH 6.1), 2 mM H₂O₂, 9 mM guaiacol, and 50 µL enzyme. An increase in absorbance due to formation of tetra guaiacohinone was measured at 420 nm (extinction coefficient of 26.6 mM⁻¹ cm⁻¹) at 30-s intervals up to 2 min using a spectrophotometer (Spectronic 20D+, Thermo Scientific, USA). Enzyme-specific activity is expressed as micromole H₂O₂ reduced per milligram protein per minute.

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 (* and **) were used to identify the level of significance of the difference between control and stress treatments as * $p\leq 0.05$ and ** $p\leq 0.01$.

Results

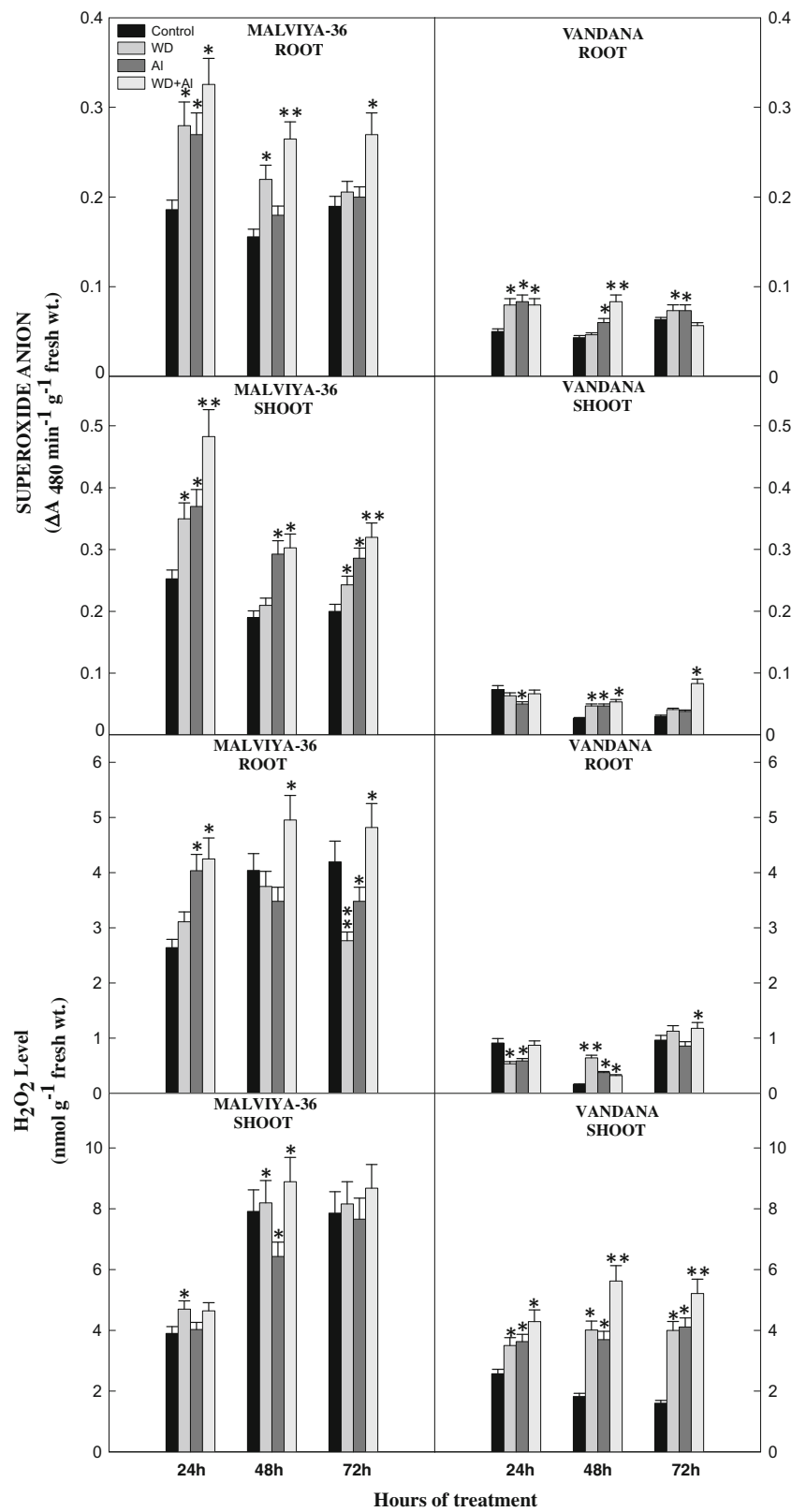
Effect of water deficit and Al on the production of O₂^{·-}, H₂O₂, and ·OH in rice seedlings

With water deficit, Al or water deficit+Al treatment for 24 to 72 h the level of O₂^{·-} increased in both roots and shoots of the seedlings of sensitive *cv.* Malviya-36 compared to controls (Fig. 1). However, in the tolerant *cv.* Vandana during 24–48-h treatments in roots and 48–72-h treatments in shoots, O₂^{·-} level increased compared to controls, but at 72 h water deficit+Al treatment in roots and 24 h of all treatments in roots, a significant decline in O₂^{·-} level was observed compared to controls. Seedlings of sensitive *cv.* Malviya-36 subjected to water deficit (15 % PEG)+Al treatment (1 mM AlCl₃) for 72 h showed nearly 42 % ($p\leq 0.05$) increased O₂^{·-} level in roots and 59 % ($p\leq 0.01$) increased level in shoots compared to controls. It was interesting to note that seedlings of tolerant cultivar in both controls and various treatments showed lower level of O₂^{·-} compared to the seedlings of sensitive cultivar.

