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ORIGINAL PAPER

In vitro rice shoot apices as simple model to study the effect of NaCl and the potential of exogenous proline and glutathione in mitigating salinity stress

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Abstract This study was conducted using in vitro rice shoot apices cultures of two Malaysian rice cultivars—MR 220 and MR 253 to investigate the effect of NaCl and the exogenous application of proline and glutathione in mitigating the salt-induced damages. The results showed that high NaCl concentrations (150, 200, 250 and 300) mM significantly impeded plant growth resulted in reduction in plant height, root length, biomass and chlorophyll content. Results showed that the supplementation of proline and glutathione effectively ameliorates salt stress induced damages. The plant height recorded at 150 mM NaCl (control) were 9.8 and 10.3 cm for MR 220 and MR 253. With the supplementation of 5 mM proline, the plant height of MR 220 and MR 253 increased to 14.8 and 15.0 cm respectively. Similarly, the plant height of MR 220 and MR 253 was further increased to 20.3 and 21.3 cm when 10 mM glutathione was added exogenously. Fresh weight was recorded as 0.06 g in 150 mM NaCl media for both cultivars and increased to 0.13, 0.26, 0.16, 0.23 g (MR 220) and 0.11, 0.14, 0.27, 0.32 (MR 253) with the supplementation of 5, 10, 15 and 20 mM proline respectively. It was noted that supplementation of 5 mM proline successfully increase the endogenous proline content from 9.05 to 58.4 and 15.8 to 70.5 μmol/g for both MR 220 and MR 253 respectively. In addition, supplementation of 5 and 10 mM glutathione increased chlorophyll content from 7.0 mg/g (NaCl) for both cultivars to 13.09, 17.06 mg/g for MR 220 and 14.7, 12.6 mg/g for MR 253 respectively. These results highlighted the potential role of exogenously applied proline and glutathione in mitigating the detrimental effect of salt stress.

Keywords Exogenous · In vitro · Salinity stress · Shoot apex · Proline · Glutathione

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Introduction

Rice (*Oryza sativa* L.) is one of the world's major staple food crops, providing more than 50 % of the daily calorie intake for three billion people in the Asian region (Khush 2005). Environmental constraints have always been major threats to agriculture as their detrimental effects can negatively influence the productivity and yield of plants (Ahmad and Prasad 2012). Among the abiotic stresses, soil salinity is considered as the most widespread soil toxicity problem that adversely affect plant growth and resulting in crop loss worldwide (Munns and Tester 2008). Moreover, the available land for cultivation is expected reduce to 30 % in the coming 25 years due to the salinity problem and the figure will be increased to 50 % at the year of 2050 (Wang et al. 2003). Plant suffered from two major stresses—osmotic and ionic stress when exposed to high saline condition. Increased concentration of salt in the soil immediately reduce the water uptake by the plant roots thus causing osmotic stress which eventually disrupt vital cellular functions such as photosynthesis and metabolism

(Horie et al. 2012). Ionic stress developed when plants absorbed and accumulated excessive amount of toxic ions resulted in stress symptoms such as chlorosis and necrosis of leaves (Munns and Tester 2008). Apart from that, overproduction of reactive oxygen species (ROS) during salinity stress salinity can induce secondary oxidative stress which eventually causes cellular damage through oxidation of lipids, protein and nucleic acids. (Pastori and Foyer 2002; Apel and Hirt 2004; Abogadallah 2010).

In the recent past, considerable efforts have been made in various economically important crop species to improve plant growth under salinity stress. One of them was through the application of exogenous protectants to overcome salt-induced damages (see review by Hasanuzzaman et al. 2013). Accumulation of proline is one of typical and vital adaptive mechanisms to counteract with the destructive effects of salt stress in many plant species (Hmida-Sayari et al. 2005; Parida et al. 2008). The inert compatible solute (proline), synthesized and accumulated in the cytosol and organelles served as a coordinator stabilizing cellular homeostasis thus protecting the subcellular structure and macromolecules under osmotic stress condition (Kavi Kishor et al. 2005). Proline is the only osmolyte that is capable of scavenging singlet oxygen and hydroxyl ions free radicals (Matysik et al. 2002). In addition, the involvement of proline in maintaining the cellular redox balance and reducing photoinhibition through the preservation of photosynthesis component and regulation of proline metabolism has been discussed comprehensively (see review by Verbruggen and Hermans 2008; Chaves et al. 2009). Research carried out by (Nounjan and Theerakulpisut 2012) showed that exogenous application of 10 mM proline successfully increased the endogenous proline level, and percentage of growth recovery in a salt sensitive Thai aromatic rice (cv. KDML 105) grown under salinity stress. The growth of rice seedlings (cv. Ratna) was also stimulated by the exogenous application of 20 and 30 mM proline at 100 mM NaCl (Roy et al. 1993).

Reduced glutathione (γ-Glu-Cys-Gly, GSH), is the most abundant source of non-protein thiols present in almost all cell compartments such as cytosol, chloroplasts, endoplasmic reticulum, vacuoles and mitochondria in plants (Noctor and Foyer 1998; Srivalli and Khanna-chopra 2008). Glutathione is an essential metabolite and a regulator in cellular defense against abiotic stress (Ogawa 2005). It is a powerful antioxidant which can function directly as a free radical scavenger and also react together with ascorbic acid in the ascorbate–glutathione cycle to remove harmful oxygen radicals, therefore protecting cell components from oxidation (Mittler 2002; Noctor et al. 2002). The protective role of glutathione in salt tolerance is through maintaining the cell's redox state. It was observed that under salt stress condition, higher GSH/GSSG (reduced glutathione/oxidized glutathione) ratio was observed for salt-tolerant rice cultivar BRRI dhan54 whilst the salt-sensitive BRRI dhan49 showed reduced GSH/GSSG ratio (Hasanuzzaman et al. 2014). In other plant species, exogenous application of 100 mM of glutathione was proven to restore the growth of two cotton lines treated with BSO (Buthionine sulfoximine), an inhibitor of glutathione under salinity stress (Gossett et al. 1996). Likewise, priming seeds with 100 mg/L resulted in increased growth and photosynthetic pigments in canola seedlings grown at 100 mM and 200 mM NaCl (Kattab 2007).

