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Research Article

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Regulation of enzymatic and non-enzymatic antioxidants contributes to salt tolerance in hitherto unknown upland farmer rice varieties

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ABSTRACT

Salt tolerance potential of the three upland farmer varieties, koduvelliyan, mullankayama and marathondi was evaluated by comparing with the released salt-tolerant pokkali variety, vytilla-2. The salt tolerance exhibited by the upland varieties was identical with the salt tolerant variety. The superoxide (O_2^-) content in the upland varieties was lower; however, the hydrogen peroxide (H_2O_2) content increased with the salt concentration. The lesser malondialdehyde (MDA) content in the koduvelliyan variety was equivalent to the vytilla-2 and slight increase was observed in mullankayama and marathondi. The ascorbate (AsA) content in the upland varieties was comparable to vytilla-2 and upon exposure to increased concentration of NaCl, the AsA level reduced in all the treatments. Reduced glutathione (GSH) content was uniform in all the varieties up to a concentration of 100mM NaCl, however, in 125-150mM NaCl, mullankayama showed a pronounced increase in GSH content. Under salt stress, due to the formation of O_2^-, the oxidation of GSH was higher, maintaining a stable GSH/GSSG ratio. Superoxide dismutase (SOD) and catalase (CAT) activity of the upland varieties was higher than vytilla-2 up to 100mM NaCl, however, in 125-150mM NaCl the SOD activity increased slightly and the CAT activity decreased. Ascorbate peroxidase (APX) activity increased in upland varieties up to 125mM NaCl, and in 150mM NaCl, maintained a steady level in all the varieties. Glutathione reductase (GR) activity increased proportionate with NaCl concentration; with highest activity in all the upland varieties. Monodehydroascorbate reductase (MDHAR) activity was uniform in all the varieties up to 100mM NaCl, however, in 125 and 150mM NaCl, vytilla-2 showed higher MDHAR activity. Dehydroascorbate reductase (DHAR) activity was lesser in upland varieties under salt stress compared to vytilla-2. The GSH/GSSG ratio decreased in marathondi and koduvelliyan varieties with the increase in NaCl concentration, however, in mullankayama and vytilla-2, the GSH/GSSG ratio was higher. The membrane stability index of all the varieties was uniform in all the concentrations of NaCl used, except marathondi. The Na^+ content in all the varieties increased in relation to NaCl concentration and the K^+ efflux was higher suggesting a higher Na^+/K^+ ratio, with increased NaCl concentration.

KEYWORDS: Salt tolerance, upland farmer varieties, enzymatic and non- enzymatic antioxidants, antioxidant system.

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INTRODUCTION

Rice (*Oryza sativa* L.) is an important crop that feeds more than half of the world's population and approximately 90% of the world's production and consumption are in Asia [1] In pursuit of biotic and abiotic tolerance in wild varieties of cultivated crop plants resulted in remarkable breakthroughs that revolutionised the agriculture sector. Kerala with its long coastal line of about 580 KM has several lagoons and backwaters covering a very large area linked to the sea. In most of the coastal land, deltaic areas at river mouths and reclaimed backwaters are either at sea level or 1.0 to 1.5 m below MSL. This leads to the intrusion of sea water up to a distance of 10 to 20 KM upstream during high tides [2]. The intrusion of seawater into the rice fields is a major threat to the richest rice-growing areas of the world [3]. In Kerala, the Pokkali and Kaipad rice cultivation system are well known for their salt tolerance potential, though not characterised and the salt tolerance potential of the upland rice varieties are not investigated so far. Salinity can be associated with the intrusion of salt water to inland fresh water and exposes more rice-growing areas to salinity [4]. Rice is very sensitive to salinity stress and is currently listed as the most salt-sensitive cereal crop with a threshold of 3 dSm^{-1} for most cultivated

varieties [5]. The identification of salt tolerance potential in the upland varieties has evolutionary significance and also can be utilised for breeding and marker-assisted selection. The inherent salt tolerance potential in the upland varieties might have arisen due to the geographical and environmental adaptations developed during evolution. Rice production in salt-affected soil is significantly reduced with an estimated annual loss of 30–50% [6]. Accretion of salt in the soil is affected by various factors in different geological and climatic regions [7]. Salinity in the soil affects the osmotic and ionic potential and oxidative stress in plants [8], creating water and nutrient balance, changes in the hormone metabolism, gaseous exchange and the production of reactive oxygen species, which will compromise cell expansion and division culminate in plant death due to senescence [9]. Strategies were developed using conventional breeding and biotechnological approaches to develop rice plants with abiotic stress tolerance using the genes related to stress tolerance identified from different plants [10].

The development of salt-tolerant rice by conventional or applied methods is a time-consuming process; hence identification of natural salt tolerant varieties can be a boost to saline land agriculture. The impact of salt exposure results in the production of superoxide radicals (O_2-) and to combat them, enzymatic and non-enzymatic antioxidants are produced. The charged O_2^- cannot move freely across the cell membranes, hence, sub-cellular compartmentalisation of the antioxidant system is necessary for efficient quenching of this anion and its immediate product H_2O_2 at their site of production. The salt tolerance mechanism in rice should focus on multiple stress tolerance with long adaptability and higher grain yield with proper compartmentalisation of stress alleviators [11].