When experiments were conducted to examine the effects of water deficit, Al or the combined treatments on H₂O₂ level in rice seedlings, it was observed that in roots and shoots of the seedlings of sensitive *cv.* Malviya-36 with 24 h of treatments, H₂O₂ level increased in the seedlings compared to controls, whereas during 48–72 h with water deficit or Al treatment, either an increase or decline in H₂O₂ level was observed (Fig. 1). However, under combined treatment of water deficit and Al, H₂O₂ level invariably increased in the seedlings compared to controls. In seedlings of tolerant *cv.* Vandana, in shoots, water deficit or Al treatment caused increase in H₂O₂ level and this level further increased with combined (water deficit+Al) treatments. However, in roots of the seedlings of tolerant cultivar, a definite trend of alteration in H₂O₂ level due to stress treatments was missing. Seedlings of sensitive *cv.* Malviya-36 subjected to water deficit+Al treatment for 72 h showed 13 % ($p\leq 0.05$) increased H₂O₂ level in roots whereas similarly stressed seedlings of tolerant *cv.* Vandana showed 18 % ($p\leq 0.05$) increased H₂O₂ level in roots and 69 % ($p\leq 0.01$) increased H₂O₂ level in shoots compared to respective controls.

The formation of ·OH was quantified in the seedlings using 2-deoxyribose oxidative degradation, after condensation of the degradation product malondialdehyde with thiobarbituric acid (TBA). In seedlings of drought/Al-sensitive cultivar when subjected to water deficit, Al or combined treatment showed higher ·OH level compared to controls (Fig. 2). However, the increased ·OH level under stress was not significant in many cases. Seedlings of drought/Al-sensitive *cv.* Malviya-36 stressed with 1 mM AlCl₃ for 72 h showed 24 % ($p\leq 0.05$) increased ·OH generation in shoots compared to controls. When seedlings of tolerant *cv.* Vandana were

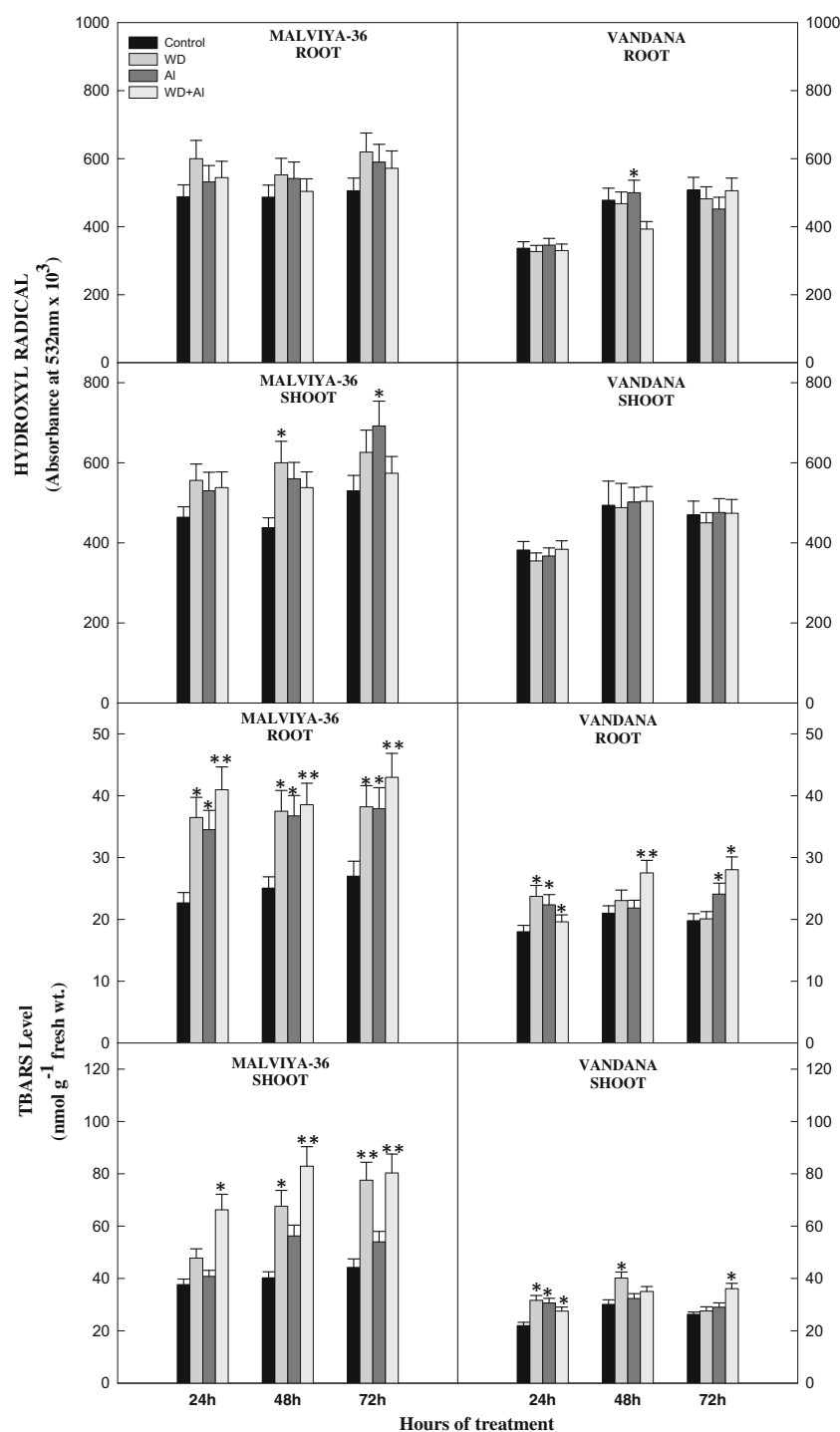
Fig. 1 Effect of water deficit (WD) created by 15 % polyethylene glycol-6000, 1 mM AlCl₃ (Al) treatment, and WD+Al treatment for 24, 48, and 72 h on contents of superoxide anion and H₂O₂ of roots and shoots of the seedlings of rice cvs. Malviya-36 (Al/drought sensitive) and Vandana (Al/drought tolerant). Rice seedlings grown for 15 days were used for the treatments. Values are mean±S.D. based on three independent determinations and bars indicate standard deviations. Asterisks * and ** represent significant differences compared to controls at $p \leq 0.05$ and $p \leq 0.01$, respectively, according to Tukey's test