The variability of field conditions in terms of soil salinity from site to site as well as the physical and chemical properties of the soils has made field experiments notoriously difficult. Due to the complex and inconsistency of natural factors present in the field, analysing the response of plants to different abiotic stresses in the field experiment could be biased (Rengasamy 2002; Benderradji et al. 2012). Using in vitro tissue culture technique is probably a good approach to study the precise physiological and biochemical responses in plants towards salt-induced osmotic and ionic stresses at the cellular level (Ahmad et al. 2007). The nutrient levels, culture condition and stress levels can be conveniently manipulated to avoid unexpected disturbance in the field experiment that could contribute to undesirable results (Pérez-clemente and Gómez-cadenas 2012). The use of the in vitro shoot apices allow us to expect a similar response with regards to the whole plant since shoot apices is a mini representative of a plant with anatomical organization that has the ability to regenerate roots and leaves (Cano et al. 1998). In rice, Fadzilla et al. (1997) demonstrated on the use of shoot cultures to study the oxidative stress and antioxidant responses under salinity stress. This approach also has been applied in other plant species including tomato (Cano et al. 1998), olive (Shibli and Al-Juboory 2002) and cucumber (Abu-Romman and Suwwan 2008).

To date, rice shoot apices culture have been rarely used to study the effect of salt stress and the effectiveness of exogenous application of proline and glutathione in mitigating the salt-induced damages. Therefore, this research was undertaken to investigate the precise responses in rice towards salt stress and also to elucidate the role of different exogenous protectants to mitigate stress-induced damages. Such in vitro model could serve as a relatively simple and less labour intensive platform to investigate either the responses of rice shoot apices towards different abiotic stresses or the mitigating effects of potential protectants against stress-induced damages.

Materials and methods

Plant material

Two widely grown Malaysia *indica* rice cultivars namely MR 220 and MR 253 mature seeds were used in this experiment. The basal media for all the experiments conducted were macro and micro nutrients of Murashige and Skoog (1962) with B5 vitamins (Gamborg et al. 1968) and supplemented with 2.75 g/L of gelrite and 30 g/L of sucrose. The pH of the media was adjusted to 5.75 with 1 M of NaOH or HCl prior to autoclaving at 121 °C and 15 psi for 20 min. For the sterilization of seeds, the de husked seeds were immersed in 90 % (v/v) ethanol for 1 min, followed by soaking in 40 % commercial Clorox (containing 5 % NaOCl) for 20 min. The seeds were rinsed five times with sterile distilled water and transferred to filter paper to absorb excess water before culturing them into MS media for germination. Four days old germinated rice seedlings were excised to produce 10 mm shoot apex which is then cultured into the media supplemented with 4 mg/L Kinetin (Kin) for multiple shoots induction. The in vitro rice shoot apices used for the subsequent experiments were derived from 1 month old multiple shoots. The leaves and roots were excised from the individual shoot leaving behind a cm long of the shoot apex region.

Treatments and culture conditions

In the first experiment, the effects of salt stress on these two cultivars were evaluated by culturing the shoot apices into media supplemented with different concentrations (0, 50, 100, 150, 200, 250 and 300) mM of NaCl. A separate experiment was carried out later to investigate the growth and responses of 150 mM of NaCl-treated rice shoot apices to exogenously supplied proline and glutathione at concentrations of 0, 5, 10, 15 and 20 mM. The MSO media was the negative control while the media supplemented with NaCl was the positive control. Three rice shoot apices were cultured into a glass bottle (12 cm height × 6 cm diameter) containing 40 ml of solidified media. There were ten replicates (3 × 10) for each treatment and the experiment was repeated twice. Results were collected after 30 day of culture.

Plant growth parameters

In order to determine the plant growth, several parameters including the plant height, the longest root length, fresh weight and dry weight were recorded to evaluate the effectiveness of exogenous proline and glutathione in recovering rice shoot apices cultured under NaCl media. The plants from each treatment were removed from culture bottles, gently washed under running tap water to remove gel residue and subsequently dried on tissue paper. Plant height, fresh weight and longest root length were determined. The samples were oven-dried at 70 °C for 1 week until a constant weight obtained in order to determine the dry weight.

Total chlorophyll content

The total chlorophyll content was determined according to the method of Arnon (1949). Briefly, 0.2 g of fresh leaves was extracted with 10 ml of 80 % (v/v) cold acetone solution. The crude extract was centrifuged at 11,000 rpm for 10 min. The absorbance was read at 645 and 663 nm. The amount of chlorophyll was determined based on the following equation.

$$\text{Total chlorophyll (mg/g)} = (20.2 * \text{Abs } 645) + (8.02 * \text{Abs } 663) \times \frac{V}{1000 * W}$$

where, V = final volume of solution and, W = weight of sample.

Proline content

The proline content was quantified using the method of Bates et al. (1973) with some modifications. Fresh whole plantlet weighing 0.1 g was homogenized with 10 ml of 3 % sulfosalicyclic acid and centrifuged at 11,000 rpm for 15 min at 4 °C. The sample mixture of 2 ml of supernatant containing 2 ml of acid ninhydrin and 2 ml of glacial acetic acid were incubated at 100 °C for one hour and then cooled in ice for 2 min. Subsequently, 4 ml of toluene was added, and the mixture was agitated for 30 s. Finally, proline was extracted from the toluene layer (top layer) and the absorbance was read at 520 nm. The proline concentration was determined from a standard curve.

Lipid peroxidation

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) content in according to Heath and Packer (1968). Fresh whole plantlet weighing 0.2 g were homogenized in 4 ml of 1 % (w/v) trichloroacetic acid (TCA) and centrifuged at 11,000 rpm for 10 min at 4 °C. Subsequently, 4 ml of 20 % (w/v) TCA containing 0.5 % (w/v) 2-thiobarbituric acid (TBA) was added into 1 ml of supernatant. The mixture was heated at 95 °C for 30 min and immediately cooled in an ice bath for 2 min. The absorbance was recorded at 532 and 600 nm for non-specific absorbance. The MDA content was calculated

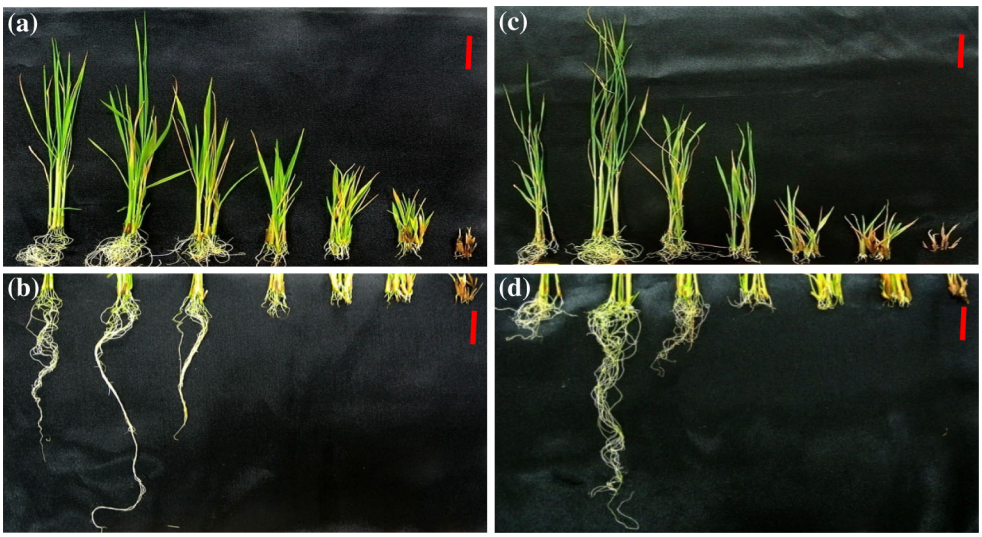

Fig. 1 Morphological appearance of MR 220 (**a**, **b**) and MR 253 (**c**, **d**) after 30 days of culture in media supplemented with (from *left* to *right*) 0, 50, 100, 150, 200, 250 and 300 mM NaCl media. (*Bar* represent 2 cm, each clump consists of 5 shoots)

using Lambert–Beer Law with extinction coefficient of 155 mM^{-1}.