Plants develop an array of ROS scavenging mechanism involving the production of antioxidant substances like ascorbate and reduced glutathione-mediated by enzymes like superoxide dismutase (SOD), peroxidases (POD), catalases (CAT) and ascorbate-glutathione cycle enzymes [12]. Studies were conducted on the up-regulation of the enzymatic and non-enzymatic antioxidants in plants exposed to abiotic or biotic stress [13]. Synchronous action of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) lead to the scavenging of ROS. APX uses the reduced ascorbate (AsA) as a reductant in the first reaction of ascorbate –glutathione cycle, which is the most important peroxidase in detoxifying H_2O_2 [14]. Correlation between the ascorbate-glutathione cycle enzymes and salt tolerance is compared in the sensitive and resistant varieties of rice [15].

The present study aimed at detecting the salt tolerance potential of the upland farmer varieties (koduvelliyan, mullankayama and marathondi) by comparing with released salt tolerant pokkali variety (vytilla-2) using the enzymatic and non-enzymatic antioxidants under different salt concentrations.

MATERIALS AND METHODS

Plant material

The upland farmer varieties koduvelliyan, mullankayama and marathondi were collected from the local farmers of Mananthavady, Wayanad district, Kerala and the released salt tolerant variety; vytilla-2 was procured from the Rice Research Station, Kerala Agricultural University, Vytilla, Ernakulam. The seeds were germinated and the seedlings were planted in pots filled with a potting mixture and immersed in tanks filled with water up to the rim of the pots. The addition of salt was initiated when the plants were 21 days old and NaCl was incremented at a concentration of 25mM per day until a final concentration of 25, 50, 75,100,125 and 150mM was attained. Leaf samples were collected from the plants after 7, 14 and 21 days of salt treatment and used for the assay.

Quantification of superoxide content

The O_2^- was measured according to Li et al. [16]. Leaf tissues (200 mg) were macerated in 65 mM phosphate buffer (pH 7.8) and centrifuged at 10,000 rpm for 10 min. The supernatant was mixed with 65 mM phosphate buffer (pH 7.8) and 10 mM hydroxylamine hydrochloride and incubated at 25ºC for 20 min. Sulphanilamide (17 mM) and 7 mM α-naphthylamine were added to the mixture, incubated for 20 min at 25º C and the absorbance was measured at 530 nm. A standard curve was prepared using nitrogen dioxide (NO_2) and the O_2^- generation rate was calculated.

Quantification of hydrogen peroxide

Hydrogen peroxide (H_2O_2) content was quantified using the method of Velikova et al. [17]. Leaf tissue (0.5g) was macerated in 2.0 ml 0.1% (w/v) trichloro acetic acid (TCA), homogenized and centrifuged at 12,000 rpm for 15 min at 4º C. Subsequently, 0.25 ml supernatant was collected, 0.75 ml buffer solution consisting of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M potassium iodide (KI) were added and the final volume was made up to 2.0 ml and the absorbance was measured at 390 nm using H_2O_2 as standard.

Determination of lipid peroxidation using MDA content

The malondialdehyde (MDA) content was determined as described by Stewart and Bowley [18]. Leaf tissue (0.5g) was macerated in LN_2 and homogenized in 5.0 ml distilled water and 5 ml 20% trichloroacetic acid (containing 0.5% thiobarbituric acid) and incubated for 30 min at 95°C. The reaction was arrested by cooling the reaction mixture and centrifuged at 10,000 rpm for 30 min. The supernatant was collected and the absorbance was recorded at 535 nm and 600 nm. TBA forms complexes with MDA, a by-product of the peroxidation reaction. The concentration of the MDA/TBA complex was calculated and expressed as the level of lipid peroxidation.

Ascorbic acid content

The AsA content was assayed using leaf sample (0.5g) macerated with 5% (v/v) trichloroacetic acid (4 ml) and centrifuged at 12,000rpm at 4ºC for 15 min. One ml of the supernatant was mixed with 100mM phosphate buffer (pH7.7) (0.5ml), 10% (v/v) TCA (1 ml), 44% (v/v) H_3PO_4 (1ml), 4% (v/v) bipyridyl (1ml) and 3% (v/v) $FeCl_3$ (0.5ml). The mixture was kept at 37º C for 1 hour and cooled down to room temperature and the OD was recorded at 525nm. A standard curve was prepared using a gradient concentration of AsA [19].

Glutathione (GSH) content

The total GSH and the reduced GSH was assayed using standard protocol [20]. Leaf sample (0.5g) was taken and macerated in 4 ml 5% (v/v) trichloroacetic acid and centrifuged at 12,000rpm at 4ºC for 15 min. Supernatant (1 ml) was taken and mixed with 100mM phosphate buffer (2.5ml, pH 7.7) and 0.6mM DTNB (0.2ml). The OD was recorded at 412nm and GSH was quantified. For quantification of total GSH, the reaction mixture was prepared by adding one ml supernatant, 100mM phosphate buffer (pH 7.7), 0.2ml 0.6mM DTNB along with 1U glutathione reductase. The absorbance was measured at 412 nm. Oxidised glutathione (GSSG) concentration was calculated using the formula: total GSH – reduced GSH.

Determination of Na^+ influx and K^+ efflux and membrane stability index (MSI)

Leaf samples from different NaCl treatments and control were collected, oven-dried and acid digested according to Allan [21]. A known volume of the dried leaf samples was digested by refluxing in a mixture of sulphuric acid and perchloric acid in 10:4 ratios, heated in a Kjeldahl's flask until the solution became colourless. The digest was filtered and transferred to a standard flask, and the volume was made up to 50 ml using deionized water and analysed for Na^+ and K^+ using a Flame photometer.