exposed to similar treatments, either an insignificant increase or decline in ·OH level was observed compared to controls, except in the roots of 48 h 1 mM Al-treated seedlings where

·OH level significantly ($p \leq 0.05$) increased compared to controls. It was pertinent to note that in both controls and stress treatments, seedlings of tolerant cv. Vandana maintained

Fig. 2 Effect of water deficit (WD) created by 15 % polyethylene glycol-6000, 1 mM AlCl_3 (Al) treatment, and WD+Al treatment for 24, 48, and 72 h on contents of hydroxyl radical and thiobarbituric acid reactive substances (TBARS) in roots and shoots of the seedlings of rice cvs. Malviya-36 (Al/drought sensitive) and Vandana (Al/drought tolerant). Rice seedlings grown for 15 days were used for the treatments. Values are mean \pm S.D. based on three independent determinations and bars indicate standard deviations. Asterisks * and ** represent significant differences compared to controls at $p \leq 0.05$ and $p \leq 0.01$, respectively, according to Tukey's test



higher level of $\cdot\text{OH}$ compared to the seedlings of sensitive cv. Malviya-36.

Effect of water deficit and Al on lipid peroxidation

When the level of lipid peroxidation products was measured in terms of TBARS, it was observed that both water deficit and Al treatments either alone or in combination caused

increase in the level of TBARS in the seedlings of both set of rice cultivars with greater level in the sensitive cultivar than the tolerant (Fig. 2). In the seedlings of sensitive cultivar, water deficit caused more increase in the level of TBARS than Al treatment, whereas a combination of water deficit and Al treatment caused still a further increase in this level. Seedlings of sensitive cv. Malviya-36 exposed to 15 % PEG, 1 mM Al, and PEG+Al for 72 h showed 44 % ($p \leq 0.05$), 42 % ($p \leq 0.05$),

and 60 % ($p \leq 0.01$), respectively, increased TBARS level in roots and 73 % ($p \leq 0.01$), 22 %, and 75 % ($p \leq 0.01$) increased level in shoots. Similarly, water deficit+Al-stressed seedlings of tolerant cultivar for 72 h showed 57 % ($p \leq 0.05$) increased TBARS level in roots and 40 % ($p \leq 0.05$) increased level in shoots compared to the level in controls. Under both controls and stress treatments, seedlings of sensitive cultivar always maintained higher level of TBARS than the seedlings of tolerant cultivar.

Localization of $O_2^{\cdot-}$, H_2O_2 , and lipid peroxidation

When seedlings of the two rice cultivars were subjected to water deficit, Al treatment, or water deficit+Al treatment for 48 h and the leaves were examined for the histochemical localization of $O_2^{\cdot-}$, in the leaves of both sets of rice cultivars, NBT-stained dark blue formazan spots appeared showing localization of $O_2^{\cdot-}$ in the leaf tissues (Fig. 3a). The intensity of spots increased in water deficit-, Al- or water deficit (WD)+Al-treated seedlings compared to controls. Leaves of the stressed seedlings of drought/Al-sensitive *cv.* Malviya-36 showed greater localization of $O_2^{\cdot-}$ in the tissues compared to the similarly stressed seedlings of the tolerant *cv.* Vandana. Localization of H_2O_2 was visualized in the leaves using DAB staining as dark brown spots (Fig. 3b). Due to water deficit, Al treatment, or combined treatments, leaves of *cv.* Malviya-36 showed greater accumulation of H_2O_2 which spread throughout the entire leaf segment, whereas in the drought/Al-tolerant *cv.* Vandana, localization of H_2O_2 was observed due to water deficit/Al treatment as dark brown scattered patches on leaves. Localization of H_2O_2 appeared to be higher in the leaves of stressed seedlings of sensitive cultivar than the tolerant. Histochemical localization of lipid peroxides was observed in the roots of both sets of control and treated seedlings using Schiff's reagent. After staining, root surfaces were stained as pink-red showing extent of lipid peroxidation in the cells (Fig. 3c). Greater formation of lipid peroxides was observed in the roots exposed to water deficit, Al, or water deficit+Al treatment compared to untreated roots. Roots of the seedlings of drought/Al-sensitive *cv.* Malviya-36 showed greater localization of lipid peroxides in the tissues compared to the similarly stressed seedlings of the tolerant *cv.* Vandana.