Statistical analyses

Statistical analyses was carried out using SPSS version 20.0, and the comparison of each treatment was based on the one-way ANOVA analysis according to Duncan's multiple test range at a significance level of 5 % ($P < 0.05$). Different letters indicate significant difference.

Results

Effect of sodium chloride on the growth of rice shoot apex

In vitro rice shoot apices responded differently at different strength of NaCl imposed. From the morphological appearances (Fig. 1), there was no visible symptom of growth inhibition for rice shoots growing in media containing 50 and 100 mM NaCl. From the results, rice shoots cultured at 50 mM NaCl produced taller plants (20.3 and 22.5 cm) than those in 0 mM NaCl (14.5 and 11.8 cm) for MR 220 and MR 253 respectively. Also, there was significant difference in terms of longest root length in which the longest root length recorded at 50 mM NaCl were 16.7 and 18.8 cm whilst the longest root length produced at 0 mM NaCl for both MR 220 and MR 253 were 9.3 and 12.3 cm respectively (Fig. 2a, b). In contrast, drastic decreases in plant height and root length were observed starting from 150, 200, and 250 NaCl for both cultivars. The plant height measured were (9.0, 3.8, and 5.5 cm) and (10.7, 4.3 and 4.4 cm) while the root length recorded were (1.3, 0.8, 0.7 cm) and (3.0, 1.3, 0.3 cm) for MR 220 and MR 253 respectively. Shoot growth appeared to cease at 300 mM whereby there is no increment in all growth parameters measured. The threshold of rice susceptibility towards NaCl was found to be at 150 mM for both cultivars separating the mild stress (50 and 100 mM NaCl) and severe stress group (200, 250 and 300 mM NaCl). Saline growth medium caused a significant reduction in the total chlorophyll content of the rice shoots. Approximately 30 units of reduction of total chlorophyll content (32.4–1.41 and 30.38–0.22) mg/g was recorded for both cultivars in 0 and 300 mM NaCl medium (Fig. 2e). However, the chlorophyll content remained high at 50 and 100 mM NaCl with a record of (22.6 and 28.4) mg/g for MR 220 and (37.27 and 27.44) mg/g for MR 253. These values were close to chlorophyll content of rice shoots in non-stressed media (32.4 and 30.38) mg/g. Results showed that the amount of proline increased under salt stress condition for both cultivars. A significant increase was observed for MR 220 cultivar at 150 mM NaCl in which the proline content was increased by fivefold as compared to control (from 27.8 to 142.2) µmoles/g and continue to increase and peaked at 200 mM NaCl (234.8) µmoles/g. Again, a similar increment happened to MR 253 whereby the value recorded at non-stressed media was 31.31 µmol/g and increased to 66.74 and 161.45 µmol/g at 150 mM and 200 mM NaCl respectively. However, the proline content dropped at the highest NaCl concentration tested which is 300 mM NaCl for both cultivars (Fig. 2f). On the other hand, the synthesis and accumulation of proline at 0, 50 and 100 was comparatively slow resulted in proline value ranging from 27.8 to 45.2 µmol/g for both cultivars. The amount of MDA produced was inconsistent along the salt