Leaf membrane stability index (MSI) was assessed according to the protocol explained by Sairam [22]. Leaves from salt treated plants were thoroughly washed in running tap water followed by washing with double distilled water. The washed leaves were heated in 10ml double distilled water at 40°C for 30 min. Electrical conductivity (C1) was recorded using Electrical conductivity (EC) meter. Subsequently, the same samples were placed in a boiling water bath (100°C) for 15 min and their electrical conductivity (C2) was also recorded. MSI can be calculated using the formula;

Membrane Stability Index (MSI) = [1 - (C1/C2)] * 100

Quantification of enzymatic antioxidants

Superoxide dismutase and CAT was extracted using the frozen sample (0.5g), powdered in LN_2, homogenized by adding 100 mM phosphate buffer (4ml, pH 7.0), 1mM EDTA (16µl) and 1% (w/v) polyvinyl pyrrolidone (PVP). The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant was collected to a fresh Eppendorf tube. APX was assayed using leaf sample (0.5 g) homogenized in extraction medium contained 50 mM potassium phosphate buffer (pH 7.0), 1mM EDTA, 2 % (w/v) PVP and 1 mM ascorbic acid. The homogenate was centrifuged at 15,000 rpm for 20 min at 4°C. For the glutathione reductase (GR) assay, the sample (0.5g) was powdered in LN_2, homogenized in 100 mM potassium phosphate buffer (pH 7.8) containing 2 mM EDTA and 1% (w/v) PVP-40 at 4°C. The homogenate was centrifuged at 15,000 rpm for 20 min at 4°C and the supernatant was collected in a fresh Eppendorf tube and used for enzyme assay. MDHAR and DHAR was assayed using powdered plant sample (0.5g) homogenized in 50mM potassium phosphate buffer (pH 7.8) and 1mM EDTA. Homogenate was centrifuged at 14,000 rpm for 20min at 4°C and the supernatant was collected for further assay.

Determination of the specific activity of antioxidant enzymes

Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured according to Giannopolitis and Ries [23] based on the photoreduction of nitroblue tetrazolium. The assay mixture contained 1.5M sodium carbonate (0.1ml), 13mM methionine (0.3ml),1mM EDTA (0.3ml), 1.3 µM Riboflavin (0.3ml) and 0.63mM nitroblue tetrazolium chloride (0.3ml, NBT). Enzyme extract (0.1ml) was added to the assay mixture as well as to the dark control. The reaction mixture was made up to 3ml using 50mM potassium phosphate buffer (pH 7.8) and incubated for 30min in the light as well as dark. OD was measured at 560nm and one unit SOD was measured as the quantity of enzyme required to inhibit 50% NBT under experimental conditions and the activity was expressed as unit enzyme mg $protein^{-1}$.

Catalase (CAT; EC 1.11.1.6) activity was measured according to Aebi [24]. 100 ml of the diluted enzyme extract was mixed with one µl 50 mM potassium phosphate buffer (pH 7.0).The reaction was initiated by the addition of 100mM H_2O_2 (100 µl) and incubated at 28°C. The blank contained distilled water in lieu of the enzyme extract. Change in absorbance at 240nm due to the degradation of H_2O_2 was recorded at an interval of 15s for 2 min. The specific activity of CAT was calculated as µmole of H_2O_2 decomposed mg $protein^{-1}$ min^{-1}.

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was measured according to Nakano and Asada [25]. The assay mixture consists of 0.5mM ascorbate (1.66ml) in 50mM potassium phosphate buffer (pH 7.0) and 0.08ml enzyme extract. The reaction was initiated by adding 0.1 ml, 2mM H_2O_2 and the decrease in absorbance due to ascorbate oxidation was recorded at 290nm at 15-s intervals for 2min and the specific activity was calculated and expressed as µmole mg $protein^{-1}$ min^{-1}.

Glutathione reductase (GR; EC 1.6.4.2) activity was determined according to Smith et al. [26] with some modifications. The reaction mixture consists of 100mM potassium phosphate buffer (pH 7.6), 1mM EDTA, 5mM NADPH, 6mM 5.5'-dithio-bis (2- nitrobenzoic acid) [DTNB] and 0.2 mM oxidised

glutathione (GSSG) and 100 μl diluted enzyme extract. The reaction was initiated by the addition of 5mM NADPH. Change in absorbance at 412 nm was recorded at 15-s intervals for 2 min due to the oxidation of NADPH and the specific activity of the enzyme was calculated and expressed as μmole mg protein^{-1} min $^{-1}$.

MDHAR (EC 1.6.5.4) activity was measured in the reaction mixture containing Tris-HCl buffer (pH 8.0), 0.2 mM NADH, 2.5 mM ascorbic acid and 49.6 μl enzyme extract. The reaction was initiated by the addition of 0.4 units of ascorbate oxidase that generated the monodehydro ascorbate radical. MDHAR activity was assayed by monitoring the decrease in absorbance at 340 nm owing to the oxidation of NADH [27]. One unit enzyme activity was calculated as the quantity of the enzyme required to oxidise 1 μmol of NADH min $^{-1}$ mg protein^{-1}.

DHAR (EC 1.8.5.1) activity was assayed at 265 nm for 1 min by following the regeneration of AsA in a 2ml reaction mixture containing 1.4ml phosphate buffer (pH 7.0), 0.2ml 20mM GSH in phosphate buffer (pH 7.0), 0.2ml 2mM DHA and 0.2ml enzyme extract [27]. DHAR activity was determined by monitoring the rate of AsA formation at 265nm. One unit enzyme activity was expressed as the quantity of enzyme required to produce 1 μmol of AsA min $^{-1}$ mg protein^{-1}.

Total protein from the samples was determined according to Lowry's protocol [28]. The absorbance was measured at 630nm using a UV-VIS spectrophotometer and the protein quantity were calculated from the standard graph prepared using gradient concentrations of BSA (1.0mg/ml). The data obtained from the 21st day's NaCl treatment is presented in the results.