Effect of water deficit and Al on activity of antioxidative enzymes

In the seedlings of both drought/Al-sensitive *cv.* Malviya-36 and the tolerant *cv.* Vandana, the activity of SOD increased in both roots and shoots with water deficit, Al, or WD+Al treatment (Fig. 4). Under a combined treatment of water deficit and Al for 72 h, in the seedlings of sensitive cultivar, 39 % ($p \leq 0.05$) increased SOD activity was observed in roots and 28 % ($p \leq 0.05$) increased activity in shoots, whereas under a similar

treatment level in the tolerant cultivar, 44 % ($p \leq 0.05$) increased SOD activity was noticed in roots and 41 % ($p \leq 0.05$) increased activity in shoots. Roots always maintained higher level of SOD activity than shoots in seedlings of both sets of cultivars. It was pertinent to note that constitutive as well as water deficit/Al-inducible SOD activity level was always higher in tolerant seedlings compared to the sensitive seedlings.

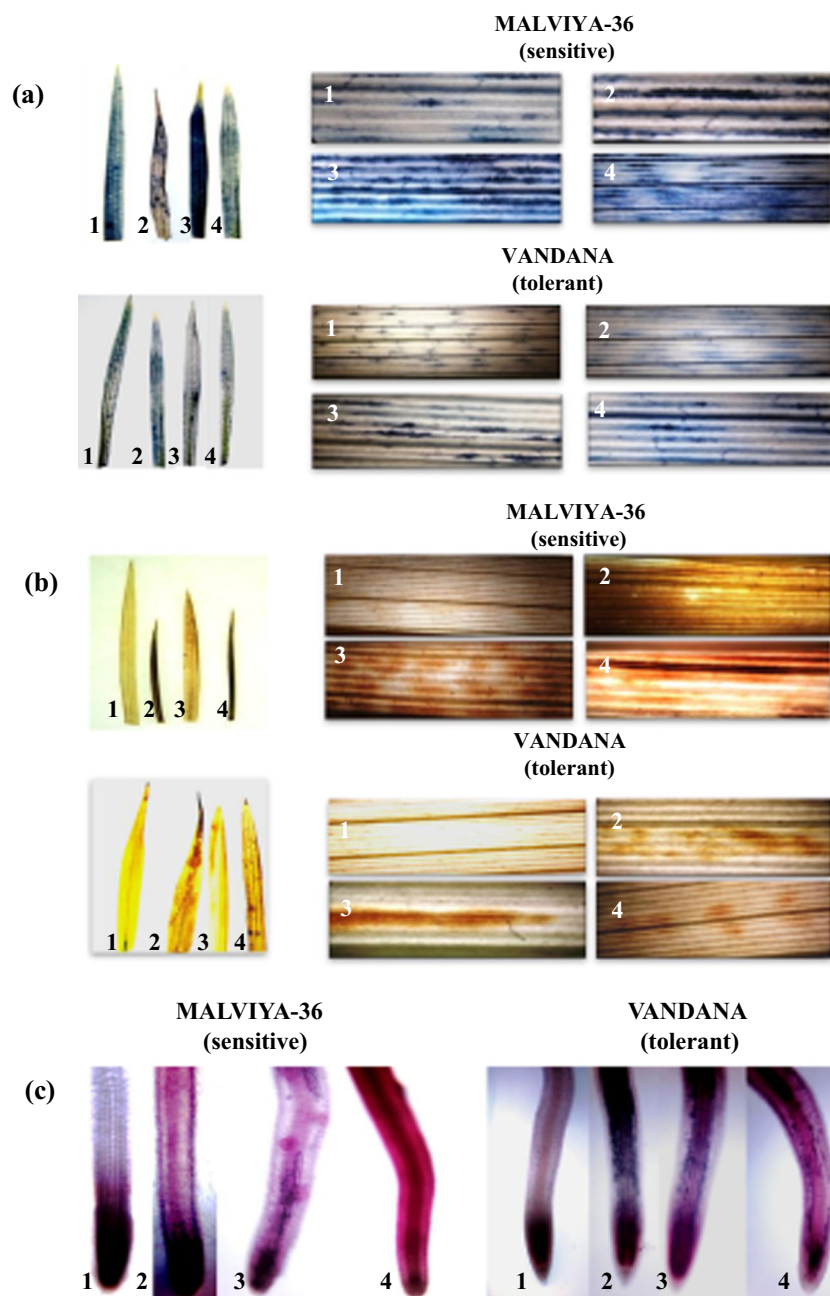
A distinct behavior of CAT activity was noticed in roots than in shoots in the seedlings of the sensitive *cv.* Malviya-36 under different treatments (Fig. 4). In the roots of this cultivar, though activity of enzyme increased under a combined water deficit and Al treatment for 24 h, but the enzyme activity declined during prolonged 48–72 h of all the treatments. However, in the shoots of this cultivar, water deficit, Al, or WD+Al treatment together caused an increased enzyme activity compared to the activity in controls. In the tolerant *cv.* Vandana, however, both in roots and shoots, CAT activity was higher in water deficit, Al, and WD+Al stress-treated seedlings compared to controls. In the sensitive cultivar, water deficit+Al treatment for 72 h led to a 55 % ($p \leq 0.01$) decline in CAT activity in roots; however, 40 % ($p \leq 0.05$) increased activity was observed in shoots, whereas in the seedlings of tolerant cultivar under similar level and duration of combined stress treatment, 20 % ($p \leq 0.05$) increased enzyme activity was observed in roots and 144 % ($p \leq 0.01$) increased activity in shoots. Similar to SOD activity level, both constitutive as well as water deficit/Al-inducible CAT activity levels were always higher in the seedlings of tolerant cultivar than the sensitive. In shoots of the tolerant *cv.* Vandana, 1 mM Al treatment led to a conspicuous greater increase in CAT activity compared to water deficit or water deficit+Al treatment. Roots maintained higher CAT activity than shoots in both the cultivars.