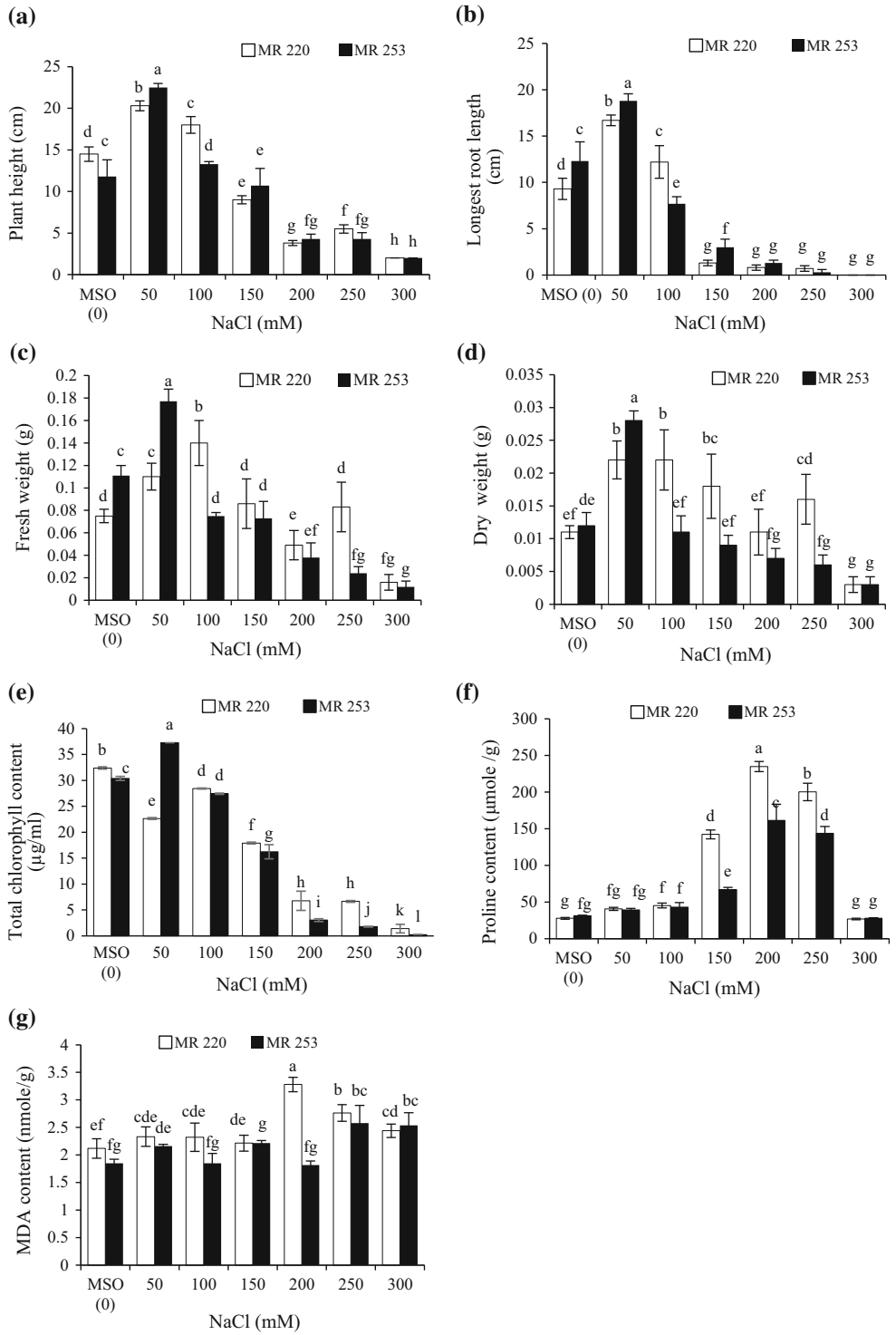

Fig. 2 Growth responses of the in vitro shoot apices on **a** plant height, **b** the longest root length, **c** fresh weight and **d** dry weight, **e** total chlorophyll content, **f** proline content and **g** MDA content after 30 days of culture under different concentrations of NaCl. Values represent mean-SE of three replication and *different letters* indicate their relative significant at $p > 0.05$ probability level

strength tested. The highest MDA value (3.2) nmoles/g was recorded at 200 mM NaCl while the other concentrations resulted in <3.0 nmoles/g for MR 220. In contrast, MR 253 rice shoots growing at 250 and 300 mM NaCl recorded the highest MDA value (2.5) nmoles/g. In addition, lower MDA content was recorded for MR 253 as compared to MR 220 in almost all tested concentrations of NaCl (Fig. 2g).

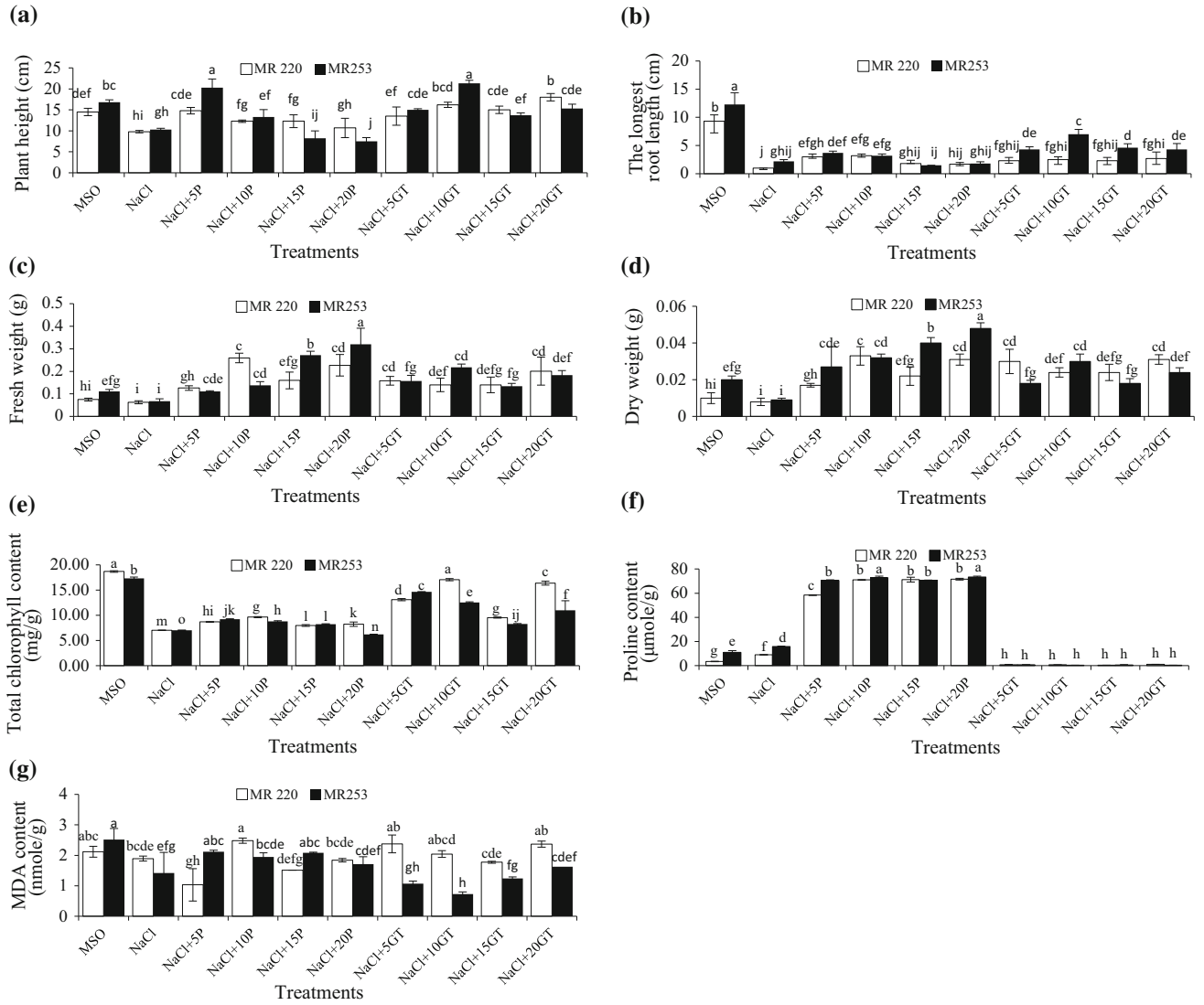

Fig. 3 Effects of exogenous application of different concentrations (mM) of proline (P) and glutathione (GT) in 150 mM NaCl media on **a** plant height, **b** the longest root length, **c** fresh weight and **d** dry weight, **e** total chlorophyll content, **f** proline content and **g** MDA content after 30 days of culture. Values represent mean-SE of three replication and *different letters* indicate their relative significant at $p > 0.05$ probability level

Effect of exogenous proline and glutathione on the growth of shoot apex under salt stress