Experimental design and Statistical analysis

The experiment was set up in a completely randomized design in 7 tanks filled equal volume of water containing NaCl concentrations of 0, 25, 50, 75, 100, 125 and 150 mM. Three replications were performed, each containing 10 seedlings. The plant samples for the enzyme assay were collected from each variety after 21days of salt application and frozen in LN_2 and stored at -80^{o}C. Statistical analysis of the different parameters were carried out according to Duncan's test at 5% probability level and Pearson's correlation were calculated using the SPSS software (Version 16.0, SPSS Inc., Chicago, USA) to analyze the effects of salt stress.

RESULTS

The salt tolerance potential of the upland varieties were similar or higher than the pokkali variety with respect to the enzymatic and non-enzymatic antioxidants and the Na^+/K^+ ratio and membrane stability index.

Superoxide and hydrogen peroxide content

All the three upland varieties and the salt tolerant pokkali variety, produced superoxide radicals proportionate with the concentration of NaCl (Fig.1.a). In the control experiment, koduvelliyan and vytilla-2 produced lower superoxide content, marathondi and mullankayama produced slightly higher superoxide content. Increase in salinity led to a progressive increase in the superoxide content in all the varieties during the 21st day of salt stress. The highest quantity of superoxide content (0.115mM gf.wt.$^{-1}$) was observed in vytilla-2 treated with 150mM NaCl, and the superoxide content in the three upland varieties were lesser compared to the salt tolerant vytilla-2, suggesting that the upland varieties have more adaptability to salt stress.

The H_2O_2 content was uniform in the upland and vytilla-2 in the control, however, in salt treatment, the upland varieties, mullankayama and marathondi, the H_2O_2 content increased and the koduvelliyan variety showed the lowest H_2O_2 content. In vytilla-2, the H_2O_2 content was the lowest among all the varieties (Fig. 1.b).

Malondialdehyde (MDA) content

In the control experiment, the vytilla-2 and koduvelliyan produced an almost equal quantity of MDA and in mullankayama and marathondi, the MDA content was lesser. Increase in NaCl concentration positively correlated with the MDA content in all the varieties, however, in koduvelliyan and vytilla-2, the MDA content remained static in all the salt concentrations tested (Fig.1.c).

Ascorbic acid and glutathione content

The ascorbic acid (AsA) content in all the varieties decreased with increasing NaCl concentrations (Fig. 1.d). In the control experiments, koduvelliyan produced a higher quantity of AsA followed by vytilla-2, marathondi and mullankayama. With increased salt concentration, the AsA content decreased in all the varieties, however, the decrease was minimal with koduvelliyan and vytilla-2 produced higher AsA content.

Glutathione content in the control was lower in vytilla-2 and marathondi varieties. Up to 100mM NaCl, all the varieties produced an equal quantity of GSH, however, in higher concentrations (125-150mM) of NaCl treatment, the GSH content increased in mullankayama and vytilla-2 (Fig. 1.e). Vytilla-2 produced a lower GSH content compared to the upland varieties. GSH content in koduvelliyan increased significantly compared to other varieties in NaCl concentration higher than 100mM. The GSH/GSSG ratio of the vytilla-2 and mullankayama was higher and decreased with increasing salt stress, suggesting, the redox homeostasis played a greater role in protecting the plants under salt stress (Fig. 1.f).

Na^+ influx and K^+ efflux and Membrane stability index

In the control, the upland varieties accumulated lesser Na^+ and the Na^+ accumulation increased in all the varieties as the NaCl concentration increased (Fig.2.b). At 150mM NaCl, all the four varieties showed a uniform level of Na^+ accumulation. In

unison with the Na^+ influx, the K^+ efflux was observed in all the four varieties (Fig.2.c), contributing to the increase in Na^+/K^+ ratio with increased NaCl treatment (Fig. 2.d). The membrane stability index of all the varieties, except marathondi showed a uniform pattern irrespective of the NaCl concentrations (Fig.2 a)

Specific activity of enzymatic antioxidants

The salt tolerant, vytilla-2 and upland varieties exhibited uniform increase in SOD activity, under salt stress (Fig.3.a). After 21 days of salinity stress, the upland varieties, koduvelliyan and mullankayama exhibited higher SOD activity than vytilla-2 up to 100mM NaCl treatment and the increase in NaCl concentration (125 and 150 mM) increased the SOD activity in vytilla-2 with highest specific activity in 150 mM NaCl (43.99U mg $protein^{-1}$). In the control experiment, the upland varieties showed a higher enzyme activity compared to vytilla-2, with a higher enzyme activity in mullankayama with a SOD specific activity of 25.26 U mg $protein^{-1}$, followed by marathondi – 23.87 U mg $protein^{-1}$, koduvelliyan – 18.75 U mg $protein^{-1}$ and vytilla-2 – 13.5 U mg $protein^{-1}$.