The activity of GPX increased in roots as well as shoots of the seedlings of both the rice cultivars due to water deficit, Al, or WD+Al treatment (Fig. 5). In controls as well as all treatments, seedlings of water deficit/Al-tolerant cultivar always showed higher level of GPX activity compared to the seedlings of the sensitive cultivar. Seedlings of the sensitive *cv.* Malviya-36 exposed to 72 h with water deficit+Al treatment showed 40 % ($p \leq 0.05$) increased GPX activity in roots and 44 % ($p \leq 0.05$) increased activity in shoots compared to controls, whereas similarly stressed seedlings of the tolerant *cv.* Vandana showed 55 % ($p \leq 0.05$) increased enzyme activity in roots and 58 % ($p \leq 0.05$) increased activity in shoots compared to the activity in controls. Roots always maintained a higher level of GPX activity than shoots, under both controls and stress treatments in both the rice cultivars.

Discussion

Aluminum toxicity and water deficit are two major abiotic stresses limiting crop productivity worldwide, more especially

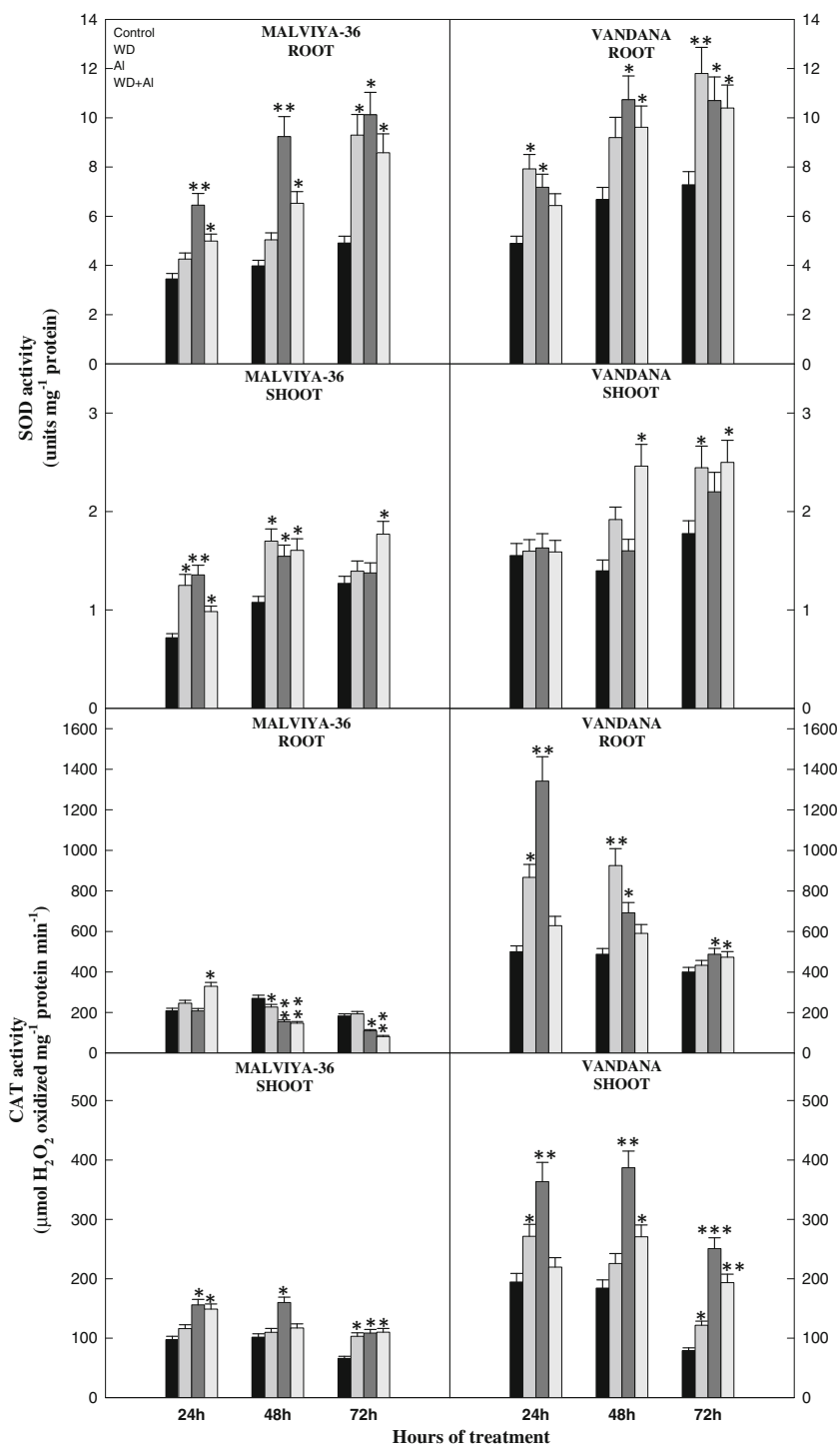
Fig. 3 Histochemical detection of **a** H_2O_2 in situ by DAB uptake and **b** $\text{O}_2^{\cdot-}$ by NBT method in rice leaves and **c** lipid peroxides in roots of rice cvs. Malviya-36 (drought/Al sensitive) and Vandana (drought/Al tolerant) after 48 h of treatments. Seedlings grown for 15 days in sand cultures were placed for 48 h in fresh sand cultures (1) containing nutrient solution which served as control, or (2) in nutrient solution containing 15 % PEG, (3) 1 mM AlCl_3 , (4) PEG+Al. *Dark blue deposits* show insoluble formazan produced by the reaction of NBT with $\text{O}_2^{\cdot-}$, whereas *dark brown spots* show reaction of DAB with H_2O_2 . *Pink-red stained root portions* represent extent of lipid peroxidation in the cells