From the previous experiment, it was found that high concentrations of salt stress demonstrated detrimental effects on the growth of rice shoots. The susceptibility threshold was found to be at 150 mM NaCl. Supplementation of exogenous proline and glutathione can significantly help to improve all the growth parameters measured as compared to the medium contained solely NaCl. Significant increase of plant height was recorded in medium supplemented with 5 mM proline and 10 mM glutathione with plant height measured at (20.3 and 21.3) cm for MR 253. These values were higher than those in both positive control (10.3 cm) as well as negative control (16.8 cm) media. Meanwhile, MR 220 responded better with the supplementation of 10 and 20 mM glutathione with plant height recorded at (16.2 and 18.0) cm respectively (Fig. 3a). The concentration of 5 mM proline was favoring the plant height. Thereafter, each increased concentration of proline was decreased the plant height. MR 220 and MR 253 recorded 14.8 and 20.3 cm of height at 5 mM proline but drop to 12.3, 17.3, 10.7 cm (MR 220) and 13.3, 8.2, 7.5 cm (MR 253) at 10, 15 and 20 mM proline respectively. The overall trend showed that the supplementation of proline and glutathione in salt stress medium slightly increased the root length as compared to the positive control media which contained solely NaCl. From the results obtained, the supplementation of 10 mM

proline produced the same root length (3.2 cm) for both cultivars. Besides that, supplementation of 5 and 15 mM of glutathione produced the same root length in MR 220 which is 2.3 cm. MR 253 showed a better response towards the supplementation of glutathione in NaCl media which produced 4.3, 7.0, 4.6 and 4.3 cm of roots at 5, 10, 15 and 20 mM glutathione respectively (Fig. 3b). The medium containing NaCl were inhibitory for biomass production of rice shoots. Whereas, medium supplemented with proline and glutathione were found to promote biomass production of rice shoots. The fresh weight and of rice shoot at 150 mM NaCl was recorded as 0.06 g for both cultivars and increased to 0.13, 0.26, 0.16, 0.23 g (MR 220) and 0.11, 0.14, 0.27, 0.32 g (MR 253) with the supplementation of 5, 10, 15 and 20 mM proline respectively. The total chlorophyll content measured was 7.0 mg/g for both cultivars at 150 mM NaCl. The supplementation of 5, 10, and 15 mM of proline increased the total chlorophyll content to 8.7, 9.6, 8.0 and 9.2, 8.8, 8.0 mg/g for MR 220 and MR 253 respectively. However, the supplementation of 20 mM of proline resulted in a significant decrease in chlorophyll content of MR 253 (6.2 mg/g). It was noted that the addition of low amount of glutathione in salt stress media effectively increased the amount of chlorophyll. The supplementation of 5 and 10 mM glutathione significantly increased the amount of chlorophyll from 7.0 mg/g for both cultivars in 150 mM NaCl media to 13.09, 17.06 mg/g for MR 220 and 14.7, 12.6 mg/g for MR 253 respectively. These values were close to 18.9 (MR 220) and 17.3 (MR 253) mg/g in negative control media. Determination of proline content indicated that there was significant increment of proline content in rice shoots after the exogenous application of proline. Results showed that the amount of proline increased under salt stress condition for both cultivars. The amount of proline quantified under non-stressed condition were 3.5 and 11.1 µmol/g and increased to 9.1 and 15.9 µmol/g under salt stress condition for MR 220 and MR 253 respectively. The proline content increased to 58.5 and 70.5 µmol/g for MR 220 and MR 253 with exogenous application of 5 mM proline. There was no significant difference in the content of proline at 10, 15 and 20 mM of proline and the amount maintained at a level higher than 70.0 µmol/g (Fig. 3f). With regards to the MDA content, a fluctuated trend was observed across all the treatment. Generally, the exogenous application of glutathione reduced the amount of MDA in MR 253 with 1.1 and 0.7 nmol at 5 and 10 mM glutathione as compared to 1.4 at 150 mM NaCl media. On the other hand, MR 220 showed a different response whereby the the MDA value decreased from 1.9 nmole (150 mM NaCl) to 1.0 nmole at 5 mM proline. Further increase in proline concentrations resulted in higher MDA value with 2.5, 1.5, 1.8 nmole at 10, 15 and 20 mM proline respectively.

Discussion

Effect of NaCl on the growth of in vitro rice shoot apex

Agriculturally important crops such as rice have a dilemma in dealing with excess amount of sodium ions because they fall into the category of glycophytes, a salt-sensitive plant. This was clearly seen from the results as all the growth parameters were impaired when the NaCl concentrations reached 150 mM and above in the growing medium. This undesirable effect was mainly due to the toxic effect of excess accumulation of Na^+ in the plant cells which also disrupts K^+ acquisition (Jose and Francisco 2002). One of the prime consequences of high concentrations of Na^+ is the loss of intracellular water which leads to the accumulation of compatible solutes (osmoprotectants) such as proline in the cytoplasm in order to counteract with the hyperosmotic condition. In this study, the drastic accumulation of proline began at 150 mM, continue to increase at 200 mM and peaked at 250 mM suggesting that the plant was suffering from osmotic stress at this level. The ability of the plant to accumulate proline under dehydrated conditions is probably due to increased biosynthesis and decreased degradation of proline (Türkan and Demiral 2009). However, this response ceased at 300 mM probably due to the halted biosynthesis of proline which eventually lead to plant death. Reduction of photosynthetic rate is a typical response in plant when confronted with salinity stress due to the reduction in water potential. The photosynthesis process will be inhibited when plant accumulated excessive toxic ions in chloroplast (Hasanuzzaman et al. 2013). Results showed the reduction in total chlorophyll content was observed in both cultivars at 100 mM NaCl and above in which the reduction was greater in MR 253 than MR 220. In contrast, at lower concentration of NaCl which is 50 mM, MR 253 showed higher total chlorophyll content as compared MR 220. There is a similar finding in *Bruguiera parviflora* whereby the rate of photosynthesis increased and the stomatal conductance remain unchanged at low salt concentrations (Parida et al. 2004). Improved growth on the plant height, root length and biomass of rice shoot apex were observed in media supplemented with 50 mM NaCl. Stimulation on increased root growth in length was observed in upland rice during drought condition (Asch et al. 2005). Both drought and salinity induced osmotic stress which could reduce water uptake. In order to prevent dehydration, it is necessary for plants to evolve some form of tolerance mechanism by producing longer roots to maximize water absorption from the surrounding. Horie et al. (2012) reviewed on the regulation of hydraulic permeability and aquaporin water channels stated that the water potential difference of rice roots was reduced but not