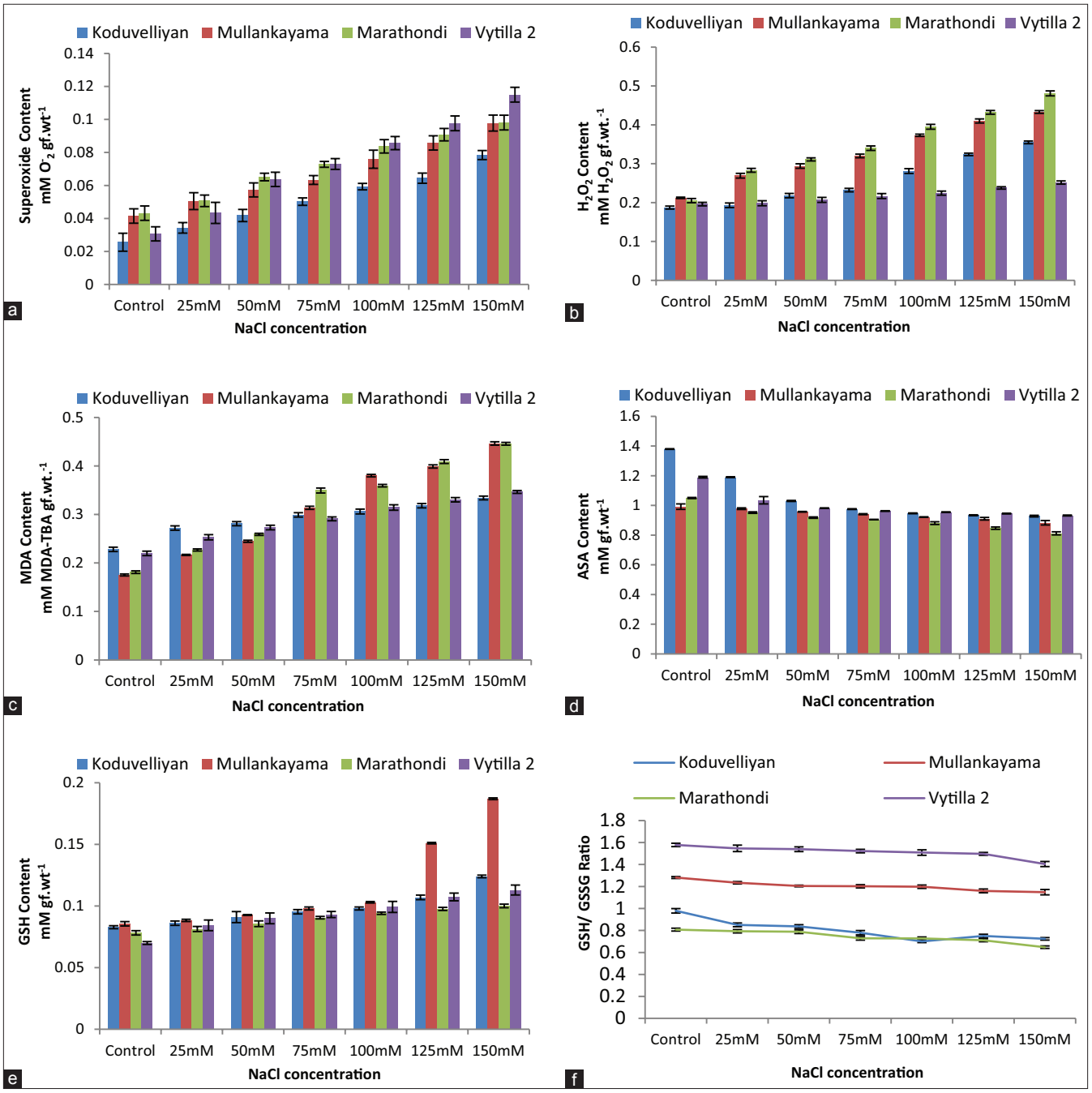

Figure 1: a) Superoxide content, b) Hydrogen peroxide content, c) MDA content, d) Ascorbate content, e) Glutathione content, f) GSH/GSSH ratio in the upland and vytilla-2 varieties treated with different concentrations of NaCl on the 21st day. The values are the mean of three independent experiments and are subjected to one-way ANOVA. The values are significant at p= > 5

CAT activity increased proportionately with the NaCl concentration with the highest activity in koduvelliyan variety. Mullankayama and marathondi exhibited an increase in the CAT activity with increased NaCl concentration. (Fig.3.b). In the control experiments, vytilla-2 showed a higher CAT activity (0.46 μM mg protein^{-1} min^{-1}), compared to the upland varieties and decreased with increased salt treatment.

Ascorbate peroxidase (APX) activity steadily increased during NaCl exposure in koduvelliyan, mullankayama, marathondi and vytilla-2 varieties (Fig.3.c) with the highest activity in 150mM NaCl. In the control experiment, mullankayama and marathondi showed the highest activity (0.221 μM mg protein^{-1} min^{-1} and 0.212 μM mg protein^{-1} min^{-1}), but under salt stress, the higher APX activity was observed in koduvelliyan variety treated with NaCl concentrations ranging from 25mM – 150mM (0.286, 0.30 , 0.31, 0.354, 0.428, 0.50 μM mg protein^{-1} min^{-1}). Vytilla-2 exhibited a lesser APX activity than koduvelliyan, in all the concentrations, except 150mM, where both the varieties exhibited uniform APX activity.

Glutathione reductase (GR) activity was higher in all the three upland rice varieties than in vytilla-2 (Fig.3.d). In the control experiment, all four varieties showed almost similar GR specific activity. However, under salt stress, the GR activity increased corresponding to salt concentration in all the varieties. In koduvelliyan, mullankayama and marathondi, the increase in the enzyme activity was much prominent than in vytilla-2. In 150mM NaCl, mullankayama showed the highest GR activity (0.628 μM mg protein^{-1} min^{-1}) followed by koduvelliyan (0.596 μM mg protein^{-1} min^{-1}), marathondi (0.566 μM mg protein^{-1} min^{-1}) and vytilla-2 (0.499 μM mg protein^{-1} min^{-1}).