in the tropics. There are ample evidences to suggest that exposure of plants to metal excess and water deficit leads to oxidative injury in the tissues (Sharma et al. 2012). Studies conducted by various groups of workers suggest that plant species tolerant to drought or metal toxicity possess better antioxidant capacity to protect themselves from oxidative damage due to stressful conditions compared to sensitive plants (Sharma et al. 2012; Mishra et al. 2013). Experiments were conducted on seedlings of two rice cultivars differing in stress tolerance, i.e., Malviya-36 (drought/Al sensitive) and Vandana (drought/Al tolerant) to investigate the correlations of ROS production, lipid peroxidation, and antioxidant

capacity of the plants with their ability to withstand water deficit and Al excess. Our earlier studies showed that rice cv. Malviya-36 appeared to be drought/Al sensitive and cv. Vandana as tolerant due to differential change in length, RWC, and chlorophyll content of the seedlings when exposed to similar levels of water deficit/Al treatment (Pandey et al. 2013). In the present study, water deficit was imposed in sand cultures using polyethylene glycol-6000 (PEG-6000) in the growth medium which is an ideal non-absorbed osmoticum to induce osmotic stress owing to its low penetration into the apoplastic and symplastic space because of its high molecular weight and size (Yang et al. 2013), and thus, it allows

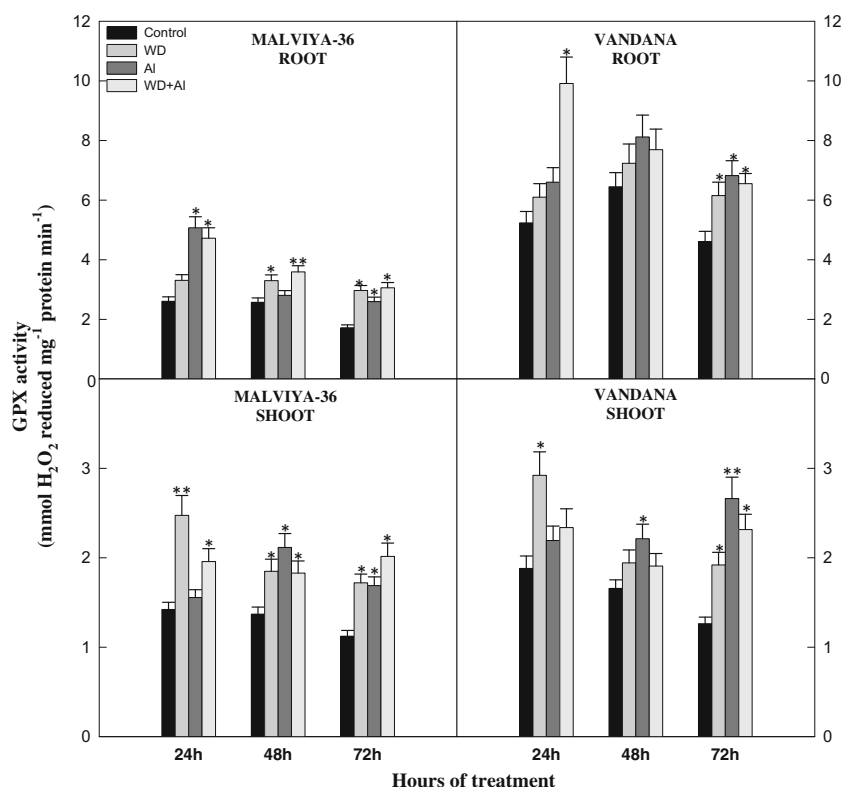
Fig. 4 Effect of water deficit (WD) created by 15 % polyethylene glycol-6000, 1 mM AlCl_3 (Al) treatment, and WD+Al treatment for 24, 48, and 72 h on the activity of superoxide dismutase (SOD) and catalase (CAT) of roots and shoots of the seedlings of rice *cv.* Malviya-36 (Al/drought sensitive) and Vandana (Al/drought tolerant). Values are mean \pm S.D. based on three independent determinations and *bars* indicate standard deviations. Asterisks *, **, and *** indicate values that differ significantly from controls at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively, according to Tukey's multiple range test