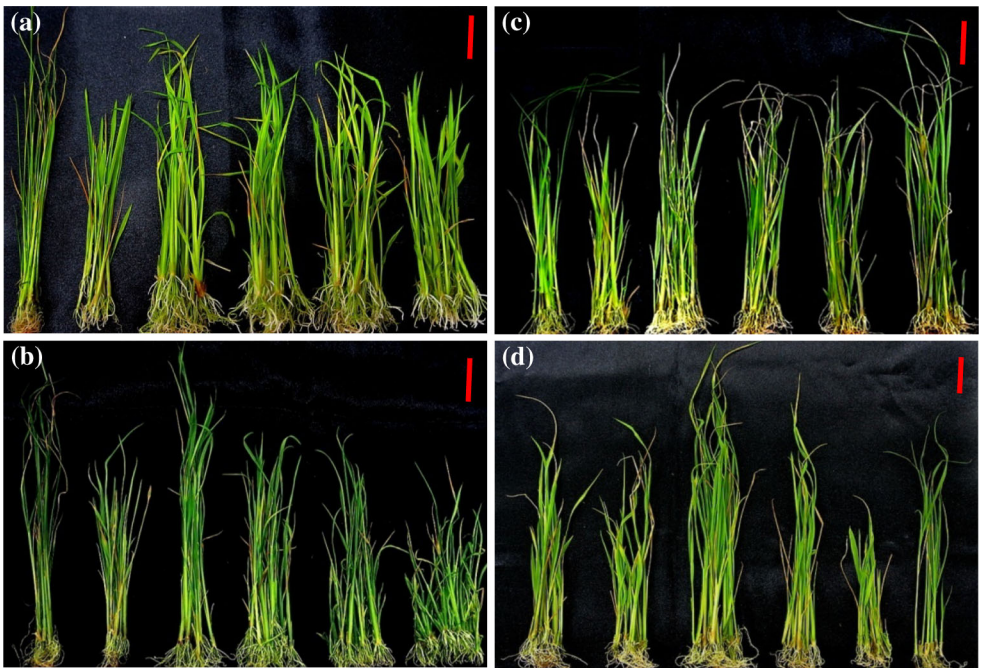

Fig. 4 Comparative effects on the rice shoots performance in proline (P) and glutathione (GT) supplemented media after 30 days of culture. **a** MR 220 and **b** MR 253 in (*left* to *right*) MSO, 150 mM NaCl, 150 NaCl+ 5 mM P, 150 mM NaCl+ 10 mM P, 150 mM NaCl+ 15 mM P and 150 mM NaCl+ 20 mM P and **c** MR 220 and **d** MR 253 in (*left* to *right*) MSO, 150 mM NaCl, 150 NaCl+ 5 mM GT, 150 mM NaCl+ 10 mM GT, 150 mM NaCl+ 15 mM GT and 150 mM NaCl+ 20 mM GT (*Bar* represent 2 cm, each clump consists of 10 shoots)

eliminated by salinity stress at 100 mM NaCl and below. Some mineral elements such as Na, Se and Si may also promote biomass production, but they are not necessary needed for the plant to survive (Subbarao et al. 2003). Besides ionic stress and osmotic stress, salinity also causes oxidative stress and nutritional imbalances in plants (Zhu 2002). MDA measures the level of lipid peroxidation which served as an important indicator on the extent of oxidative damage in cell membranes (Sharma et al. 2005). This study showed that there are no significant differences in the MDA content in the rice shoots for both cultivars at all tested concentrations of NaCl. Generally, MR 253 contained a lower amount of MDA as compared to MR 220. The lower level of lipid peroxidation in MR 253 under salinity stress condition suggested that they may have better protection against oxidative damage. In study conducted using pigmented rice, it was reported that the MDA levels in the salt-tolerant group cultured under salt stress condition were lower than in salt-sensitive group (Chutipaijit et al. 2011).

Effect of exogenous application of proline and glutathione

It has been reported that exogenous applications of plant growth regulators, fertilizers, and non-enzymatic antioxidants have been proven to minimize the detrimental effects of salinity on plant growth and yield (Kaya et al. 2010). The impressive growth of rice shoot apices in 150 mM NaCl supplemented with different concentrations of proline (5–20) mM was eye-catching (Fig. 4) in which the tiny shoot apex successfully regenerated into multiple adventitious shoots with densely grown roots. Plant counteract with ionic stress by sequestrate excessive amount of Na^+ into vacuoles to lower the amount of cytosolic Na^+ (Yamaguchi and Blumwald 2005). However, this has caused imbalance in the osmotic pressure between the cytosol and the vacuole. Proline was then synthesized and accumulated in the cytosol and organelles. This osmolyte served as a coordinator to maintain osmotic balance in plant cell thus protecting the subcellular structure and enzyme functions under stress condition (Kavi Kishor et al. 2005). From the results, it is suggested that the exogenous application of proline served as reservoir for rice resulting in the increase of the endogenous proline level. It was reported that salt stress led to stomatal closure to minimize the loss of water through transpiration. However, this resulted in restriction on the availability of carbon dioxide for carbon fixation which in turn expose the chloroplasts to excessive excitation energy and the production of undue amount of ROS (Parida and Das 2005; Ahmad and Sharma 2008). Proline is the only osmolyte reported to be involved in scavenging harmful oxygen radicals. This was explained in a study where

singlet oxygen was induced photochemically and detected as the formation of stable nitroxide radical (TEMPO). It was observed that the production of TEMPO decreased in the presence of 5 and 10 mM proline and completely undetected at 20 mM proline while glycine and other types of sugar have no effect on the singlet oxygen level (see review by Matysik et al. 2002). In addition, proline also acts as a source of carbon, nitrogen and energy during and recovery from stresses (Kavi Kishor et al. 2005). Determination of a suitable concentration of proline to apply is extremely important because high concentrations of proline can lead to adverse effect in plant growth and disruption on cellular metabolisms (Ehsanpour and Fatahian 2003; Nanjo et al. 2003). Reduction of photosynthetic rate is the prime response in plant when confronted with stress environment. Thus, it is important to investigate whether the exogenous application of proline can preserve the photosynthesis activity. From the result obtained, significant reduction in total chlorophyll content was observed in the media supplemented with 20 mM proline. The excessive exogenous application of proline is hypothesized to cause damage to ultra-structures of chloroplast and mitochondria (Hare et al. 2002). Higher fresh weight resulted from vigorous shoot growth was recorded in proline supplemented media could be another possibility that contribute to decrease chlorophyll content as energy was diverted to form new shoots. No further increment was observed in the endogenous proline content after 10 mM suggesting that a saturation point was reached. Moreover, supplementation of 20 mM of proline resulted in decreased of plant height, root length as well as total chlorophyll content. In rice, exogenous application of 40 mM proline resulted in reduced growth of early seedlings (Roy et al. 1993). Deivanai et al. (2011) found that pre-treatment of rice seeds with 1 mM proline effectively increased the germination rate under salt stress condition while the addition of high concentration of proline impair various cellular functions. Exogenous application of 10 mM proline also promoted tobacco suspension cell growth under salt stress (Okuma et al. 2000). The presented information from different research suggested that optimal concentrations of proline may be species or genotype dependent. Hence, there is a need to determine the optimal concentration before commercial application can be applied in large scale to improve salt stress tolerance.