The monodehydroascorbate reductase (MDHAR) activity (Fig.3.e), showed significant increase in activity (1.48 μM mg protein^{-1} min^{-1} and 1.67 mM mg protein^{-1} min^{-1} respectively) in vytilla- 2 treated with 125mM and 150mM NaCl concentrations, however, in the upland varieties the MDHAR activity was identical in the control and salt treatments. Among the upland varieties, the highest MDHAR activity was observed in marathondi (1.02 μM mg protein^{-1} min^{-1}) followed by koduvelliyan (0.983 μM mg protein^{-1} min^{-1} in 150mM) and mullankayama (0.957 μM mg protein^{-1} min^{-1}).

Dehydroascorbate reductase (DHAR) activity showed a proportionate increase in vytilla-2 under salt stress up to 150mM NaCl (Fig. 3.f). In the upland rice varieties the DHAR activity is comparatively lesser than vytilla-2. Among the upland varieties,

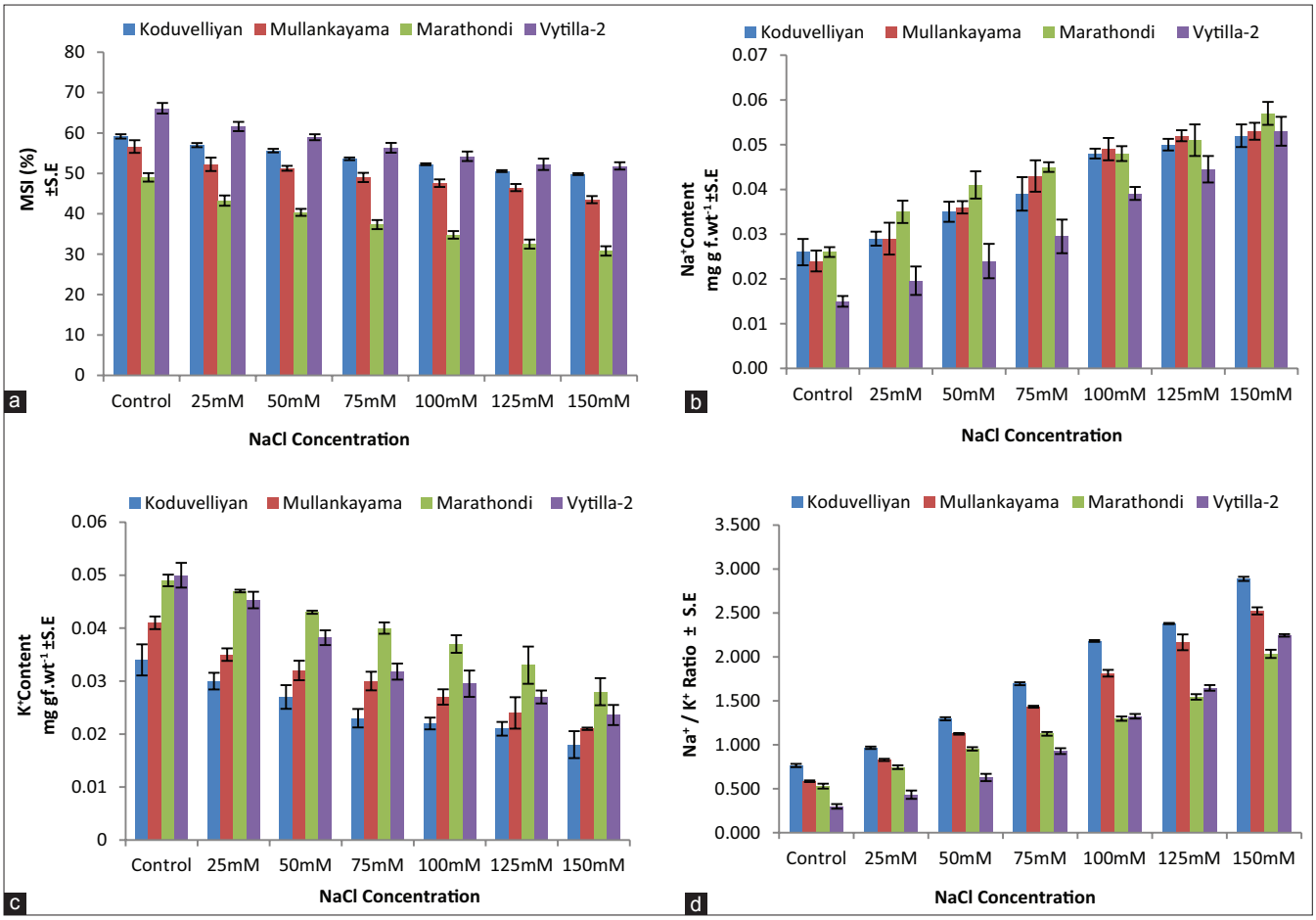

Figure 2: a) Membrane stability index (%), b) Na^+ Content, c) K^+ Content, d) Na^+/K^+ Ratio in the upland and vytilla-2 varieties treated with different concentrations of NaCl on the 21st day. The values are the mean of three independent experiments and are subjected to one-way ANOVA. The values are significant at p= > 5