mimicking the response of plants to water deficit in the fields (Basu et al. 2010). Our earlier studies revealed that similar level of water deficit and Al treatment caused greater decline in the lengths as well as fresh weights of roots and shoots of the seedlings of *cv.* Malviya-36 as compared to *cv.* Vandana, suggesting tolerant nature of *cv.* Vandana compared to *cv.* Malviya-36 against these two stresses (Pandey et al. 2013).

Most of the stressful conditions of the environment elicit a common response involving the overproduction of ROS such as $\text{O}_2^{\cdot-}$, H_2O_2 , and $\cdot\text{OH}$ in plant cells. ROS cause a serious damage to vital organelles such as chloroplasts, mitochondria, and plasma membrane by peroxidation of membrane lipids, oxidation to proteins, and fragmentation of nucleic acids (Gill and Tuteja 2010). The present study indicated higher level of generation of the ROS, $\text{O}_2^{\cdot-}$, H_2O_2 , and $\cdot\text{OH}$ as well as

Fig. 5 Effect of water deficit (WD) created by 15 % polyethylene glycol-6000 and 1 mM AlCl₃ (Al) treatment for 24, 48, and 72 h on activity of guaiacol peroxidase (GPX) of roots and shoots of the seedlings of rice cvs. Malviya-36 (Al/drought sensitive) and Vandana (Al/drought tolerant). Values are mean±S.D. based on three independent determinations and bars indicate standard deviations. Asterisks * 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



increased lipid peroxidation in the seedlings of sensitive cultivar due to water deficit, Al toxicity, or combined treatments. Whereas in the seedlings of tolerant cultivar, the level of O₂^{•-}, H₂O₂, [•]OH, and lipid peroxidation was much lower under similar treatment conditions. The histochemical analysis results for O₂^{•-}, H₂O₂ detection in leaves, and lipid peroxides in roots of control and stressed seedlings of the two cultivars also revealed increased production of ROS and increased lipid peroxide level in the sensitive seedlings than the tolerant when subjected to similar level of stress treatments. Earlier studies have also suggested that rice plants tolerant to abiotic stresses such as salinity, drought, and metals show lower production of ROS and lower level of lipid peroxidation compared to the sensitive plants when subjected to similar level of stresses (Mishra et al. 2013; Pyngrope et al. 2013). A higher level of lipid peroxidation was seen in the seedlings of sensitive cultivar than tolerant under water deficit, Al, or combined treatment, leading to disintegration of plasma membrane, increased electrolytic leakage, and more oxidative damage to cellular organelles in the sensitive cultivar ultimately leading to reduced plant growth. Under water stress, oxidative damage to cell components is primarily mediated by O₂^{•-} (Carvalho 2008) which further causes formation of [•]OH through H₂O₂ that triggers primary symptoms observed in water-stressed plants (Wang et al. 2005). In the present study, it is evident that both water deficit and Al stress caused increased production of O₂^{•-}, H₂O₂, and [•]OH in the tissues of rice seedlings. Ali et al. (2015) have also reported a higher rate of ROS

production and lipid peroxidation in Al-stressed *Brassica napus* plants. Hernandez et al. (2001) have shown that salinity stress causes leakage of electrons from electron transport chain in the chloroplast and mitochondria to molecular oxygen resulting to overproduction of reactive oxygen species.

The overproduced ROS are scavenged enzymatically through a complex and elaborate coordination of antioxidative enzymes (Shapiguzov et al. 2012). Among these enzymes, SOD plays a prime role in protecting cells against oxidative damage by dismutating O₂^{•-} to H₂O₂ and O₂ (Wang et al. 2005). Changes in the activity of SOD are used as indicators of changes in O₂^{•-} production (Wang et al. 2005). Activities of the SOD isoforms have been shown to increase in plant cells under stressful conditions, which appear to be a part of defense mechanism of the cells under oxidative stress induced in cytosol, mitochondria, and chloroplasts (Ushimaru et al. 1995). This reaction is the first step in ROS scavenging system. Our results showed enhanced SOD activity in both the rice cultivars *vs.* Malviya-36 and Vandana under all the treatment conditions. Increased activity of SOD under water deficit/Al toxicity alone or in combination in seedlings of sensitive cultivar might be involved in scavenging increasingly produced O₂^{•-}. Whereas a substantially high activity level of SOD in the seedlings of tolerant cultivar under control as well as treatments indicates that the tolerant cultivar intrinsically maintains a high level of SOD activity irrespective of production of O₂^{•-} for an efficient scavenging of O₂^{•-}. Similar to our results, higher Al-induced SOD activity in Al-tolerant

cultivar than in Al-sensitive cultivar has been reported in soybean, maize, rice, and ryegrass (Du et al. 2010; Meriga et al. 2010; Pandey et al. 2013). Srivastava et al. (2014) have also reported enhanced SOD activity in Cd- and Pb-stressed rice seedlings. Gill et al. (2015) have shown that transgenic plants with overexpressed SOD possess higher efficiency of ROS elimination and render the plants stress tolerant.