Plants, when encountering with abiotic stresses including salinity, drought and metal toxicity, generate ROS. Glutathione is a powerful reducing agent which play an important roles during the elimination of ROS either as individual molecule or through the ascorbate–glutathione cycle (Noctor et al. 2012). The water soluble glutathione protects plants from oxidative damage in all cell compartments as their antioxidative effect is not restricted in chloroplast as that of carotenoids and α-tocopherol (Heyneke et al. 2013). These might have contributed to the low amount of proline content due to the action of glutathione as powerful ROS quencher which reduced stress in cultured rice shoots. The results of this study showed that enhanced plant growth in terms of plant height and biomass as well as lower MDA value were recorded by exogenous application of glutathione for MR 253. MR 220 responded similarly in terms of plant height and biomass but contained higher amount of MDA suggesting that the oxidative stress level might be higher. The beneficial effects of exogenous glutathione in alleviating salinity stress was demonstrated by Wang et al. (2014) using both salt-tolerant rice cultivar, Pokkali and salt-sensitive rice cultivar, Peta. Enhanced in antioxidant enzymes activities and reduced MDA content in the chloroplast was reported. In other monocots, maize seedlings treated with 100 μM glutathione resulted in dramatically diminished leaf hydrogen peroxide and MDA (Sun et al. 2012). The protective mechanism of glutathione in conferring salt tolerance is through maintaining the cell's redox state. It was observed that under saline condition, the transgenic tobacco overexpressing glyoxalase pathway enzymes showed minimal salt stress induced oxidative stress because the transgenic plants contained higher amount of glutathione and higher GSH/GSSG ratio than wild type plant (Yadav et al. 2005). The results showed that the increase in total chlorophyll content was found in both cultivars supplemented with glutathione. This finding was in agreement where priming of canola seeds with GSH significantly increased the photosynthetic pigments in 3 weeks old seedlings grown under 100 and 200 mM NaCl (Kattab 2007). The protective role of glutathione against metal-induced abiotic stresses has been frequently discussed in these few years. It was reported that pre-treatment of *Japonica* rice seedlings (Xiushui 63) with 100 μM of glutathione has effectively reduce the inhibition of growth due to cadmium toxicity (Cao et al. 2013). Besides regulating ROS levels, glutathione takes part in the regulation of growth, development, the cell cycle, gene expression, and protein activity due to its effect on the redox state of the cells (Ogawa 2005; Shao et al. 2008). Despite that, the involvement of glutathione in plant development and regulation still remain elusive, which calls for further investigations. Perhaps by understanding the function of glutathione on a molecular biology basis, one could exploit the potential use of exogenous application glutathione in mitigating the detrimental effect of abiotic stresses to plant.

In conclusion, the study indicated that the growth of rice shoot apices was significantly affected at high concentrations of NaCl and the supplementation of proline and glutathione at 5 and 10 mM has successfully mitigated the effect of salt stress at 150 mM NaCl.

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References

Abogadallah GM (2010) Antioxidative defense under salt stress. Plant Signal Behav 5:369–374

Abu-Romman S, Suwwan M (2008) Influence of NaCl salinity on growth and physiology of cucumber microshoots grown on rooting medium. Dirasat: Agric Sci 35:73–80

Ahmad P, Prasad MNV (2012) Abiotic stress responses in plants: metabolism, productivity and sustainability. Springer Science Business Media LLC, New York

Ahmad P, Sharma S (2008) Salt stress and phyto-biochemical responses of plants. Plant Soil Environ 54:89–99

Ahmad MSA, Javed F, Ashraf M (2007) Iso-osmotic effect of NaCl and PEG on growth, cations and free proline accumulation in callus tissue of two *indica* rice (*Oryza sativa*. L) genotypes. Plant Growth Regul 53:53–63

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress and signal transduction. Annu Rev Plant Biol 55:373–399

Arnon DI (1949) Copper enzymes in isolated chloroplasts Polyphenoloxidase in Beta vulgaris. Plant Physiol 24:1–15

Asch F, Dingkuhn M, Sow A, Audebert A (2005) Drought-induced changes in rooting patterns and assimilate partitioning between root and shoot in upland rice. Field Crop Res 93:223–236

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water stress studies. Plant Soil 39:205–207

Benderradji L, Brini F, Kellou K, Ykhelf N, Djekoun A, Masmoudi K, Bouzerour H (2012) Callus induction, proliferation, and plantlets regeneration of two bread wheat (*Triticum aestivum* L.) genotypes under saline and heat stress conditions. ISRN Agron Article ID 367851

Cano EA, Perez A, Moreno V, Caro M, Bolarin M (1998) Evaluation of salt tolerance in cultivated and wild tomato species through in vitro shoot apex culture. Plant Cell Tissue Organ Cult 53(1):19–26

Cao F, Liu L, Ibrahim W, Cai Y, Wu F (2013) Alleviating effects of exogenous glutathione, glycinebetaine, brassinosteroids and salicylic acid on cadmium toxicity in rice seedlings (*Oryza sativa*). Agrotechnol 2:1

Chaves MM, Flexas J, Pinheiro C (2009) Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Ann Bot 103:551–560

Chutipaijit S, Cha-um S, Sompornpailin K (2011) High contents of proline and anthocyanin increase protective response to salinity in *Oryza sativa* L. spp. *indica*. Aus J crop Sci 5(10):1191–1198

Deivanai S, Xavier R, Vinod V, Timalata K, Lim OF (2011) Role of exogenous proline in ameliorating salt stress at early stage in two rice cultivars. J Stress Physiol Biochem 7(4):157–174

Ehsanpour AA, Fatahian N (2003) Effects of salt and proline on *Medicago sativa* callus. Plant Cell Tissue Organ Cult 73:53–56

Fadzilla NM, Finch RP, Burdon RH (1997) Salinity, oxidative stress and antioxidant responses in shoot cultures of rice. J Exp Bot 48:325–331

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–168

Gossett DR, Banks SW, Millhollon EP, Lucas MC (1996) Antioxidant response to NaCl stress in a control and a NaCl-tolerant cotton cell line grown in the presence of paraquat, buthionine sulfoximine and exogenous glutathione. Plant Physiol 112:803–809

Hare PD, Cress WA, van Staden J (2002) Disruptive effects of exogenous proline on chloroplast and mitochondrial ultrastructure in Arabidopsis leaves. S Afr J Bot 68:393–396

Hasanuzzaman M, Nahar K, Fujita M (2013) Plant response to salt stress and role of exogenous protectants to mitigate salt-induced damages. In: Ahmad P, Azooz MM, Prasad MNV (eds) Ecophysiology and Responses of Plants under Salt Stress. Springer, New York, pp 25–87

Hasanuzzaman M, Alam MM, Rahan A, Hasanuzzaman M, Nahar K. Fujita M (2014) Exogenous proline and glycine betaine mediated upregulation of antioxidant defense and glyoxylase systems provides better protection against salt-induced oxidative stress in two rice (*Oryza sativa* L.) varieties. Biomed Res Int 2014, Article ID 757219

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

Heyneke E, Luschin-Ebengreuth N, Krajcer I, Wolkinger V, Müller M, Zechmann B (2013) Dynamic compartment specific changes in glutathione and ascorbate levels in Arabidopsis plants exposed to different light intensities. BMC Plant Biol 13:104