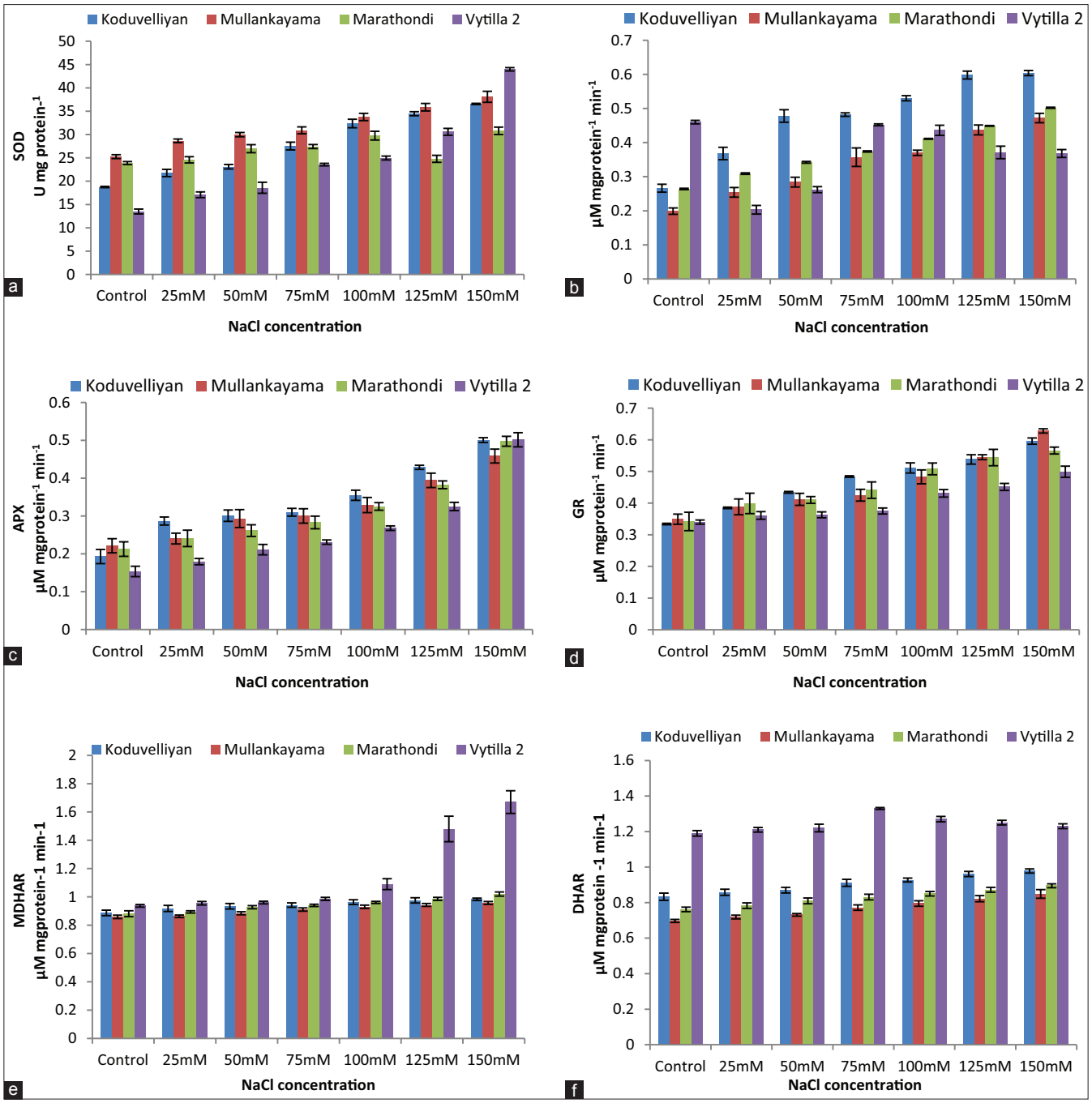

Figure 3: a) Superoxide dismutase activity, b) Catalase activity, c) Ascorbate peroxidase activity, d) Glutathione reductase activity, e) Monodehydroascorbate reductase activity, f) Dehydroascorbate reductase activity in the upland and vytilla-2 varieties treated with different concentrations of NaCl on the 21st day. The values are the mean of three independent experiments and are subjected to one-way ANOVA. The values are significant at p= > 5

koduvelliyan showed the higher activity with an enzyme activity of 0.833µM mg protein^{-1} min^{-1} in control and 0.858, 0.87, 0.911, 0.927, 0.961, 0.978μM mg protein^{-1} min^{-1} respectively in concentrations from 25mM up to 150mM.

DISCUSSION

The salt tolerance potential of hitherto unknown upland rice varieties was investigated by comparing the quantity of antioxidants, the specific activity of the antioxidant scavenging and AsA-GSH cycle enzymes with a salt tolerant released pokkali rice variety, vytilla-2 for a period of 21days under NaCl concentrations ranging from 25-150mM. The salt stress studies on the rice varieties attempted so far were for a shorter duration, however, the present study was carried out for 21days in 25-150mM NaCl using 21 days old plants. Increased or similar activities of enzymatic and non-enzymatic antioxidants were observed in upland farmer varieties compared to salt-tolerant

pokkali variety. The specific activities of ROS scavenging enzymes SOD, CAT and AsA-GSH cycle enzymes APX, GR, MDHAR and DHAR were upregulated in the upland varieties under salt stress. The salt tolerance capacity of the upland varieties will provide a better candidate for planting stock improvement for interspecific breeding and marker-assisted selection [29]. The lesser superoxide content in the upland varieties treated with 150mM NaCl for 21 days indicate their tolerance is better than Vytilla-2. In contrast to this study, increased O_2^- radicals were produced under salt stress in plants, due to the excitation or incomplete reduction of molecular oxygen that forms the harmful byproducts of cellular metabolism [30]. The higher ionic intake is correlated with the higher ROS production such as O_2^- and H_2O_2 might be due to salt-induced ionic toxicity and osmotic stress [31]. The increase in O_2^- content is directly proportionate with the SOD specific activity. The highest SOD activity was observed in 150mM NaCl, reflecting the diffusion-limited rate to produce H_2O_2 [32]. In most of the plants, perturbation in the ROS steady - state is observed during salt stress, however, in the upland varieties, the ROS production, as well as it's scavenging, is unknown and the plants survived in high salt concentration pointing to the existence of an efficient ROS scavenging mechanism. The higher inherent concentration of H_2O_2 in the upland and vytilla-2 varieties is explained by the higher specific activity of SOD and APX and the increase in SOD/APX ratio are correlated with the survival of the upland and vytilla-2 varieties under salt stress. The increase in the MDA content is proportionate with the salt concentration, suggests that the breakdown product of membrane lipids accumulate in all the varieties under salt stress, though the determination of MDA content is not a direct measure of lipid peroxidation, it is referred as an indicator of lipid peroxidation [33]. In the upland variety, koduvelliyan, the MDA content is at par with the salt tolerant vytilla-2, suggesting that the upland varieties have an inherent mechanism to protect against lipid peroxidation; these results are in congruence with the MDA content in *Leymus chinensis* under salt stress [34].