Accumulation of H_2O_2 is toxic for the tissues, and therefore, it needs to be eliminated from the cells. Catalase and peroxidase decompose overproduced H_2O_2 in the cells due to higher SOD activity. The marked increase of CAT activity in seedlings of tolerant cultivar Vandana grown under water deficit, Al, or WD+Al treatment possibly indicates enhanced removal of H_2O_2 . The enhanced CAT activity shows its efficient role in antioxidative defense providing greater stress tolerance to the rice seedlings. Among the enzymes considered to play an important role in the cellular defense strategy against oxidative stress, CAT plays an important role as it decomposes H_2O_2 to water and O_2 . CAT has one of the highest turnover rates among the antioxidative enzymes (Sharma et al. 2012). Tolerant seedlings were characterized by high level of CAT activity under both controls as well as stress treatments as compared to the seedlings of sensitive cultivar. A decline in CAT activity in the roots of sensitive cultivar beyond 24 h of water deficit, Al, or combined treatments indicates that in this cultivar, the H_2O_2 scavenging mechanism by CAT is less effective under stress compared to the tolerant cultivar. Varying observations have been reported for alterations in CAT activity under abiotic stresses (Srivastava and Dubey 2012). The activity of CAT increased in plants subjected to salinity and heavy metal stresses (Kumar et al. 2012), whereas some plant species, when exposed to toxic concentrations of heavy metals Pb, Cd, Cu, Ni (Srivastava et al. 2014; Hussain et al. 2013; Fahr et al. 2013), drought (Sairam and Saxena 2000; Sharma and Dubey 2007), and senescence (Toivonen and Brummell 2008), showed a decline in CAT activity. Decline in CAT activity in drought-stressed or metal-exposed plants could be attributed to either inactivation of enzyme due to its direct interaction with metal ions or ROS (Dat et al. 2000) or due to its decreased synthesis or impaired protein assembly (Sahu et al. 2012).

Peroxidases are regarded as stress enzyme, and increase of peroxidase activity in stressed plants is attributed to oxidative reactions occurring in the cells due to an increase in the level of peroxides and free radicals (Sharma et al. 2010). Peroxidases have an important role in scavenging H_2O_2 in plants and have been used as a biomarker for various types of abiotic and biotic stresses (Sharma et al. 2010). Elevated activity of GPX has been reported in a wide range of environmental stresses including drought, salinity, and toxicity due to metals (Sharma et al. 2012). When the seedlings of both the rice cultivars were subjected to water deficit, Al, or WD+Al treatment, a significant elevation in the activity of GPX was observed with higher activity level in the tolerant cultivar than

the sensitive one. This suggests the crucial role of GPX in scavenging H_2O_2 in rice plants exposed to both water deficit and Al toxicity. An expressive enhancement of peroxidase activity was also observed in soybean plants exposed to 150 μ M Al (Shamsi et al. 2008) and in rice plants exposed to 421 μ M Al (Bhoomika et al. 2013). High level of GPX activity in control and stressed seedlings of tolerant cultivar suggests the contributory role of GPX in the tolerant cultivar towards providing protection against H_2O_2 .

In the present study, it was pertinent to note that roots always had higher SOD, CAT, and GPX activities than shoots under both controls and treatments, whereas production of O_2^- , H_2O_2 , and lipid peroxidation was always higher in shoots than in roots. This shows that in rice plants, roots have a more efficient antioxidative system to scavenge ROS, whereas in shoots, due to a lesser activity of antioxidative enzymes, ROS are not sufficiently scavenged leading to increased lipid peroxidation. As roots are in direct contact with the soil environment, the effects of abiotic stresses such as salinity, drought, and metal toxicity are initially experienced by roots. Therefore, an efficient ROS scavenging system in roots provides an adaptive mechanism to the plants to overcome the effects of abiotic stresses.

In conclusion, results of the present study suggest that in rice seedlings, water deficit/Al toxicity induces ROS production; oxidative stress and antioxidative enzymes SOD, CAT, and GPX appear to play an important role in scavenging ROS and withstanding oxidative stress induced by water deficit/Al toxicity. Rice cultivars differing in water deficit/Al tolerance show varying levels of ROS production and different activity levels of antioxidative enzymes when subjected to water deficit/Al toxicity treatments. Tolerance in rice plants to both of these stresses appears to be associated with higher constitutive and induced activities of the antioxidative enzymes. Therefore, breeding of crops possessing a combination of water deficit and Al tolerance in drought-prone acid soils should include the criteria of better ROS scavenging capacity marked by high activity level of antioxidative enzymes.

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