Hmida-Sayari A, Gargouri-Bouzid R, Bidani A, Jaoua L, Savoure A, Jaoua S (2005) Overexpression of D1-pyrroline-5-carboxylate synthetase increases proline production and confers salt tolerance in transgenic potato plants. Plant Sci 169:746–752

Horie T, Karahana I, Katsuhara M (2012) Salinity tolerance mechanisms in glycophytes: an overview with the central focus on rice plants. Rice 5(1):11

Jose MP, Francisco JQ (2002) Plants and sodium ions: keeping company with the enemy. Genome Biol 3(6):1017.1–1017.4

Kattab H (2007) Role of glutathione and polyadenylic acid on the oxidative defense systems of two cultivars of canola seedlings grown under saline condition. Aust J Basic Appl Sci 1:323–334

Kavi Kishor PB, Sangam S, Amrutha RN, Laxmi PS, Naidu NR, Rao KRSS, Rao Sreenath, Reddy KJ, Theriappan P, Sreenivasulu N (2005) Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: its implications in plant growth and abiotic stress tolerance. Curr Sci 88:424–438

Kaya C, Tuna AL, Dikilitas M, Cullu MA (2010) Responses of some enzymes and key growth parameters of salt stressed maize plants to foliar and seed applications of kinetin and indole acetic acid. J Plant Nutr 59:1–6

Khush GS (2005) What it will take to feed 5.0 billion rice consumers in 2030? Plant Mol Biol 59:1–6

Matysik J, Bhalu AB, Mohanty P (2002) Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants. Curr Sci 82:525–532

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7:405–410

Munns R, Tester M (2008) Mechanisms of salinity tolerance. Annu Rev Plant Biol 59:651–681

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15:473–497

Nanjo T, Fujita M, Seki M, Kato T, Tabata S, Shinozaki K (2003) Toxicity of free proline revealed in an *Arabidopsis* T-DNA-tagged mutant deficient in proline dehydrogenase. Plant Cell Physiol 44:541–548

Noctor G, Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. Annu Rev Plant Biol 49:249–279

Noctor G, Gomez L, Vanacker H, Foyer CH (2002) Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signalling. J Exp Bot 53:1283–1304

Noctor G, Mhamdi A, Chaouch S, Han Y, Neukermans J, Marquez-Garcia B, Queval G, Foyer CH (2012) Glutathione in plants: an integrated overview. Plant, Cell Environ 35:454–484

Nounjan N, Theerakulpisut P (2012) Effects of exogenous proline and trehalose on physiological responses in rice seedlings during salt-stress and after recovery. Plant Soil Environ 58(7):309–315

Ogawa K (2005) Glutathione-associated regulation of plant growth and stress responses. Antioxid Redox Signal 7:973–981

Okuma E, Soeda K, Tada M, Murata Y (2000) Exogenous proline mitigates the inhibition of growth of *Nicotiana tabacum* cultured cells under saline conditions. Soil Sci Plant Nutr 46:257–263

Parida AK, Das AB (2005) Salt tolerance and salinity effects on plants: a review. Ecotox Environ Saf 60:324–349

Parida AK, Das AB, Mohanty P (2004) Investigations on the antioxidative defense responses to stress in a mangrove, *Bruguiera parviflora* differential regulations of isoforms of some enzymes. Plant Growth Regul 42:213–226

Parida AK, Dagaonkar VS, Phalak MS, Aurangabadkar LP (2008) Differential responses of the enzymes involved in proline biosynthesis and degradation in drought tolerant and sensitive cotton genotypes during drought stress and recovery. Acta Physiol Plant 30:619–662

Pastori GM, Foyer CH (2002) Common components, networks, and pathways of cross-tolerance to stress. The central role of "Redox" and abscisic acid-mediated controls. Plant Physiol 129:460–468

Pérez-Clemente RM, Gómez-cadenas A (2012) *In vitro* tissue culture, a tool for the study and breeding of plants subjected to abiotic stress conditions. In Leva A, Rinaldi MRR (eds), Recent advances in plant in vitro culture (pp 92–108). ISBN: 978-953-51-0787-3

Rengasamy P (2002) Transient salinity and subsoil constraints to dryland farming in Australian sodic soils: an overview. Aust J Exp Agr 42:351–361

Roy D, Basu N, Bhunia A, Banerjee SK (1993) Counteraction of exogenous L-proline with NaCl in salt-sensitive cultivar of rice. Biol Plant 35:69–72

Shao HB, Chu LY, Lu ZH, Kang CM (2008) Primary antioxidant free radical scavenging and redox signalling pathways in higher plant cells. Int J Biol Sci 4:8–14

Sharma N, Gupta NK, Gupta S, Hasegawa H (2005) Effect of NaCl on photosynthetic rate, transpiration rate and oxidative stress tolerance in contrasting wheat genotypes. Photosynthetic 43:609–613

Shibli RA, Al-Juboory K (2002) Comparative responses of 'Nabali' olive microshoot, callus and suspension cell culture to salinity and water deficit. J Plant Nutr 25:61–74

Srivalli S, Khanna-chopra R (2008) Role of glutathione in abiotic stress tolerance. In: Khan NA, Singh S, S Umar (eds) Sulfur assimilation and abiotic stress in plants. Springer, Berlin, pp 207–225

Subbarao GV, Ito O, Berry WL, Wheeler RM (2003) Sodium- a functional plant nutrient. Crit Rev Plant Sci 22(5):391–416

Sun HY, Wang XY, Dai HX, Zhang GP, Wu FB (2012) Effect of exogenous glutathione and selenium on cadmium-induced changes in cadmium and mineral concentrations and antioxidative metabolism in maize seedlings. Asian J Chem 25(6):2970–2976

Türkan I, Demiral T (2009) Recent developments in understanding salinity tolerance. Environ Exp Bot 67:2–9

Verbruggen N, Hermans C (2008) Proline accumulation in plants: a review. Amino Acids 35(753):759

Wang WB, Vinocur Altman A (2003) Plant response to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta 218:1–14

Wang RL, Liu SH, Zhou F, Ding CX, Hua C (2014) Exogenous ascorbic acid and glutathione alleviate oxidative stress induced by salt stress in the chloroplast of *Oryza sativa* L. Z Naturforsch 69c:226–236

Yadav SK, Singla-Pareek SL, Reddy MK, Sopory SK (2005) Transgenic tobacco plants overexpressing glyoxalase enzymes resist an increase in methylglyoxal and maintain higher reduced glutathione levels under salinity stress. FEBS Lett 579:6265–6271

Yamaguchi T, Blumwald E (2005) Developing salt-tolerant crop plants: challenges and opportunities. Trends Plant Sci 10:615–620

Zhu JK (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol 53:247–273