The slight decrease in AsA content in the upland varieties and in the salt tolerant vytilla-2, proportionate with the lesser activity of the AsA- GSH cycle enzymes mediating the stress alleviation as reported in other plants [35]. It is presumed that AsA is crucial in stress perception; act as a connecting link between stress perception and downstream signaling [36]. Glutathione levels were upregulated in 150mM NaCl in mullankayama, koduvelliyan and vytilla-2 with higher levels of reduced glutathione and the upland varieties expressed a higher GSH content than in vytilla-2 and the protection provided by GSH assisting in maintaining the photosynthetic efficiency of the chloroplasts. The AsA and GSH steady state suggests their role in maintaining the stability of cellular organelles and attaining the redox homeostasis, which plays a prominent role in salt tolerance by reacting with the ROS and generating AsA [37]. The increase in the GSH levels under salt stress suggests that the upland varieties develop better tolerance and maintain an increased GSH and GSSG suggesting the increased oxidation of GSH due to the action of the overproduced ROS. The lower GSH/GSSG ratio in all the varieties suggesting that the redox buffering system is operational in these varieties under salt stress thus affecting the alleviation of ROS by the GSH to convert it into the oxidised state [38]. The Na^+ influx and subsequent K^+ efflux suggests that the regulatory mechanism is operational in maintaining a stable Na^+/K^+ ratio. The concerted action of non-selective cation channels and some high-affinity K^+ transporters helps in maintaining the Na^+/K^+ balance in the plants under salt stress [39]. The higher Na^+/K^+ ratio in the upland varieties is coordinated by the higher MSI inhibited by these varieties suggesting their salt tolerance, contrary to the reports on lesser Na^+/K^+ ratio to higher MSI in rice varieties [40].

The higher quantity of inherent O_2^- and H_2O_2 generation in a higher concentration of NaCl in the salt tolerant variety, vytilla-2 and in the upland varieties and subsequent coordinated upregulation of SOD and CAT activity suggests that all these varieties have inherent salt tolerance potential. A distinctive increase in SOD activity was observed in the vytilla-2 and the upland varieties; nevertheless, the CAT activity was increased only in the upland varieties. In vytilla-2, the activity of CAT decreased attributing to the lower levels of H_2O_2 content in the vytilla varieties; contradicting the earlier reports that increased NaCl concentration upregulated the H_2O_2 content and CAT activity [41].

The increased GR activity is correlated with the buffering mechanism of increased GSH under salinity stress and the maintenance of redox potential by maintaining a stable GSH/GSSG ratio. In most of the cases, the homeostasis coupled with Na^+/K^+ ratio under salt stress shows a decreasing trend, where as in the present study, the GSH/GSSG ratio reduced, coupled with an increase in MSI and Na^+/K^+ ratio, contrary to the earlier results [42].

In response to the increased salinity, the total APX activity in the salt tolerant variety and the upland varieties increased in 125-150mM NaCl, suggests that the upregulation of APX activity leads to the amelioration of salt stress in the upland varieties as reported in *L. pennellii* [43]. The lesser CAT activity in the upland and vytilla varieties is compensated by the upregulation of APX. Such a balancing act by the APX for the detoxification of H_2O_2 is reported in various other plants under salt stress [44], and the increased APX activity may be responsible for the synthesis of uniform levels of AsA and GSH under salt stress in the salt-treated upland and pokkali varieties, leading to higher photosynthesis and survival [45,46]. The MDHAR and DHAR specific activities remained in a steady-state in the upland varieties, however, in vytilla-2, the MDHAR expression was upregulated in 125-150mM NaCl, correlated with the ascorbate generation by MDHAR, relating this to the steady-state ascorbate and GSH production under stress in these varieties.

The increase in DHAR activity in the vytilla-2 suggests the normalised GSH/GSSG ratio in the variety. In the upland varieties, the DHAR activity was slightly up-regulated, but not at par with the vytilla-2. This may be due to the redox potential exhibited by these varieties as the GSH to GSSG ratio remain stable and the ASA content is proportionate with the DHAR production suggesting their interdependence.

CONCLUSIONS

The present study underlines the long-term survivability of the upland farmer rice varieties, koduvelliyan, mullankayama and marathondi when compared with the released salt tolerant variety, vytilla-2 under salt stress. Studies on salt tolerant rice are mostly concentrated on short term low salt concentration exposure, but the present study is focused on long-term exposure. Quantification of the enzymatic and non-enzymatic antioxidants showed the salt tolerance potential of the upland varieties at par with the vytilla-2. This shows that the upland varieties have inherent salt tolerance mechanism and can be successfully utilised for breeding purpose as they have a higher disease resistance and drought tolerance capacity.

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